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**RESPONSIBLE EDITOR** Prof. Ugo Oliviero, Department of Translational Medical Sciences, Federico II University, Via pansini 5, Napels, Campania, 80131 Italy, e-mail: ugo.oliviero@unina.it

**PUBLISHER** Walter de Gruyter GmbH, Berlin/Boston, Genthiner Straße 13, 10785 Berlin, Germany

**JOURNAL MANAGER** Katharina Appelt, De Gruyter, Genthiner Str. 13, 10785 Berlin, Germany, Tel.: +49 (0)30 260 05-325, e-mail: jbcpp.editorial@degruyter.com

**RESPONSIBLE FOR ADVERTISEMENTS** Kevin Göthling, De Gruyter, Genthiner Straße 13, 10785 Berlin, Germany, Tel.: +49 (0)30 260 05-170, e-mail: anzeigen@degruyter.com



**TYPESETTING** TNQ Technologies, Chennai, India

Yohana Febriani Putri Peu Patty, Mufarrihah and Yunita Nita*

# Cost of illness of diabetes mellitus in Indonesia: a systematic review

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**Abstract**

**Objectives:** Diabetes Mellitus (DM) is a group of insulin metabolism disorder that affects the socio-economic conditions of the community. The cost of treating diabetes in 2019 was USD 760 billion and by 2045 there are predicted to be 700 million people living with diabetes. The purpose of this systematic review was to provide an overview of the economic burden caused by Diabetes Mellitus for the government, health care providers, and for the community.

**Methods:** This systematic review was carried out by considering the related studies about the cost of illness, evaluation of disease costs, or therapeutic costs for various types of diabetes mellitus that were published in both English and Indonesian. The search engines PUBMED, DOAJ, SCOPUS, SCIENCE DIRECT, and GOOGLE SCHOLAR were used without date published restrictions.

**Results:** A systematic search identifies 18 eligible studies conducted in various regions in Indonesia. The study was retrospective with variation in their perspectives and methods to estimate the diabetes cost. Drug cost was the major contributor to direct medical cost followed by complications cost while other cost was affected by transportation cost, productivity losses, and time spent by family accompanying patients.

**Conclusions:** Diabetes mellitus creates a significant financial burden and affects the health care system as well as the individual and society as a whole. Research about the cost of diabetes in the future should be carried out on a large scale in order to get a more specific cost estimation.

**Keywords:** diabetes mellitus; economic burden of disease; illness costs.

## Introduction

Diabetes Mellitus (DM) is a group of metabolic disorders as a result of the inability to insulin secretion, insulin sensitivity, or both and can have an impact on chronic microvascular, macrovascular, and neuropathic complications [1]. Diabetes is a global disease which is a problem for both low- and middle-income countries [2]. The latest data estimated that in 2019, 463 were diabetic worldwide. The number of diabetics will increase to 578 and 700 million by 2030 and 2045, respectively [3]. This condition made diabetes and its complications cause 11.3% (4.2 million) deaths worldwide that occur in the age group of 20–79 years [4].

Studies related to the cost of diabetes mellitus have been carried out in various countries, and several studies. Costs for diabetes care in 2019 amounted to USD 760 billion, it's increase of 4.5% from 2017 (USD 727 billion). This shows that the effects of diabetes affect the socio-economic conditions of the community and threaten national productivity and economy, especially in low- and middle-income countries [2, 5].

Based on the doctor's diagnosis, the highest prevalence of diabetes mellitus in Indonesia was experienced by women (1.8%), urban residents (1.9%), and groups aged between 45 and 75 years (3–6%) [6]. Several studies were conducted on the cost of illness of diabetes mellitus in Indonesia but based on a literature search, no systematic review has been carried out. Cost of illness analysis enables health policymakers to understand the financial burdens of diabetes mellitus and making decisions such as control programs to improve the health of future diabetes patients [7].

Therefore, this study aimed to conduct a systematic review of the study related to diabetes cost of illness that has been carried out in Indonesia and summarize important findings from previous studies. The results of this systematic review can provide an overview of the economic

***Corresponding author: Yunita Nita**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Kampus C, UNAIR, Mulyorejo, Surabaya, 60115, Indonesia,
E-mail: yunita-n@ff.unair.ac.id
**Yohana Febriani Putri Peu Patty,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Mufarrihah,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

burden caused by diabetes mellitus for the government, health care providers, and for the community.

# Materials and methods

### Search strategy

To answer the purpose of this study, a systematic literature search for journal articles using several databases such as PUBMED, DOAJ, SCOPUS, SCIENCE DIRECT, and GOOGLE SCHOLAR was conducted. The keywords search terms were "economics", "cost", "cost of illness", "cost of disease", "cost analysis", "Diabetes", and "Indonesia". The search was carried out from October to December 2019 without date published restrictions and published in both English and Indonesian. The references were downloaded in RIS format and then transferred to EndNote.

### Inclusion and exclusion criteria

The studies were eligible if the direct and/or indirect cost of type 1 and type 2 diabetes, gestational diabetes including costs of diabetes complications was presented in the results section. However, we excluded the economic evaluation studies besides the cost of illness analysis (e.g., Cost-effectiveness analysis). Review papers, conference abstracts, case reports, editorial letters to the editor, and studies for which the full text could not be retrieved or only an abstract was available were also excluded.

### Data filtering and extraction

After duplicates were removed, titles with the words "cost", "direct medical cost", and "diabetes" or similar words were included by one researcher and then the second researcher checked the process. Both researchers independently read and evaluated the abstract and also the full text of the article if necessary. The primary outcomes were the total costs, direct and/or indirect costs, diabetes complications, and medicines used in diabetes therapy. Extracted information also included the year of publication, the city or region studied, type of diabetes, setting (outpatient or inpatient) studied, sample size, the data source, the time horizon, design studied, and perspective as well. Data extraction was carried out using an extraction table (Tables 1 and 2).

The quality of the studies was critically assessed following the previous studies [26]. The checklist used in this study is the one based on the 10-point checklist (Additional file 1). Each point was given a score of 2 if it meets the criteria, score of 1 if it was partial, and low with a score of 0. The maximum score is 20 with 16 studies has average quality while two studies have poor quality.

### Standardization of costs

The reported costs were transferred from the local currency in the year of the costs to the inflated values in local currency for the year 2019 using the consumer price index (CPI) [27]. The costs data then converted into USD using 2019 purchasing power parity (PPP) estimates [28].

# Results

## Studies characteristics

An initial search using a database and specific keywords resulted in 3,266 articles (Figure 1). After eliminating the duplicates, the titles, and abstracts, finally 18 articles were eligible for the systematic review process. The articles were published between 2006 and 2019, with only one study in diabetic retinopathy extrapolated the cost estimates to the national population. A total of 17 of the selected studies were carried out at the regional level which was conducted limited to one specific health service such as central public hospital, regional public hospital, private hospital. The time horizon conducted by each study ranged from 1 month to 24 months, while seven studies used a time horizon of less than 6 months [8, 14, 16–18]. More than half of the studies include type 2 diabetes patients (n=11) with two studies conducted in diabetic retinopathy and diabetic foot patients, respectively.

Most of the studies were retrospective designs (n=12) using medical records, financial data, and payment receipts as the data source. Hospitals perspective was the most widely used method to collecting data on the cost of diabetes and the sample size ranging from 37 [20] to 1,396 [11] followed by societal perspective (n=3), payer perspective (n=2), and patient perspective (n=1) (Table 1).

## Cost of diabetes mellitus

Studies in this systematic review have a variation among the cost components that estimated the direct and indirect cost. The average cost of type 2 diabetes outpatients without complications was around USD 91.77–USD 175.60/patient/month [8, 9, 14, 17]. Cost incurred by type 2 diabetes outpatients could reach USD 300.28–USD 349.38/patient/month [8, 14]. The average cost for type 2 diabetes outpatients based on INA-CBGs claim during January 2016–December 2017 (2 years) was USD 9,574.77/7 days of treatment. In 2017, there was a period when the hospital real costs were still low compared with INA-CBGs [23]. Another study also found the hospital real cost for outpatient and inpatient with type 2 diabetes was higher than the cost of INA-CBGs [11].

The burden of costs was clearly seen in diabetic patients who are hospitalized with complications (Table.3). Macrovascular and microvascular complications received a total average cost of around USD 489.36–USD 3,508.46/episode [14, 16]. In 2025, it is predicted that the total cost

**Table 1:** Characteristics of research articles.

| Study | City | DM type | Setting | Sample size | Data source | Time horizon | Study design | Perspective |
|---|---|---|---|---|---|---|---|---|
| [8] | JOG | 2 | O | 100 patients | Interview, medical record, pharmacy, and receipt | July–August 2005 | P | H |
| [9] | JOG | 2 | O | 100 patients | Medical record, pharmacy, and receipt | January–December 2004 | R | H |
| [10] | SKT | 1 & 2 | I | 101 patients | Medical record, hospital financial data, and pharmacy | January–December 2010 | R | H |
| [11] | JOG | 1 & 2 | O & I | 1.396 patients | Medical record, detail of direct medical cost, and INA CBGs claim data | January–June 2014 | R | H |
| [12] | JOG | 1 & 2 | O & I | 587 episodes | INA CBGs, medical record, and financial data | January–June 2014 | R | H |
| [13] | JOG | 1 & 2 | O & I | 697 patients | Medical record and hospital data | January–June 2014 | CS/R | H |
| [14] | JOG | 2 | O & I | 77 patients | Medical record, receipt, and finance | October–December 2014 | R | PY |
| [15] | SKW | 2 | O | 200 patients | Medical record, and social security administrator for health (BPJS) prolanis data | January–December 2013 | R | H |
| [16] | DJB | 2 | I | 35 patients | Medical record, administration, pharmacy, and patient interview | August–October 2016 | P | S |
| [17] | BDJ | 2 | O | 45 patients | Medical record | March 2016 | R | H |
| [18] | BKS | 2 | O | 110 patients | Medical record, questionnaire, supporting data, and patient interview | March–May 2014 | CS/P | S |
| [19] | BDO | 2 | O | 417 patients | Medical record, financial data, and drug ceiling price list | 6 months therapy | R | S |
| [20] | BTJ | DF | I | 37 patients | Medical record | 1 February 2017–12 February 2018 | P | H |
| [21] | JOG | 1 & 2 | O & I | 158 pasien/1.531 visits | Medical record, financial data, and pharmacy | January–June 2014 | R | H |
| [22] | IDN | DR | – | – | National insurance standard | – | Model from previous population-based DR study | H & PX |
| [23] | JKT | 2 | O | 443 patients | Medical record, hospital financial data | January 2016–December 2017 | Time series | H |
| [24] | JOG | 2 | O | 200 patients | Medical record, hospital financial data | July–September 2017 | CS/R | H |
| [25] | JOG | 2 | O | 123 patients | Medical record, detail of direct medical cost, and INA CBGs claim data. | August–September 2018 | R | PY |

JOG, Yogyakarta; SKT, Surakarta; SKW, Singkawang; DJB, Jambi; BDJ, Banjarmasin; BKS, Bengkulu; BDO, Bandung; BTJ, Banda Aceh; IDN, Indonesia; JKT, Jakarta; DF, Diabetic Foot; DR, Diabetic Retinopathy; O, Outpatient; I, Inpatient; CS, Cross Sectional; P, Prospective; R, Retrospective; H, Hospital; PY, Payer; PX, Patient; S, Society.

caused by retinopathy complications will be USD 8.9 million compared to 2017 which was only around USD 2.4 million [22]. Patients with diabetic foot who had amputations spent an average of 15 days of stay with an average cost of USD 10,178.22 [20]. Patients were hospitalized with complications who had Public Health Insurance (JAMKESMAS) had an average total cost of USD 1,312.68/month while patients holding Civil Servants Health Insurance (ASKES PNS) had an average total cost of USD 1,217.61/month [10].

The total cost of type 1 and type 2 diabetes outpatient and inpatients was estimated over six months with differences based on the number of patients, total visit episode, or total treatment episode [11–13, 21]. An episode is defined as a series of consultation meetings between patients and doctors and supporting examinations according to medical indications and medications given on the same day (outpatients). Another definition is one series of services if the patient is treated for >6 h or if the patient has received an inpatient facility <6 h but has been administratively declared

**Table 2:** Data extraction of research articles.

| Study | Hospital type | Gender | Age, years | Duration of diabetes, years | Length of stay, days | Diabetes complication | Drugs |
|---|---|---|---|---|---|---|---|
| [8] | Central Public Hospital | Female = 44%<br>Male = 56% | 41–85 | <1 = 5%<br>1–5 = 28%<br>5–10 = 28%<br>10–15 = 26%<br>>15 = 13% | – | Hypertension = 69%<br>Neuropathy = 21.5%<br>Hipoglycemia = 12.3% | Biguanid = 37.5%<br>Sulfonylureas = 35.8%<br>α-glucosidase = 17.5%<br>Insulin = 17.5% |
| [9] | Regional Public Hospital | Female = 71%<br>Male = 29% | 61.2 ± 13.7 | – | – | – | Sulfonylureas = 41.7%<br>Biguanid = 42.2%%<br>α-glucosidase = 16.1% |
| [10] | Regional Public Hospital | Female = 52.5%<br>Male = 47.5% | <45–>65 | <5 = 84.16%<br>>5 = 15.84% | <10 = 56.4%<br>>10 = 43.5% | 1 complication = 43.5%<br>2 complication = 34.6%<br>>2 complication = 21.8% | Oral = 76.92%<br>Insulin = 51,35%<br>Oral + insulin = 70.97% |
| [11] | Central Public Hospital | Male = 57%<br>Female = 43% | 45–64 | – | 1–3 = 21.43%<br>5 = 26.7%<br>>10 = 32.7% | Neuropathy; Ulcer; Multiple complication | – |
| [12] | Private Hospital | – | 25–44<br>45–64 | – | – | Neuropathy; Nephropathy; Retinopathy; Peripherals; Unspecified | Biguanid = 39.3%<br>Sulfonylureas = 26.84%<br>Insulin = 21%<br>Thiazolidinedione Pioglitazone and Acarbose, Amitriptyline, Meloxicam, Paracetamol, Tramadol, Diclofenak Sodium, Antalginine |
| [13] | Private Hospital | Male = 15.6%; 39.4%<br>Female = 83.3%; 51% | 25–44<br>45–64 | – | – | Neuropathy; Peripherals | – |
| [14] | Private Hospital | Male = 56.7%<br>Female = 43.3% | <45–>45 | – | – | Hypertension; Dyslipidemia; Chronic heart failure; Neuropathy; Ulcer | Insulin Novorapid (43.3%)<br>Insulin Novorapid + Metformin (19%) |
| [15] | Regional Public Hospital | Male = 48%<br>Female = 52% | <40–≥60 | – | – | MIC 10.5%<br>MAC 45.5%<br>Both 26% | Oral = 92.5%<br>Insulin + Oral = 7.5% |
| [16] | Regional Public Hospital | – | – | – | 2–10 | Stroke; Ulcer; Hipoglycemia; Hypertension; Chronic kidney disease | – |
| [17] | Regional Public Hospital | Male = 66%<br>Female = 34% | 35–>64 | 1–18 | – | – | Sulfonylureas + a-Glukosidase = 40%<br>Sulfonylureas + Biguanide = 35%<br>Biguanide + a-Glukosidase = 17% |
| [18] | Regional Public Hospital | Male = 66%<br>Female = 34% | 18–≥65 | 1–≥7 | 0–≥ 1 | – | – |
| [19] | No information | – | – | – | – | | Insulin + Metformin = 41.4% |

**Table 2:** (continued)

| Study | Hospital type | Gender | Age, years | Duration of diabetes, years | Length of stay, days | Diabetes complication | Drugs |
|---|---|---|---|---|---|---|---|
| [20] | Regional Public Hospital | Male = 59% Female = 41% | 56.9 ± 9.4 | 7.7 ± 4.1 | 14.8 ± 7.5 | Neuropathy; Vasculopathy; Infection | – |
| [21] | Regional Public Hospital | Male = 32% Female = 68% | <45–>60 | – | 1–>10 | Diabetic ketoacidosis; Hipoglycemia; Chronic heart failure; Strokes; Retinopathy; Neuropathy; ulcer | – |
| [22] | – | – | – | – | – | – | – |
| [23] | Central Public Hospital | Male = 41%; 40% Female = 59%; 60% | 55–64 | – | – | Renal Ophthalmic neurological peripheral circulatory | Analog Insulin, Analog Insulin + Human Insulin, Analog Insulin + Oral Antidiabetic, Analog Insulin + Oral Antidiabetic + Non Diabetes drugs |
| [24] | Regional Public Hospital | Male = 43.5% Female = 56.5% | ≥60 | 5–10 | <5–>10 | Microvascular = 15% Macrovascular = 19.5 % Both = 2% | Oral = 11.7% Insulin = 14.6% Oral + Oral = 24.3% Oral + Insulin = 49.4% |
| [25] | Private Hospital | Male = 35.8% Female = 64.2% | 45–64 | – | – | Microvascular = 13% Macrovascular = 46 % Both = 39% | Sulfonylureas + Biguanid = 21.13% Sulfonylureas + Biguanid + Acarbose = 3.25% Insulin = 4.06% Insulin + Oral = 1.81% |

as an inpatient [30]. The total cost of the disease around USD 116,949.94–USD 3,909,877.48 [11, 13, 21].

## Direct medical costs and indirect costs

Drugs became a cost component which was very influential on direct costs (Table 4). The direct medical cost for outpatients was around USD 63.0–USD 446.53/patient/month [8, 9, 17, 24, 25], USD 193.84–USD 684.98/episode [12, 13, 21], 234.71–608.86/patient/6 month [19] and USD 654.65–1,268.05/patient/year [15, 18]. While for patients who are hospitalized the direct medical cost was around USD 335.28–USD 2,302.99/episode. Biguanides and sulfonylurea were frequently prescribed as monotherapy or combination. A combination of sulfonylurea and $\alpha$-glucosidase was also observed in the combination of 2 OHA. Analog insulin was prescribed in monotherapy and combination with OHA. The average cost of insulin therapy could reach USD 608.86/patient/6 months. Whereas, the average cost of the combination insulin and OHA was USD 234.71/6 months [19].

Besides drugs, the other biggest cost that also affected was the cost of complications and costs due to laboratory examinations. Diabetic patients with complications were spent direct medical cost around USD 349.38–USD 12,539.74/episode [10, 12, 13]. Laboratory tests were such as fasting blood sugar level, 2-h after meal sugar level, $HbA_{1C}$, HDL, LDL, TGA, creatinine level, and other laboratory tests. The purpose of this examination is to evaluate the results of therapy at a cost of USD 301.04/patient–USD 354.58/patient [10, 19].

The majority of the studies have focused on direct medical costs, with relatively little emphasis on the measurement of transportation costs, productivity costs, premature mortality costs, and the value of pain. These conditions may have underestimated the actual socioeconomic cost of diabetes. If diabetes cost analysis took all aspects of cost, the findings may create a complete picture of the economic burden of diabetes mellitus [31, 32]. Cost due to lost productivity and the cost of accompanying patients estimated between USD 140.56 while cost due to lost productivity and cost of accompanying patients estimated between USD 244.81–USD 305.37 per patient, carried out with the human capital approach [18, 19].

# Discussion

The global burden of diabetes has a huge economic impact with reaching 1–8% of world GDP, and 34.7% of this

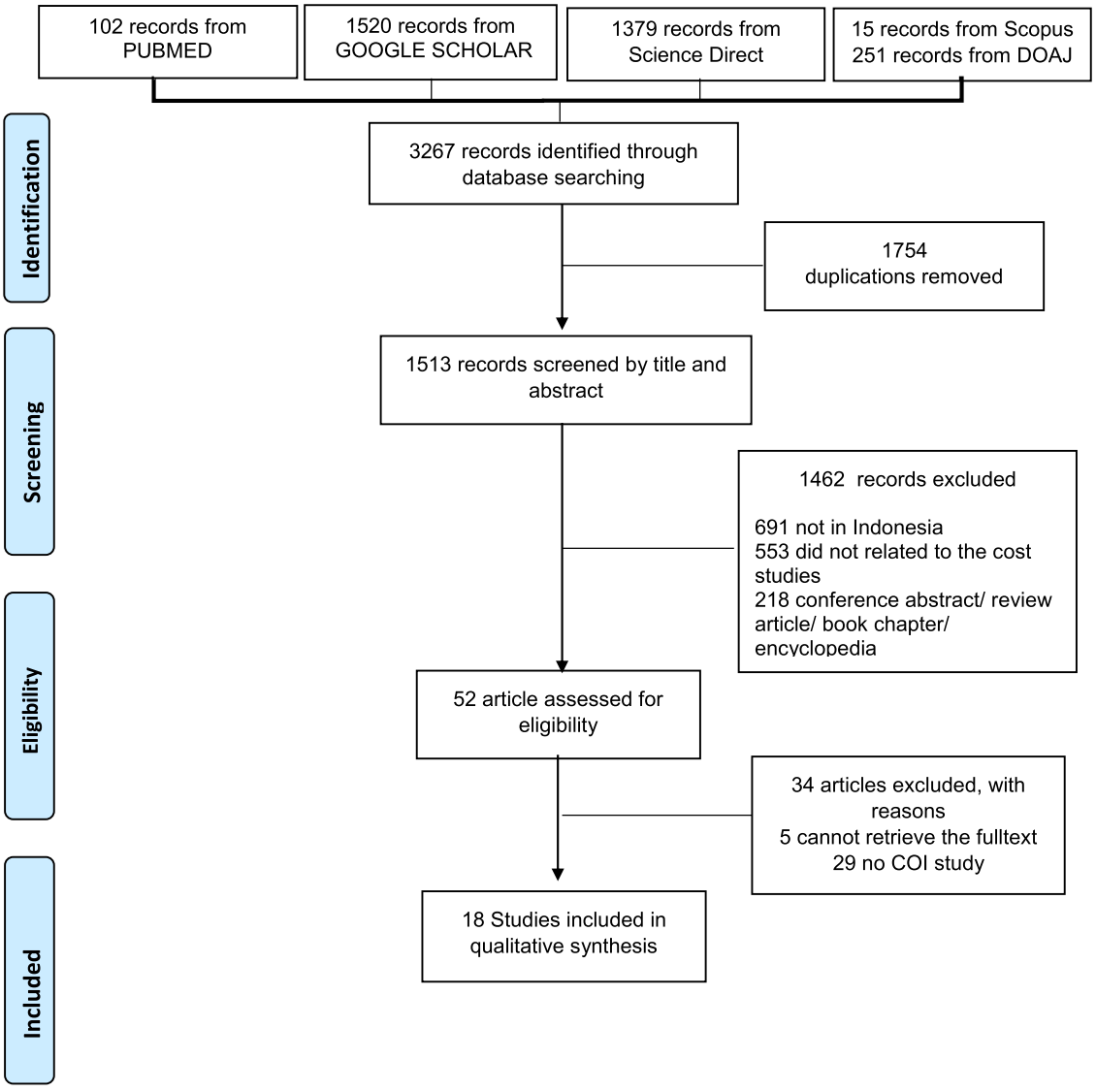


**Figure 1:** PRISMA flow chart through the phases of the systematic review [29].

burden attributable to indirect costs [33]. To our knowledge, this study is the first systematic review about the cost of illness diabetes mellitus in Indonesia. This can be caused by the lack of research in Indonesia related to pharmacoeconomics especially cost of illness study in diabetes mellitus with only 18 studies over the last 15 years.

An important finding in this review is that there is a wide variation in the methodology used to estimate any cost component of diabetes mellitus treatment (Table 1). 17 studies in this review were conducted on a relatively small sample and limited to a specific group of health service (a hospital) and were generally representative of that particular diabetes population (Diabetes type 2). According to IDF, about 45.8% of diabetes patients are undiagnosed. Estimating the cost of health care should ideally take into account the undiagnosed diabetes mellitus patients [34]. This is based on the fact that individuals with diabetes mellitus at the time of diagnosis already have complications. So, if this group does not participate in the calculation of costs, the estimated total cost generated can be lower than the actual economic burden of diabetes mellitus [32, 34, 35].

The majority of diabetes patients in these studies are female (Table 2), although there are no significant differences with male patients. In a study of the USA and China reported that males had more hospitalizations, longer stays, higher expenditures and costs than females [36, 37]. In Finland, the prevalence of microvascular complications such as neuropathy and retinopathy is more common in female patients. Likewise, the average cost of diabetes care in females was higher than males [38]. These differing

**Table 3:** Total cost of diabetes mellitus.

| Study | Year of cost estimate | Total cost estimate for resource (IDR) | Today's cost – CPI 2019 (IDR) | 2019 USD PPP |
|---|---|---|---|---|
| [9] | 2004 | 179,430 ± 123,184/patient/month$^{M}$ | IDR 436,211 ± 299,472/patient/month$^{M}$ | 91.77 ± 63.01 |
| [8] | 2005 | 208,500–754,500/patient/month$^{M}$ | 458,912–1,660,668/patient/month$^{M}$ | 96.55–349.38 |
| [10] | 2010 | 4,127,180/patient/6 months$^{M1a}$ | 6,239,333/patient/6 months$^{M1a}$ | 1,312.68 |
| | | 3,828,282/patient/6 months$^{M1b}$ | 5,787,469/patient/6 months$^{M1b}$ | 1,217.61 |
| [19] | 2013 | 2,598,991/patient/6 months$^{M2a}$ | 5,128,945/patient/6 months$^{M2a}$ | 1,079.07 |
| | | 3,966,381/patient/6 months$^{M2b}$ | 3,360,767/patient/6 months$^{M2b}$ | 707,07 |
| [14] | 2014 | 2,247,550–3,853,084/patient/episode$^{IN}$ | 2,731,631–4,682,968/patient/episode$^{IN}$ | 574.70–985.24 |
| | | 1,913,800–9,499,936/patient/episode$^{IC}$ | 2,325,997–11,546,050/patient/episode$^{IC}$ | 489.36–2,429.15 |
| | | 247,309–686,753/patient/month$^{ON}$ | 300,574–834,667 patient/month$^{ON}$ | 63.24–175.60 |
| | | 128,143–1,174,342/patient/month$^{OC}$ | 155,742–1,427,274 patient/month$^{OC}$ | 32.77–300.28 |
| [18] | 2014 | 6,081,571/patient/year$^{M}$ | 7,391,431/patient$^{M}$ | 1,555.07 |
| [11] | 2014 | 15,290,740,745/6 months (Inpatient & Outpatient) | 18,584,089,503/6 month (Inpatient & Outpatient) | 3,909,877.48 |
| [13] | 2014 | 816,967,440/6 months (Inpatient & Outpatient) | 992,927,437/6 months (Inpatient & Outpatient) | 208,900.45 |
| [21] | 2014 | 1,203,799,389/6 months (Inpatient & Outpatient) | 1,463,075,985/6 months (Inpatient & Outpatient) | 307,814.26 |
| [12] | 2014 | 457,367,600/6 months (Inpatient & Outpatient) | 555,876,301/6 months (Inpatient & Outpatient) | 116,949.94 |
| [17] | 2016 | 500,743/patient$^{M}$ | 552,698/patient$^{M}$ | 116.28 |
| [16] | 2016 | 15,108,522/episode | 16,676,121/episode | 3,508.46 |
| [23] | 2016 | 41,231,910/7 days$^{M}$ | 45,509,967/7 days$^{M}$ | 9,574.77 |
| [20] | 2017 | 45,500,000/15 days$^{M}$ | 48,378,267/episode$^{M}$ | 10,178.22 |
| [25] | 2018 | 3,164,732/month$^{Mi}$ | 3,260,641$^{M1}$ | 686.00 |
| | | 9,984,566/month$^{Ma}$ | 10,287,156$^{M2}$ | 2,164.29 |
| | | 11,534,060/month$^{Mia}$ | 11,883,609$^{M3}$ | 2,500.17 |
| [22] | 2025 | 8.9 billion/2025* | – | – |

*Cost prediction in 2025; $^{M1a}$Average cost based on Public Health Insurance & Underprivileged; $^{M1b}$Average cost based on General & Civil Servants Health Insurance; $^{M2a}$Average cost in group with insulin used; $^{M2b}$Average cost in group with combination insulin-OHA used; $^{IN}$Inpatient without complication; $^{IC}$Inpatient with complication; $^{ON}$Outpatient without complication; $^{OC}$Outpatient with complication; $^{M}$Average cost per patient; $^{Mi}$Average cost per patient in microvascular group; $^{Ma}$Average cost per patient in macrovascular group; $^{Mia}$Average cost per patient in both microvascular and macrovascular group.

results may be due to differences between health care systems, cost definitions, and the methodologies [37].

The published research estimates of diabetes mellitus cost vary greatly between sources. Some studies in this review reported that drug cost contribution to total illness costs was much higher than other costs component. More appropriate and effective use of diabetes drugs might reduce total drug costs. A study related to drug costs reported that only 18% of the total cost was for insulin and OHA, while amount 16% was spent on non-diabetes drugs for managing diabetes complications [39]. People with diabetes mellitus-related complications spent two times more on non-diabetes mellitus-related medications than those without complications. This found suggesting that a significant amount was spent on medications used to manage complications [40]. There is a result where diabetes mellitus without complications has a higher average cost than diabetes mellitus with complications. This is due to the different types of therapy that are obtained. Patients without complication spent higher costs when they used branded drugs either single insulin or a combination of insulin and OHA. Meanwhile, patients with complications can have lower costs because the therapy obtained was generic drug therapy, either single insulin or a combination of OHA and insulin.

Patients with diabetes mellitus and complications had significantly higher hospitalization events with total costs were twice as much as those without complications [41–43]. The type of diabetes and accompanying complications were found to be important determiners for diabetes treatment costs [44]. It is important to identify which types of complications that greatly increase the economic burden

**Table 4:** Direct medical and nonmedical cost of diabetes mellitus.

| Study | Year of cost estimate | Direct medical cost estimate for resource (IDR) | Today's cost – CPI 2019 (IDR) | 2019 USD PPP | Direct nonmedical cost Indirect cost (IDR) | Today's cost – CPI 2019 (IDR) | 2019 USD PPP |
|---|---|---|---|---|---|---|---|
| [9] | 2004 | 354,368/patient/month[a] (medicine) | 861,502 | 181.25 | – | – | – |
| [8] | 2005 | 571,000/patient/month (medicine)[a] | 1,256,781 | 264.41 | – | – | – |
| | | 754,500/patient/month (complications)[a] | 1,660,668 | 349.38 | | | |
| [10] | 2010 | 1,062,677/patient (medicines)[b] | 1,606,519 | 337.99 | – | – | – |
| | | 1,114,832/patient (laboratory)[b] | 1,685,365 | 354.58 | | | |
| | | 709,741/patient (actions)[b] | 1,072,962 | 225.74 | | | |
| | | 1,054,164/patient (medicine)[c] | 1,593,650 | 335.28 | | | |
| | | 946,493/patient (laboratory)[c] | 1,430,876 | 301.04 | | | |
| | | 727,361/patient (actions)[c] | 1,099,600 | 231.34 | | | |
| [19] | 2013 | 862,750[e]–2,238,000[d]/ patient/6 months (medicines) | 1,115,626–2,893,968 | 234.71–608.86 | 538,146 (Transportation) 516,650 (Loss Productivity) | 668,082 695,879 | 140.56 146.40 |
| | | 17,925[e]–55,058[d]/patient/ 6 months (complications) | 23,178–71,195 | 4.88–14.98 | | | |
| | | 957,235[e]–1,030,833[d]/ patient/6 months (laboratory) | 1,237,805–1,332,974 | 260.42–280.44 | | | |
| [15] | 2013 | 1,804,743/patient/year (medicines)[a] | 2,333,721 | 490.98 | – | – | – |
| | | 577,518/patient/year (others)[a] | 746,791 | 157.12 | | | |
| [18] | 2014 | 4, 959,114/patient/year[a] | 6,027,217 | 1,268.05 | 1,122,457/patient | 1,451,454 | 305.37 |
| [13] | 2014 | 2,678,844/episode outpatient (medicines)[a] | 3,255,818 | 684.98 | – | – | – |
| | | 1,107,250/episode outpatient (actions)[a] | 1,345,731 | 283.13 | | | |
| | | 8,539,206/episode outpatient (medicines)[a] | 10,378,396 | 2,183.5 | | | |
| | | 14,021,357/episode outpatient (actions)[a] | 17,041,303 | 141.19–352.04 | | | |
| [12] | 2014 | 758,077/episode out patient (medicines)[a] | 921,353 | 193.84 | – | – | – |
| | | 814,087–2,711,400/ episode inpatient (medicines)[a] | 989,426–3,295,386 | 208.16–693.31 | | | |
| | | 552,169–1,376,750/ episode inpatient (medical supports)[a] | 671,096–1,673,277 | 141.19–352.04 | | | |
| [16] | 2016 | 10,946,355/episode[a] | 12,082,104 | 2,302.99 | 1,054,247 (Loss Productivity) | 1,163,631 | 244.81 |
| [17] | 2016 | 266,745/patient/month (medicines) | 299,421 | 63.0 | | | |
| | | 194,774/patient/month (laboratory) | 203,945 | 43.0 | | | |
| [20] | 2017 | 42,300,000 (neuropathic wounds)[a] | 46,688,878 | 9,822.80 | – | – | – |

**Table 4:** (continued)

| Study | Year of cost estimate | Direct medical cost estimate for resource (IDR) | Today's cost – CPI 2019 (IDR) | 2019 USD PPP | Direct nonmedical cost Indirect cost (IDR) | Today's cost – CPI 2019 (IDR) | 2019 USD PPP |
|---|---|---|---|---|---|---|---|
| | | 41,100,000 (vasculopathic wounds)[a] | 45,364,371 | 9,544.14 | | | |
| | | 54,000,000 (wound infection)[a] | 59,602,823 | 12,539.74 | | | |
| [24] | 2017 | 478,600[f]; 600,203[g] (medicines) | 508,875; 638,171 | 107.06; 134.26 | – | – | – |
| | | 234,125[f]; 142,236[g] (complications) | 248,935; 151,233 | 52.37; 31.81 | | | |
| [25] | 2018 | 408,567/month (medicines)[h] | 420,948 | 88.56 | – | – | – |
| | | 1,245,987/month (complications)[i] | 1,283,747 | 270.08 | | | |
| | | 2,059,959/month (medicines)[j] | 2,122,387 | 446.53 | | | |

[a]Average cost, [b]Cost based on Public Health Insurance & Underprivileged, [c]Cost based on General & Civil Servants Health Insurance, [d]Cost in group with insulin used, [e]Cost in group with combination insulin-OHA used, [f]Average cost based on monotherapy OHA & insulin, [g]Average cost based on combination of OHA & insulin, [h]Average cost based on microvascular group, [i]Average cost per patient in macrovascular group, [j]Average cost per patient in both microvascular and macrovascular group.

so that the policymaker can be considered in diabetes mellitus management programs that can reduce the cost burden.

Age and diabetes duration were positively associated with macrovascular and microvascular complications [45]. The ranged age of diabetes patients in this study was 35–65 years, with diabetes duration between 5 and 10 years (Table 2). Other studies suggest poor glycemic control which may be due to poor adherence therapy or delay in initiating drug therapy associated with diabetes complications [46, 47]. Chronic disease costs show a strong association with the increasing effects of diabetes with aging and the medical cost increases in older diabetes patients [48].

There was also a variability reported about inpatient costs and outpatient costs between studies. Cost variations possibly due to caring utilization at different levels of the health system or from different sectors [49]. It's the same with previous studies which found there was a high economic burden on the patients with diabetes getting care from the private sector as compared to public hospitals [50]. There was a strong relationship between the length of stay and diabetes treatment cost. The greater length of stay caused higher costs. It indicates that the more healthcare utilization, the higher healthcare costs the patients significantly had [43].

The hospital financial performance has increased using the INA-CBGs system along with studies that show INA-CBGs claims are higher than hospital rate claims [51]. Another study shows that the average value of hospital charges for treating diabetes patients with the severity level I are much lower while severity level II and III are larger when compared with rates of INA-CBGs [52]. With the variety of complications experienced by patients, the need for drugs was greater which tends to affect the total cost of diabetes mellitus. It is important for every hospital to have a strategy to overcome the cost difference caused by these things as if making a clinical pathway consisting of protocol therapy and the standard care ranging from admission to hospital charge [51, 52].

A limitation of this analysis is the heterogeneity in data reporting among articles included in this review. The cost data reviewed were derived from different methodologies of each study. The difficulties faced when conducting reviews were due to the fact that several studies did not specify which cost components were included in their analyzes. For example, some studies present the costs in a detailed way, while other studies combine particular costs to one cost position. This is may impact some of our findings.

Diabetes mellitus creates an economic burden on healthcare system as well as on individuals and society as a whole. The cost of drugs and the cost of complications imposed the largest burden on the total cost of diabetes mellitus. The results of this review can be used as a reference for health policymakers to focus on reducing the burden of diabetes mellitus and the factors associated with increasing diabetes costs. Indonesia still lacks and future

needs much more research in the field of pharmacoeconomics about the cost of illness in diabetes mellitus. Focus on increasing the methodological principles and carried out on a large scale, especially at the national level so that economic analysis of diabetes costs may be more specific.

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Riza Alfian, Umi Athiyah* and Yunita Nita

# Social media health interventions to improve diabetes mellitus patient outcome: a systematic review

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**Abstract**

**Objectives:** The use of modern technology and social media has revolutionized the way health information is distributed to diabetes mellitus patients. Social media can be used as a medium of providing health interventions to improve patient health outcomes. Social media is able to provide a more intensive communication facility between healthcare professionals and patients. We aim to systematically review and describe the effect of social media interventions on health outcomes of patients with diabetes mellitus.

**Methods:** A systematic review was carried out from three electronic databases (Pubmed, Scopus, and Medline). Eligible publications are studies that describe the application of social media interventions on the health outcomes of patients with diabetes mellitus.

**Results:** Fourteen studies were selected for this systematic review, 10 studies with a randomized controlled trial design, and 4 studies with a nonrandomized controlled trial design. Six studies only used interventions using social media, A blend of face-to-face social media intervention was used in 6 studies, 2 studies used a combination of telephone and social media intervention. One study had treatment behavior outcomes with improvement in treatment behavior, 6 studies had clinical outcomes (an improvement in HbA1c values in the four studies), 6 studies had treatment behavior outcomes and clinical outcomes (1 study had improved treatment behavior and clinical outcomes, 3 studies had improved treatment behavior outcome only), and 1 study had medication adherence outcome (no improvement in medication adherence).

**Conclusions:** These findings indicate that the intervention using social media can improve the health outcomes of diabetes mellitus patients.

**Keywords:** diabetes mellitus; health intervention; patient outcome; social media.

## Introduction

Diabetes mellitus is often referred to as a silent killer because the prognosis of the disease takes a long time to be detected [1, 2]. Patient with blood glucose level above normal limit persistently has the potential for complications of microvascular and macrovascular diseases, increased health costs, mortality, and decreased quality of life [3–5]. In 2013, the number of people with diabetes mellitus worldwide was 382 million people and in 2019 it increased to 463 million people. If this increasing trend continues, the number of individuals with diabetes mellitus worldwide is expected to exceed 700 million by 2045. The costs spent for handling diabetes mellitus worldwide in 2019 reached USD 760 billion, an increase of 4.5% compared to expenditure in 2017 [6–8].

The development of information technology allows health care not only to be done face-to-face but also when patients and health care providers are not meet at the same location. One form of technological advancements that has been used to support health care is an online social media platform. Facebook, Instagram, Twitter, Telegram, and WhatsApp are examples of online social media platforms that have been used to support communication between health care providers and patients in managing chronic diseases and their treatment [9–12].

Recent studies have shown that there is a growing trend regarding the application of social media for the management of the chronic disease [13]. The emergence of online social media can be used as a medium of supporting existing face-to-face traditional health care. Online social media allows patients and health care

***Corresponding author: Umi Athiyah**, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +628123249338, E-mail: umi-a@ff.unair.ac.id
**Riza Alfian,** Sekolah Tinggi Ilmu Kesehatan ISFI Banjarmasin, Banjarmasin, Indonesia; and Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Yunita Nita,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

providers to communicate quickly, efficiently, affordably, and in real-time [14–16]. The practitioners can provide counseling and education using online social media so as to increase patient awareness in order to optimize disease management and treatment [17–19].

The intervention using social media to increase health outcomes still becomes a new study so that there are still many gaps from the various evidence that have been produced. Existing systematic reviews show that the social media application as a medium of intervention can improve lifestyle, self-care, and clinical outcomes for chronic disease patients [20, 21]. However, the positive or negative impacts of using social media to support health services cannot be concluded definitively. Thus, this systematic review presents new evidence regarding the use of social media as an intervention method to improve the health outcomes of diabetes mellitus patients.

# Methods

A systematic review was carried out with the aim of identifying the literature describing interventions using social media to improve treatment behavior outcomes, clinical outcomes, and adherence in diabetes mellitus patients. Two research questions on this systematic review are: (1) Is there any evidence of the interventions using social media aimed at affecting the health outcomes of diabetes mellitus patients? and (2) What are the reported results of the intervention? e.g., the impact on treatment behavior outcomes, clinical outcomes, or another impact.

### Search strategy

An electronic literature search was carried out in February 2020. The three databases used in this study were PubMed, Medline, and Scopus. The keywords used are "social media", "social networking", "Facebook", "WhatsApp", "WeChat", and "Twitter" combined with the keyword "diabetes" contained in the title or abstract of the articles. The searching strategy for literature was restricted to publications in English. There was no limitation on the reference year in this systematic review. Searching on electronic databases was also complemented by manual searches from the results of reference reviews and other systematic reviews.

### Study selection

Articles that were included in this systematic review must meet the criteria in the form of a research article (primary study), which are using social media as a medium of intervention, reporting the outcome of the intervention, and the research sample was diabetes mellitus patients. Articles that did not meet these criteria were not included in the systematic review.

### Data extraction and analysis

All search results were uploaded to EndNote X9. Duplicate articles were removed. The selection of articles to be included in the systematic review was carried out through three stages. In the first stage, one reviewer screened all titles and abstracts, then consulted two other reviewers when there were any problems in article selection. After that, articles that had the potential to be selected in the first stage were further screened by assessing the full-text articles. The third stage involved compiling a list of articles to be reviewed and data extraction. Extracted data on systematic review consisted of the research design, intervention model, type of social media, duration of intervention, direction of communication, sample, sample size, and outcomes.

# Results

## Search result

The article search from three databases resulted in 1,502 publications, 887 duplicate articles removed, 615 titles and abstracts were screened. After screening them, 29 articles met the inclusion criteria, and further exploration was carried out by reading the full text. Twenty-nine articles were then subjected to further screening, leaving only 13 articles to be used as data sources for systematic review. Output in the form of unclear intervention and an assessment of the feasibility of the intervention method were the two main reasons these articles were excluded. The search also used other systematic reviews to find articles that did not appear from the search strategy that has been conducted. A search using another systematic review resulted in an additional 1 article. This systematic review included a total of 14 articles (Figure 1).

## Research design

Ten studies used a randomized intervention design [22–31]. Four studies used a nonrandomized intervention design [32–35]. The sample size in each article varied from 45 to 998 patients with diabetes mellitus.

## Characteristics of intervention

Six studies used a blend of face-to-face social media intervention [23–27, 31]. Three studies that used a blend of face-to-face social media intervention were able to improve treatment behavior outcomes in diabetes mellitus patients [23, 25, 26], but only one study was able to decrease HbA1c

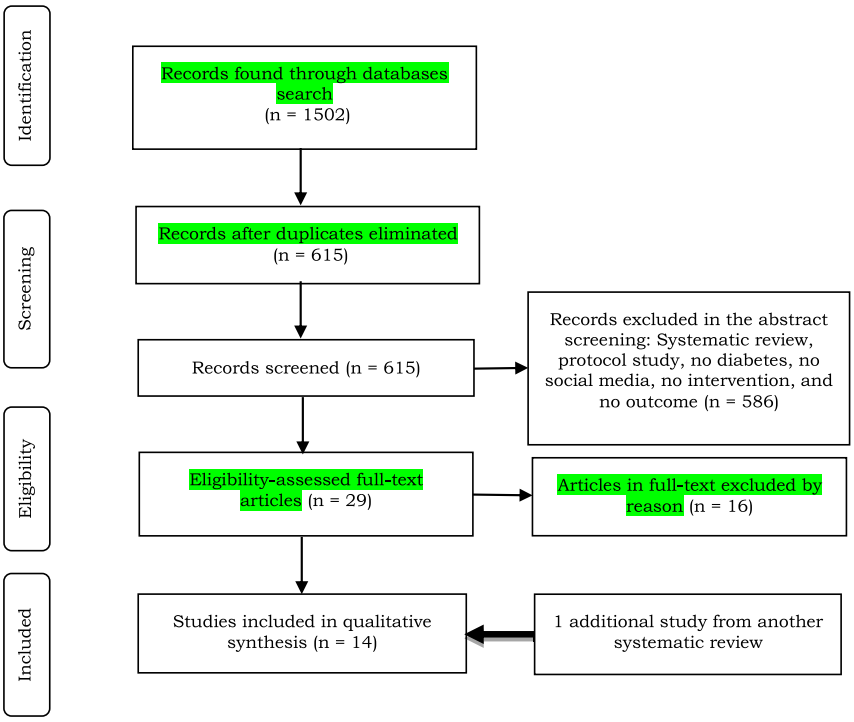


**Figure 1:** Flowchart of study selection.

[26]. Two studies used a blend of social media and telephone interventions [32, 33]. Both studies were able to significantly reduce $HbA_{1c}$ levels. Social media as the main intervention method were used in six studies [22, 28–30, 34, 35]. Two studies succeeded in improving treatment behavior outcomes [22, 34] and only one study was able to improve $HbA_{1c}$ value [30].

Communication using social media was carried out both privately between individual patients and the health care provider and in the form of a group containing the health care provider and all patients who became the study samples. The social media used as the medium of providing intervention were WhatsApp [22, 23, 33–35], Telegram [24], WeChat [26, 30, 31], Facebook [25, 29], a combination of Facebook and Skype [28], and particular social media application [27, 32]. Ten studies used two-way communication between health care providers and patients [22, 24–27, 29–31, 33, 34]. The remaining 4 studies used one-way communication from health care providers to patients [23, 28, 32, 35] (Table 1).

The most frequent communication with social media by health care providers was once per week [22, 25–27, 30, 33, 34], twice per week [32], every day [24], and once per month [23, 29, 31]. The more intensive the communication frequency between the practitioner and the individual patient, the more health outcomes will be achieved. The duration of use of social media for health interventions in patients performed was less than or up to 3 months [22, 24, 25, 27, 30, 32, 34, 35], 6 months [33], and more than 6 months [23, 26, 28, 31].

### Health outcomes

One study had treatment behavior outcomes with improvement in treatment behavior [22]. Six studies had clinical outcomes [28–33], but only four of them had improvement in $HbA_{1c}$ value [30–33]. Six studies had treatment behavior outcomes and clinical outcomes [23–27, 34], but only one study had improved treatment behavior and clinical outcomes [26], meanwhile, three studies had improved treatment behavior outcome only [23, 25, 34]. One study had medication adherence outcome, but no improvement in medication adherence [35].

## Discussion

This review aims to assess the use of social media online as a medium of intervention to improve the health outcomes of diabetes mellitus patients and to see its impact on health outcomes. The research that was included in this systematic review used randomized intervention and non-randomized intervention. The study used two sample groups, by which the treatment group sample received an intervention which implementation involved the use of

**Table 1:** Studies meeting inclusion criteria.

| References | Research designs | Types of intervention | Types of social media | Duration of intervention | Direction of communication | Sample size | Results and discussion |
|---|---|---|---|---|---|---|---|
| Alanzi et al. [22] | Randomized intervention | Social media | WhatsApp every week | 2 months | 2 directions | 84 patients | Treatment behavior outcome: The level of knowledge and self-efficacy of the post-study of the intervention and control groups was different. ($p<0.001$). |
| Alghafri et al. [23] | Randomized intervention | Face to face and social media | WhatsApp every month | 12 months | 1 direction | 174 patients | Treatment behavior outcome: The increase in physical activity scores in the intervention and control groups was different ($p=0.003$). Clinical outcome: The decrease in HbA1c, cholesterol, LDL, and HDL levels of the two groups was not different ($p>0.05$), but was different in triglycerides and blood pressure ($p<0.05$). |
| Aligholipour 2019 [24] | Randomized intervention | Face to face and social media | Telegram every day | 3 months | 2 directions | 63 patients | Treatment behavior outcome: The change in the intervention group's self-care score and the control group was not different ($p=0.24$). Clinical outcome: The decrease in the level of FBG between the two groups did not differ ($p=0.09$) |
| Bender et al. [25] | Randomized intervention | Face to face and social media | Facebook every week | 3 months | 2 directions | 45 patients | Treatment behavior outcome: The change in body weight in the treatment and control groups was different ($p=0.001$). Clinical outcome: The decrease in HbA1c and FBG of the two groups was not different |
| Berman et al. [32] | Nonrandomized intervention | Phone and social media | Unspecific social media every 2 weeks | 3 months | 1 direction | 101 patients | Clinical outcome: After the intervention, there was a significant decrease in the level of HbA1c ($p<0.001$). |
| Döğer 2019 [33] | Nonrandomized intervention | Phone and social media | WhatsApp every week | 6 Months | 2 directions | 82 patients | Clinical outcome: In the group with rare communication, there was an insignificant decrease in HbA1c level ($p=0.172$) and was significant in that group that often communicates ($p<0.001$). |

**Table 1:** (continued)

| References | Research designs | Types of intervention | Types of social media | Duration of intervention | Direction of communication | Sample size | Results and discussion |
|---|---|---|---|---|---|---|---|
| Dong et al. [26] | Randomized intervention | Face to face and social media | WeChat every week | 12 months | 2 directions | 119 patients | Treatment behavior outcome: The total post study self-efficacy score of the treatment and control groups was different ($p<0.05$). Clinical outcome: In the post study, there was a difference in HbA1c levels in the two groups ($p<0.05$), but there were no differences in FBG and PBG levels ($p>0.05$). |
| Kim and Utz [27] | Randomized intervention | Face to face and social media | Unspecific social media every week | 2 months | 2 directions | 151 patients | Treatment behavior outcome: After follow-up in the three groups, there was no difference in the score for self-care behavior ($p=0.581$). Clinical outcome: There was no difference in HbA1c level after the follow-up in the three groups ($p=0.840$). |
| Petrovski et al. [28] | Randomized intervention | Social media | Facebook and Skype | 12 months | 1 direction | 56 patients | Clinical outcome: In the intervention and control groups, there was no difference in the reduction in HbA1c levels. |
| Petrovski and Zivkovic [29] | Randomized intervention | Social media | Facebook once a month | NR | 2 directions | 67 patients | Clinical outcome: The reduction in the levels of HbA1c in the intervention and control groups did not differ. |
| Sap et al. [34] | Nonrandomized intervention | Social media | WhatsApp every week | 2 months | 2 directions | 52 patients | Treatment behavior outcome: The increase in knowledge score about DM disease and its therapy in the intervention and control groups differed significantly Clinical outcome: The change in HbA1c level in the two groups was not different ($p=0.99$). |
| Sartori et al. [35] | Nonrandomized intervention | Social media | WhatsApp | 3 months | 1 direction | 499 patients | Adherence outcome: The proportion of patients adhering to taking medication in the treatment and control groups was not different ($p>0.05$). |
| Zhang 2018 [31] | Randomized intervention | Face to face and social media | WeChat every month | 24 months | 2 directions | 998 patients | Clinical outcome: The proportion of patients with post study levels of HbA1c, LDL, and normal blood pressure was different in the treatment and control groups ($p<0.01$), but for a BMI of 25kg/m$^2$, it was not different ($p=0.38$). |

**Table 1:** (continued)

| References | Research designs | Types of intervention | Types of social media | Duration of intervention | Direction of communication | Sample size | Results and discussion |
|---|---|---|---|---|---|---|---|
| Chen et al. [30] | Randomized intervention | Social media | WeChat every week | 3 months | 2 directions | 90 patients | Clinical outcome: The level of HbA1c, FBG, and PPG in the post study group were different in the treatment and control groups ($p<0.05$). |

DM, diabetes mellitus; PPG, post prandial blood glucose; FBG, fasting blood glucose; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NR, not reported.

social media and the control group only received usual care [22–31, 34, 35].

Face-to-face health care is supported by online social media and health care online social media only as the main medium of intervention without face-to-face are able to improve treatment behavior and clinical outcomes [22, 23, 25, 26, 30–34]. These findings indicate that the application of online social media as the main and supporting medium of providing health care interventions has a positive impact on treatment behavior and clinical outcomes of diabetes mellitus patients.

Even though the health care provided by health care providers to patients face-to-face is quite effective, constraint in the form of too short communication time hinders the achievement of more optimal health outcomes [36, 37]. Patients often have difficulty in understanding too much information provided by health care providers at one time. This occurs in chronic diseases patients such as diabetes mellitus, the treatment of which is lifelong and complex [38, 39]. Social media can be used as an alternative medium of providing health care because it can increase the intensity of communication between health care providers and patients [40–42]. The more intensive the communication is, the greater the patient's involvement in disease management and treatment is. This intensive communication also makes it possible to achieve more optimal health outcomes [38, 43, 44]. Patients who have more intensive communication with health care providers have better treatment behavior and decreased $HbA_{1c}$ levels than those who rarely communicate with health care providers [22, 26, 30, 33].

The providing of health care by application of social media online as a medium to diabetes mellitus patients is a promising thing [14, 45, 46]. However, the providing of health care by application of social media online has not been utilized optimally by health care providers. It is important to empower health care providers with the ability to use social media and the ability to communicate effectively and efficiently online [16].

The review was conducted with a broad approach, and comes to include all studies that discuss interventions to improve the health outcomes of patients with diabetes mellitus using social media online. The studies included took place in different settings and that evaluated the effectiveness of different types of interventions and outcomes, which increased the level of heterogeneity of the results. However, the authors have done their best to ensure proper identification of intervention and outcomes components using the information provided.

## Summary and outlook

Most of the online social media interventions identified in this review were able to influence treatment behavior and clinical outcomes. Online social media has the ability to be used as a source of support in the providing of health care. The application of online social media in providing health care should be considered by health care providers to improve the health outcomes of patients with diabetes mellitus.

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I Komang Prawira Nata Nugraha, Anita Purnamayanti*, I Gusti Ngurah Made Suwarba and Nani Parfati

# Developing pharmacokinetics–pharmacodynamics model of valproic acid syrup based on prediction of population pharmacokinetics parameter and seizure frequency in Indonesian pediatric epilepsy outpatients

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**Abstract**

**Objectives:** Valproic acid (VPA) is a broad-spectrum antiepileptic drug with known efficacy profile in pediatric patients, despite of its narrow therapeutic index. There is lack of VPA's pharmacokinetics profile in Indonesian pediatric subjects, partly due to limited pediatric blood volume taken for conducting therapeutic drug monitoring. This study aimed to determine the correlation between VPA pharmacokinetics parameters based on population data and seizure frequency in pediatric epilepsy outpatients.

**Methods:** This observational study was conducted at Sanglah General Hospital during June–December 2019. The subjects of this research were 38 pediatric epilepsy patients who adhered to VPA syrup monotherapy for at least 3 weeks. Five subjects randomly selected for blood sample collection. Thus, VPA concentration level in the blood being analysed as a comparison to its concentration predicted from Yukawa's steady state equation. Monolix2019R2® software was used to identify VPA population pharmacokinetics–pharmacodynamics (PK–PD) parameters at steady state level.

**Results:** Population PK–PD of VPA syrup at steady state level were ka_pop = 6.25/h, Vd_pop = 3.36 L, Cl_pop = 3.17·$e^{-11}$ mL/min, $IC_{50}$_pop = 1.85·$e^{-6}$, correlation of Vd_pop and Cl_pop = 0.966. Kendall Tau Correlation of predicted VPA steady state concentration and frequency of seizure was –0.66. Mean prediction error between predicted steady state concentration of five subjects and their related blood levels was ≤25% and considered as within clinically acceptable limit.

**Conclusions:** It needs further study to develop best matched PK–PD model of VPA syrup at steady state condition in pediatric epilepsy.

**Keywords:** pediatric; population pharmacokinetics; prediction of concentration; seizure frequency; valproic acid.

## Introduction

According to International League Against Epilepsy (ILAE), Commission on Therapeutic Strategies, situations in which anti epilepsy drug (AED) measurements are most likely to be of benefit include to guide dosage adjustment in situations associated with increased pharmacokinetic variability (e.g., children, the elderly, patients with liver or renal impairment, and drug formulation changes) [1].

Pediatric is a special population group associated with increased pharmacokinetics variability, as well as drug toxicities due to limited capability of the developing physiological system. Pediatric epilepsy patients rely on their parents in administering the medicines for treating their disease. Valproic acid (VPA) is a broad-spectrum first line antiepileptic therapy in pediatric which means it can be used in almost all type of epileptic seizures due to its well establish efficacy. Indeed, VPA has a narrow therapeutic

***Corresponding author: Anita Purnamayanti**, Department of Clinical Pharmacy, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia, Phone: +62 81336444888,
E-mail: anita_p_rahman@yahoo.com. https://orcid.org/0000-0003-4544-7052
**I Komang Prawira Nata Nugraha,** Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia
**I Gusti Ngurah Made Suwarba,** Department of Paediatric Health, Sanglah General Hospital, Denpasar, Indonesia
**Nani Parfati,** Department of Pharmaceutics, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia

index and high protein binding, which means discrepancies between the minimum concentration of VPA to be able to effectively act as AED with its minimum toxic concentration level is relatively near. Thus pediatric patients have increased risk of sub- or supra-optimal VPA therapy [2, 3].

Ideally it is necessary to apply AED measurement, also known as therapeutic drug monitoring (TDM). Blood samples should be taken from the subjects to identify AED concentration, as well as pharmacokinetics parameter in specific clinical conditions; in order to analyzed inter- and intra-subject variability that could lead to sub- or supratherapeutic concentration of AED. Subtherapeutic concentration means that there was no or minimal effect of the drug, while supratherapeutic one means that there was increased risk of toxicity of AED therapy. The main objective of pharmacokinetic monitoring of antiepileptic drugs is to optimize treatment by studying drug levels in biological matrices. Inter- and intra-subject variability in blood VPA concentration is not uncommon in pediatric epilepsy. Adapting individual doses is complicated, due to the presence of factors including: (a) the considerable interindividual pharmacokinetic variability of antiepileptic drugs; (b) the use of these drugs as prophylactics for long-term epileptic seizure control, and (c) having no defined correlation between efficacy and a biological marker that could help with decision-making [3, 4].

Incidence epilepsy in pediatric patients remains high in Sanglah General Hospital Bali, Indonesia. There were 276 incidences of epilepsy with average of 69 cases per year, mostly in age group less than one year (46.0%) during period of January 2007–December 2010 [5]. There is lack of pharmacokinetics data population for AEDs in Indonesian pediatric epilepsy as guidance in individualizing dosage regimens. There are several obstacles in performing TDM to pediatric patients, for example limited blood volume, technically difficult in accessing the pediatric vein, and patient discomfort. One of the strategies to overcome these limitations is to calculate the individual blood concentration prediction from the equation derived from population pharmacokinetics data [6–9]. Yukawa et al. conducted pharmacokinetics–pharmacodynamics (PK–PD) study of VPA administered to pediatric patients and developed an equation for calculating the clearance of VPA from population data [7]. In this research, we used Yukawa's equation for calculating VPA clearance, due to similarities in research design which is conducted for assessing VPA syrup in its steady state condition among pediatric epilepsy patients. Aim of this research was to analyze the estimated concentration of VPA and its correlation with seizures frequency in pediatric outpatients. This research was aimed to determine the correlation between VPA pharmacokinetics parameters based on population data and seizure frequency in order to develop PK–PD analysis in the future research. As far as our knowledge, this is the first PK–PD study of VPA syrup in the steady state condition in Indonesian pediatric epilepsy subjects.

## Materials and methods

The design of this research was as an observational study conducted prospectively. All patient were treated in Sanglah General Hospital – Bali, Indonesia from June to December 2019, and fulfilled all of the following inclusion criteria: (1) aged 6–18 years, (2) had been receiving VPA syrup monotherapy for at least 3 weeks before the research, (3) had not discontinued administration of VPA because of the side effect, (4) were not take any drugs that might alter clearance of VPA, and (5) patients and their family was willing to be involved in this research. We excluded pediatric patients with abnormal liver and renal function tests. This research complies with all national relevant regulations and had been given ethical clearance from Ethics Committee of Faculty Medic and Health Sciences, Udayana University/Sanglah General Hospital.

Recruited subjects were followed every 2 weeks on their visit schedule to Outpatient Department. We performed twice weekly oral and written education session starting on the day of recruitment up to one month in order to improve concordance between subject's parents and health professionals, thus to maintain VPA monotherapy adherence. We supplemented written education material (booklet) for each subjects. Subjects had been asked to bring their VPA syrup bottle in every visit, and its volume measurement conducted by comparing the volume left in the subject's bottle to a standardized scaled VPA syrup bottle. All of the subjects included in this research had 100% VPA syrup adherence during this stage of research, so that drug adherence was not the confounding factor.

Estimated steady state concentrations of VPA was calculated based on pharmacokinetics population data using Yukawa's equation for VPA clearance as follow [7]:

$$\begin{aligned}\mathrm{Cl}\left(\mathrm{mL}\cdot\mathrm{kg}^{-1}\cdot\mathrm{h}^{-1}\right) = {} & 18.9\times\text{body weight (kg)}^{-0.276}\\ & \times\text{VPA daily dose}\left(\mathrm{mg}\cdot\mathrm{kg}^{-1}\cdot\mathrm{day}^{-1}\right)^{0.142}\\ & \times\text{gender (which is 1 for male, and 0.887 for female)}\end{aligned} \quad (1)$$

Predicted VPA steady state concentration of each subjects then was calculated from this estimated clearance, thus the results were so called "predicted VPA steady state concentrations based on population data". Correlation of the predicted valproic acid steady state concentration based on population data with the seizure frequency as a pharmacodynamics parameter of VPA's efficacy in seizure control was analyzed. Five subjects were then randomly selected to follow therapeutic drug monitoring procedure. Blood samples had been taken after the third visit to Outpatient Department and were analyzed as the subject's steady state plasma concentration of valproic acid and compared it to its predicted VPA steady state concentrations based on population data. The comparative results between predicted VPA steady state concentrations based on population data with the VPA plasma concentration of the five subjects randomly selected for blood sampling were further calculated as prediction error (PE) and weighted prediction error (WPE) thus tested

them for biases. Monolix2019R2® software was used to process pharmacokinetics and pharmacodynamics parameters based on population data as fixed effects. Parameters of PK–PD derived from fixed effects modelling using Monolix2019R2® software comprise of pharmacokinetics parameters of population clearance (Cl_pop), population volume of distribution (V_pop), population absorption rate constant (ka_pop); as well as pharmacodynamics parameter of 50% inhibition concentration ($IC_{50}$), and population inter individual deviation (lambda0pop).

## Results

A total of 42 potential subjects (6–14 years old) in the September–December 2019 period were recruited; and 38 out of 42 subjects had been included in this research (Figure 1). The subjects came from difference tribes in around Indonesia, for example from Java, Bali, East Nusa Tenggara, as well as Chinese descent ethnicity. The average body weight of 38 subjects was 32.32 ± 16.05 (14–76 kg), the total dose of valproic acid syrup administered was 19.55 ± 6.19 (8–37 mg/kg body weight/day). There were a great intersubject variability in the mean predicted valproic acid steady state concentration of 38 subjects which was 74.87 ± 18.06 (43.89–121.51 mg/L); in comparing to serum valproic acid steady state concentration of five subjects which was 104.20 ± 36.05 (57.00–138.00 mg/L). Thirty six subjects had no seizure at all and one subject experienced one seizure per month at the end of research period, in comparing to 3–10 seizures per month at the baseline; another subject constantly had seizures as frequent as in the beginning of research. The subjects' characteristics at baseline and the end of the research period were presented in Table 1. The result of Wilcoxon sign rank test showed that there was statistically significant median differences between frequency of seizure per month at baseline form and at end of this research (p=0.04). The correlation between predicted valproic acid steady state concentration of 38 subjects and their frequency of seizures was calculated with Kendal Tau test. The results of the Kendall's Tau test was the predicted VPA steady state concentration of 38 subjects and their frequency of seizures had a very weak association (r=–0.067), in contrast to the correlation coefficient of five subjects and their frequency of seizures that had a strong association (r=–0.62).

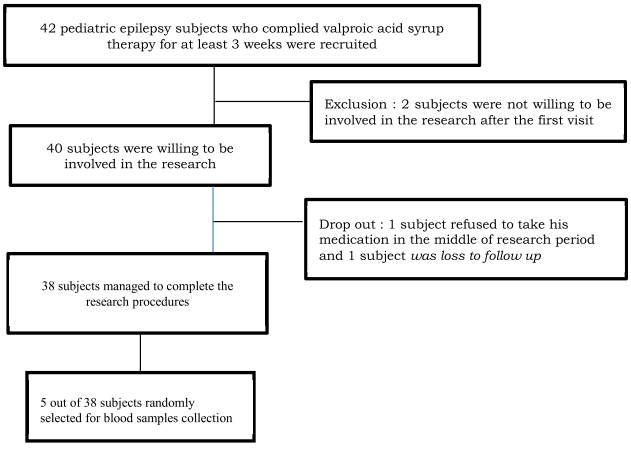


**Figure 1:** Patient selection process.

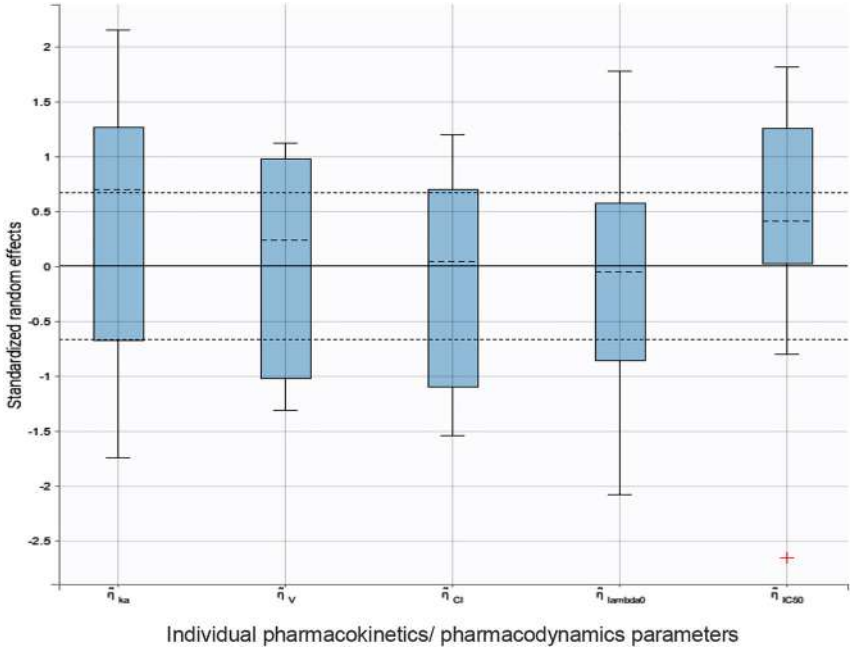


**Figure 2:** Random effects of pharmacokinetics and pharmacodynamics parameter of valproic acid syrup.

**Table 1:** Adherence to valproic acid syrup and the seizure frequency at the baseline and the end of the research period.

| Drug adherence, % | No. of the subjects | Percentage, % | Baseline seizures, times/month | No. of the subjects | Percentage, % | Seizures during the research period, times/month | No. of the subjects | Percentage, % |
|---|---|---|---|---|---|---|---|---|
| 100 | 38 | 100.00 | 3 | 23 | 60.53 | 0 | 36 | 94.74 |
| | | | 5 | 14 | 36.84 | 1 | 1 | 2.63 |
| | | | 10 | 1 | 2.63 | 10 | 1 | 2.63 |
| Total | 38 | 100.00 | | 38 | 100.00 | | 38 | 100.00 |

Population PK–PD parameter estimates based on predicted vs. serum VPA steady state concentration were performed using SAEM algorithm of Monolix2019RA® software (Figure 2). Correlation value between VPA's volume of distribution (Vd_pop) and clearance (Cl_pop) of 38 subjects estimates by Monolix2019R2® software was corr_V_Cl = 0.23, which means there was a weak correlation between Vd_pop and Cl_pop of 38 subjects. Correlation between valproic acid's volume of distribution and clearance of five subjects estimates by Monolix2019R2® software was corr_V_Cl = 0.97, which means there was a strong correlation between Vd_pop and Cl_pop of five subjects. This correlation coefficient was markedly improved in five subjects, comparing to its correlation in 38 subjects (Tables 2 and 3).

**Table 2:** Population pharmacokinetics–pharmacodynamics parameter estimates from 38 subjects adhered to valproic acid syrup monotherapy at steady state level using SAEM algorithm of Monolix2019RA® software.

| Parameter | Value of fixed effects | | Standard deviation of random effects |
|---|---|---|---|
| ka_pop | $2.18 \cdot e^{3}$ | Omega ka_pop | 2.32 |
| V_pop | 3.78 | Omega V_pop | 0.30 |
| Cl_pop | $3.19 \cdot e^{-15}$ | Omega Cl_pop | 1.55 |
| Lambda0 | 3.94 | Omega lambda0 | 0.32 |
| $IC_{50}$ | $7.29 \cdot e^{-6}$ | Omega $IC_{50}$ | 6.7 |
| Correlation | | | |
| Corr_V_Cl | 0.23 | | |

ka_pop, estimated absorption rate constant based on population data (/hours); V_pop, estimated distribution volume which is hypothetical volume where drug dissolved and being distributed throughout the body based on population data (Liters); Cl_pop, estimated drug removal from the certain volume of the blood per unit time based on population data; Lambda0, intersubject variability; $IC_{50}$, amount of concentration which needed to controlled of seizure in 50% subjects; Corr_V_Cl, correlation coefficient between distribution volume and its clearance of the drug; Omega_ka_pop, standard deviation of random effect of absorption rate constant; Omega_V_pop, standard deviation of random effect of volume of distribution; Omega_Cl_pop, standard deviation of random effect of clearance; Omega_lambda0, standard deviation of random effect of lambda0; Omega_$IC_{50}$, standard deviation of $IC_{50}$.

The comparison of predicted valproic acid steady state concentration derived from Yukawa's clearance equation and its plasma concentration of five subjects showed that 60% prediction in three subjects were closed to its related

**Table 3:** Population pharmacokinetics–pharmacodynamics parameter based on valproic acid concentration in the blood of five randomly selected subjects adhered to valproic acid syrup monotherapy at steady state level using SAEM algorithm of Monolix2019RA® software.

| Parameter | Value of fixed effects | | Value of standard deviation of random effects (between-subject variability) |
|---|---|---|---|
| ka_pop | 6.25 | Omega ka_pop | 1.03 |
| V_pop | 3.36 | Omega V_pop | 0.32 |
| Cl_pop | $3.17 \cdot e^{-11}$ | Omega Cl_pop | 9.68 |
| Lambda0 | 2.81 | Omega lambda0 | 0.09 |
| $IC_{50}$ | $1.85 \cdot e^{-6}$ | Omega $IC_{50}$ | 4.46 |
| Correlation | | | |
| Corr_V_Cl | 0.97 | | |

ka_pop, estimated absorption rate constant based on population data (/hours); V_pop, estimated distribution volume which is hypothetical volume where drug dissolved and being distributed throughout the body based on population data (Liters); Cl_pop, estimated drug removal from the certain volume of the blood per unit time based on population data; Lambda0, intersubject variability; $IC_{50}$, amount of concentration which needed to controlled of seizure in 50% subjects; Corr_V_Cl, correlation coefficient between distribution volume and its clearance of the drug; Omega_ka_pop, standard deviation of random effect of absorption rate constant; Omega_V_pop, standard deviation of random effect of volume of distribution; Omega_Cl_pop, standard deviation of random effect of clearance; Omega_lambda0, standard deviation of random effect of lambda0; Omega_$IC_{50}$, standard deviation of $IC_{50}$; Fixed effect, the model which assume that the true effect size for all studies is identical, and the only reason the effect size varies between studies is sampling error (error in estimating the effect size); Random effect, the model which assume that the true effect size for all studies is not identical. Each of the study might has a different effect size. The estimate provided by small study may be imprecise, but it is information about an effect that no other study has estimated.

plasma concentrations, and there were only one subject with higher and one subject with lower predicted concentration (20%, respectively). We then calculated the mean PE as well as mean weighted prediction error (MWPE) with a cut off =25% deviation from standard deviation (SD) between the predicted VPA steady state concentration and its plasma concentration. The value of mean difference was 5.11 (CI 95% – 11.1173–21.3414; p=0.431) which means that bias effect of mean PE had a low grade of bias. The value of mean WPE was 8.20 (CI 95% – 17.4637–33.8637; p=0.425) which means that the bias effect of mean WPE had a low grade of bias.

## Discussion

Pediatric patient is a special care group which needs special attention due to the great variability in their physical conditions. Children grow up fast physically as well as hormonally from the newborn up to adolescent, despite of suboptimal physiology of most of their organs. This conditions leads to variability in pharmacokinetics and therapeutic response profile to numerous drug administered to the pediatric patient – in comparation to the adults. Thus knowledge of PK–PD properties of medication used to treat pediatric patients is utmost important [1, 2]. Previous study at Sanglah General Hospital showed a relatively high incidence of epilepsy in children and the needs of drug information services assisted by pharmacist in order to reach concordance between parents and the healthcare professionals. Parental concordance to their children therapy will further lead to drug adherence and compliance, thus it will improved clinical outcome – which in the case of valproic acid represents by its seizure control [10].

Underlying brain disorders and structural abnormalities, type of seizure, etiology, genetics, physicochemical properties of drug therapy, and drug compliance are factors that may affect seizure outcome. These factors could not fully explain wide variability of seizure outcome demonstrated in pediatric epilepsy patients. Wide variability of response to anticonvulsant therapy is not uncommon – especially in relation to older generation of antiepileptic drugs, such as valproic acid [10, 11]. Parents role is important in administering anticonvulsant therapy to their children who suffered from epilepsy. Thus this research controlled this potential bias with maintaining parental adherence to drug dosage regimen giving to their epilepsy children over a period of 1 month, so that there was 94.74% seizure free subjects at the end of research period. In general there always be difficult to control seizure in about 40% of the epilepsy patients, despite of their compliance to take the antiepileptic drugs [1]. This emphasized the importance of pharmacists role in improving parents' knowledge and behavior to adhere in administration of VPA syrup to their children. Inter professional collaboration between healthcare professionals as a solid healthcare team in maintaining chronic drug therapy adherence and to improve clinical outcome, especially in younger children, is utmost important [12, 13].

The result of this research was population PK–PD parameters which derived from multi ethnicity of the subjects voluntary participating in this research. The subjects referred to Sanglah General Hospital which serves as tertiary care for patients in Central Indonesia. However, the results cannot be considered as national representation of Indonesian pediatric epilepsy due to limited number of research subjects. Further research should be conducted as multicenter study across Indonesia in order to represent national population. As far as our knowledge, this is the first VPA PK–PD research in pediatric epilepsy patient conducted in Indonesia. So, the results of this study are important data to guidance clinical judgement of dosing adjustment in selected pediatric epilepsy at Sanglah General Hospital in the near future; which guidance had never existed before.

Population PK–PD parameter was successfully determined using free access of Monolix2019R2® software (Tables 2 and 3). Clearance of the drug and volume of distribution at steady state are considered to be the primary pharmacokinetic measurements obtained from *in vivo* experiments. Steady state level is a state where concentration of the drug is maintained with the lowest fluctuation between the minimum and its maximum level. In that situation the effect of the VPA is stabilized, and optimal as the steady state concentration maintained in the minimum effective concentration (MEC) range of 50–100 mg/L. Ninety nine percent of drug concentration after multiple dose therapy will normally reach the steady state level in around seven times the drug's half-life ($t_{1/2}$, 6–8 h in pediatric patients receiving VPA monotherapy); which was 42–56 h [13]. By ensuring drug adherence for at least one month, we convince that valproic acid steady state level had been achieved. In this research, pharmacokinetics parameters predicted under steady state level; which means that the concentration of valproic acid in the blood was constant at all the time. This phenomenon is due to the equal rate of drugs absorbed from its absorption site into the blood circulation and the rate of drugs distributed from the blood vessels into the tissue. This reflected the pharmacodynamics effect of valproic acid, which is its efficacy in controlling the seizures. In this research there was no sign or symptom of valproic toxicity during the research

period. This is clinically important, due to there is no need to adjust the dosage, once the steady state level is achieved in the MEC range.

Relatively small volume of distribution of VPA determined in this research means that there was a greater concentration of VPA in the plasma bound to albumin than the unbound VPA in the tissue. As a small free fatty acid, VPA is largely hydrophobic, imparting efficient entry to the central nervous system (CNS) with good oral bioavailability. Approximately 90% VPA binds to albumin in the blood vessels, with the unbound fraction increasing linearly from approximately 10% at concentration of 50 mg/L up to approximately 30% at 200 mg/L [14]. Instead, small concentrations of unbound VPA in the CNS tissue which bound to its receptor was adequately inhibit the seizure. This finding was in concert to the relatively small of 50% inhibition concentration ($IC_{50}$) resulted in this research, which was the smallest valproic acid concentrations needed to inhibit 50% of the seizure incident. The strong correlation (r=0.97) between VPA's volume of distribution with its clearance of five subjects randomly selected for determination of serum VPA concentration in this research reflected that the smaller concentration of VPA in CNS tissue at any particular time would distributed back into the systemic circulation which then underwent elimination from the blood VPA mostly cleared from the body through hepatic metabolism [15].

Our research shared similarity with Yukawa study in the subjects' profile, including sex and age of the subjects, as well as average body weight, dosage and predicted concentration of valproic acid at steady state condition. In this research we only managed to draw blood samples from five subjects, due to the limited time in every subject's visit to Outpatient Department. The comparison of predicted VPA steady state concentration derived from Yukawa's clearance equation and its plasma concentration of five subjects showed that there was one subject who had higher and one subject who had lower predicted concentration. Higher concentration potentially lead to higher risk of adverse events or toxicity, while lower one could lead to seizure attacks [16]. In fact, the five subjects had experienced no seizure attack at all during our research period, which means that the individual valproic acid concentration was in the optimum level for these pediatric subjects. Despite of these differences, the bias effect of mean PE and WPE were at low grade of bias [17]. Mean prediction error between predicted steady state concentration levels of five subjects and their related blood levels was ≤25% and considered as within clinically acceptable limit. According to Bondareva et al. (2011) the cut off ≤25% deviation is considered to be "clinically good"; while mean difference cut off ≤35% is considered as clinically acceptable [18]. Thus, the results of this research are robust enough to guide dosing adjustment of valproic acid syrup monotherapy in pediatric patients. The lack of PK–PD model of antiepileptic drugs in pediatric appears to hinder identification of suitable dosing regimens for pediatric patients with epilepsy [19]. Volume of distribution for Indonesian epilepsy pediatrics succesfully determined in this research. The volume of distribution at steady state is considered to be one of the primary pharmacokinetic measurements obtained from *in vivo* experiments [20]. Based on these results of the research, valproic acid syrup therapy administered to pediatric epilepsy patients safely managed to reach steady state level, thus effective in controlling the seizure without any adverse events; so that no dosage adjustment needed. In other word, the valproic acid syrup was a safe and effective therapy for pediatric epilepsy patients; thus the whole treatment was "on the right track".

Conducting an ideal therapeutic drug monitoring (TDM) is cost consuming, so this impractical to be developed in everyday practice. VPA concentration in this research measured in the blood as a total VPA, instead of unbound VPA; thus we could not determine the possibility of its level which out of MEC range. Sub therapeutic concentration would lead to less VPA's efficacy in controlling the seizure frequency. In contrast, the valproic acis concentration higher than MEC range would leads to toxic effect that could be life threatening. Difficult to control seizure in one subject who still experienced seizures 10 times per month at the baseline as well as at the end of this research period would probably due to VPA's sub therapeutic concentration.

Pharmacokinetics and pharmacodynamics parameters based on population data had successfully determined, but we have not developed a PK–PD modelling yet due to our research limitations. There were several limitations of the study. The design of this research was observational study with a limited number of subjects who were involved in the research, due to unwillingness of the potentially eligible pediatric epilepsy patients to performed blood sampling. We tried to overcome this limitation by randomly assigned blood sampling between the 38 subjects who managed to adhere to VPA therapy for one month. The other obstacle in this research was the limited blood volume of the pediatric subjects, so that we could not performed several blood sampling on the estimated peak and through concentration at steady state level. The last limitation was that the analysis procedures could only measure total VPA concentration, instead of unbound VPA; thus conducting a therapeutic drug monitoring is cost consuming to be performed in daily practice. Unbound VPA is reflecting the amount of VPA which can interact with the receptor in order to actively control the seizures. This limitation leads to less accuracy in

measuring VPA plasma concentration for pharmacokinetics parameters determination. These obstacles should be overcome in the future research.

## Conclusions

Developing PK–PD model of VPA syrup at steady state condition based on population data in pediatric epilepsy subjects had been undertaken, and some of the parameters were sets. It needs further study to develop best matched PK–PD model of VPA syrup at steady state condition in Indonesian pediatric epilepsy outpatient due to limitation of the study.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.
**Ethical approval:** Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors' institutional review board or equivalent committee.

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Suciati*, Debora Poerwantoro, Aty Widyawaruyanti and Kornkanok Ingkaninan

# Acetylcholinesterase inhibitory activity of extract and fractions from the root of *Rauvolfia serpentina*(L.) Bth.ex Kurz

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**Abstract**

**Objectives:** Alzheimer's disease (AD) is a degenerative brain disease characterized by confusion, behavior changes, decline in memory and cognitive skills. One of the strategies in the treatment of AD is to use acetylcholinesterase (AChE) inhibitors. The current study aims to determine the AChE inhibitory activities of the extract and fractions of the root of *Rauvolfia serpentina*.

**Methods:** Extraction was carried out by maceration method using ethanol, followed by liquid–liquid partition using *n*-hexane, ethyl acetate and *n*-butanol. Further fractionation was conducted by using vacuum liquid chromatography (VLC). The AChE inhibitory assays were performed by using Ellmann's method. Phytochemical screening was carried out by TLC method.

**Results:** The ethanolic extract of *R. serpentina* showed inhibition against AChE enzyme with an $IC_{50}$ value of 7.46 μg/mL. The extract and fractions showed higher inhibition against butyrylcholinesterase (BChE) compared to AChE. Amongst three fractions obtained, the *n*-butanol fraction showed the strongest inhibition with an $IC_{50}$ value of 5.99 μg/mL against AChE. VLC fractionation of the *n*-butanol fraction yielded 13 subfractions (VLC 1–VLC 13). Four out of 13 subfractions gave more than 80% inhibition against AChE, namely subfractions 4–7, with $IC_{50}$ values ranging from 4.87 to 47.22 μg/mL. The phytochemical screening of these subfractions suggested the presence of alkaloids.

**Conclusions:** The ethanolic extract, as well as fractions of *R. serpentina* root, are potential for AChE inhibitor. The alkaloid compound may be responsible for this activity.

**Keywords:** acetylcholinesterase inhibitor; Alzheimer's disease; *Rauvolfia serpentina*.

## Introduction

Alzheimer's disease (AD) is a degenerative brain disease characterized by confusion, behavior changes, decline in memory and cognitive skills. This disease has been recorded as the most common cause of dementia in the elderly population [1, 2]. AD often begin with minor symptoms and grow into severe brain damage or even death. The onset of AD usually occurs in population at the age of 65 years or above [3]. The increase in life expectancy means that the number of people suffering from AD is anticipated to increase each year if there is no effective medication found. The pathophysiology of AD is credited to several factors including the acetylcholine (ACh) deficiency due to neuronal loss in the brain. ACh is a neurotransmitter, produced in the nerve ending of the presynaptic nerve, which is associated with memory and cognitive functions. Therefore one of the target treatment of AD is the use of acetylcholinesterase (AChE) inhibitors, which can prevent the hydrolysis of ACh to choline and ethanoic acid [4]. There are three AChE inhibitors approved to be used by FDA for AD, namely galantamine, rivastigmine and donepezil. Donepezil is of a synthetic origin, while rivastigmine is a derivative developed from the natural compound physostigmine [5]. Galantamine is an alkaloid isolated from natural sources, *Galanthus nivalis* (Amaryllidaceae) [6]. The AChE inhibitors are beneficial to improve cognitive, functional and behavioral effects on AD patients. However, studies have shown the presence of several side effects and limited effectiveness of these medications [7, 8]. A sesquiterpenoid alkaloid Huperzine A, isolated from *Huperzia* spp., has also been reported as a potential

***Corresponding author: Suciati**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute of Tropical Diseases, Universitas Airlangga, Surabaya, Indonesia, E-mail: suciati@ff.unair.ac.id
**Debora Poerwantoro,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Aty Widyawaruyanti,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute of Tropical Diseases, Universitas Airlangga, Surabaya, Indonesia
**Kornkanok Ingkaninan,** Bioscreening Unit, Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand

therapy for AD. The product containing extract of *Huperzia* spp. has been commercialized as a food supplement for memory improvement in China [1].

Plants containing alkaloids have been the target of screening for AChE inhibitors. Recently, we have reported the potency of several *Cassia* spp. as well as several marine sponges as AChE inhibitor [9, 10]. In the current study, investigation on the AChE inhibitory activities of extract and fractions from the root of *Rauvolfia serpentina* was conducted. The root of *R. serpentina* possesses high therapeutic properties. It is traditionally used as a tranquillizer for nervous and mental disorders [11]. The root also provides high protein, starch and micronutrient which is useful for treating malnutrition [12–14]. More than 60 indole alkaloids have been reported from the root of *R. serpentina* [11, 15]. These alkaloids, especially reserpine and rescinnamine have an important role in the well-known hypotensive activity of this plant [11]. The presence of alkaloid compounds in this plant made this worth to be investigated for AChE inhibitory assay.

# Materials and methods

### Reagents

AChE from electric eel (AChE typeVI-S), horse-serum butyrylcholinesterase (BChE), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), and bovine serum albumin (BSA), tris buffer and galantamine were purchased from Sigma–Aldrich.

### Plant collection

The dried root of *R. serpentina* was obtained from the local market in Surabaya, East Java, Indonesia. The sample specimen (voucher number: PSR 06) was kept at the Institute of Tropical Diseases Universitas Airlangga. The identification of the plant material was conducted by Purwodadi Botanic Garden (Identification letter number: 814/IPH.06/HM/VIII/2019).

### Extraction and fractionation

The dried root was grinded to obtain powdered material. One kilogram of the powdered root was extracted with ethanol 96% by using the maceration method. The sample was submerged in the solvent (2 L) for 24 h, followed by filtration. The residue was re-extracted with ethanol using the same procedure three times. The filtrate was then evaporated in the rotary evaporator to obtain a crude ethanolic extract (18.23 g). A portion of the ethanolic extract (18.0 g) was dissolved with a mixture of ethanol and $H_2O$ (1:1) (220 mL), then further separated by a liquid–liquid partition with *n*-hexane (250 mL), ethyl acetate (250 mL), and followed by *n*-butanol (50 mL). Fractionation with each solvent was conducted three times. Each of the fractions obtained was evaporated *in vacuo*, and yielded *n*-hexane (3.61 g), ethyl acetate (6.80 g), and *n*-butanol (2.63 g) fractions. Further fractionation of the *n*-butanol fraction was conducted by using vacuum liquid chromatography (VLC). Sample (2.6 g) was mixed with silica gel (2.6 g) to obtain a dried powdered sample, which was then applied to the VLC. The sample was eluted with a combination of dichloromethane and methanol in order of increasing polarity, and yielded 13 subfractions namely VLC 1–VLC 13.

### Phytochemical screening

Samples were dissolved in ethanol. The solutions were applied on the TLC plate (Silical gel $F_{254}$), which was then developed with a combination of dichloromethane:methanol (1:3). The plate was visualized under UV 254 and 366 nm, followed by Dragendorff spray to indicate the presence of alkaloid.

### Cholinesterase inhibitory assay

The assay was performed according to the modified Ellman's method [9, 10, 16]. The solution of plant extract and fractions were made in methanol at a concentration of 10 mg/mL, which was then diluted to 1 mg/mL with 50 mM Tris buffer. The final test concentration is 100 µg/mL after the dilution of samples in the microplate well. Sample solutions were added to a 96-well microplate, followed by the addition of 1.5 mM ATCI or 1.5 mM BTCI (25 µL), 3 mM DTNB (125 µL), and Tris buffer (50 µL). The substrate was then hydrolyzed by the addition of 25 µL of 0.22 U/mL of either *Ee*AChE or BChE.The solutions were shaken for 30 s in a microplate reader (Bio-Tek Instrument, USA) before measurement. The product, 5-thio-2-nitrobenzoate, indicated by a yellow color, was measured at 405 nm every 5 s for 2 min. Every experiment was carried out in triplicates. Galantamine was used as a positive control, and 10% methanol was used as a negative control. For the $IC_{50,}$ measurement serial concentrations of the samples were prepared ranging from 0.2–200 µg/mL. The enzyme activity was calculated as a percentage of the velocity of the test sample, compared with that of the nontreated control. The inhibitory activity was calculated as:

$$\%\text{Inhibition} = \frac{(\text{mean velocity of control} - \text{mean velocity of sample})}{\text{mean velocity of control}} \times 100$$

### Data analysis

The 50% inhibitory concentration ($IC_{50}$) was determined using GraphPad Prism 7.04 software by plotting log concentrations as axis and % inhibition as ordinate. The inhibition data of the samples against AChE and BChE were analyzed using unpaired *t*-tests in GraphPad Prism 7.04.

# Results

The ethanolic extract of the root of *R. serpentina* showed inhibition against the AChE enzyme with an $IC_{50}$ value of 7.46 µg/mL. To investigate the selectivity against cholinesterase enzyme, the sample was also tested against both

AChE and BChE at a concentration of 100 µg/mL. The results shown in Figure 1 indicated that the extract inhibited both enzymes; however, the inhibition was higher against BChE compared to AChE. Likewise, the *n*-hexane, ethyl acetate, and *n*-butanol fractions also gave higher inhibition against BChE than against AChE enzyme (Figure 1). Amongst the three fractions, the *n*-butanol fraction gave the strongest inhibition against AChE with an $IC_{50}$ value of 5.99 µg/mL (Table 1). The determination of the $IC_{50}$ value was not conducted on the *n*-hexane fraction since it only gave low inhibition against the AChE enzyme.

Subfractionation of the *n*-butanol fraction by using VLC yielded 13 subfractions. All subfractions were subjected to AChE inhibitory assay. The results presented in Figure 2 showed that four subfractions, namely subfractions 4–7 gave more than 80% inhibition against the tested enzyme. The $IC_{50}$ values of these subfractions were also determined. The results (Table 2) showed that subfraction 5 gave the strongest inhibition against the AChE with $IC_{50}$ value of 4.87 µg/mL. Subfractions 4–7 were subjected to phytochemical screening by using TLC method. The results showed that after spraying with Dragendorff dye, dark orange color spots on a yellowish-orange background were observed. This suggested that subfractions 4–7 of *R. serpentina* roots contain alkaloid.

## Discussion

ACh is a neurotransmitter, produced in the nerve ending of the presynaptic nerve, that has been discovered to be involved in the pathogenesis of AD. This neurotransmitter plays an important role in memory and learning function [17]. In AD patients, the ACh that is released has a very short half-life due to the presence of large amounts of the cholinesterase enzymes, namely AChE and BChE [4, 18].

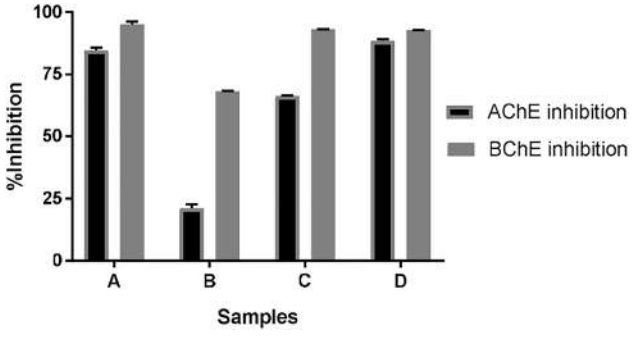


**Figure 1:** Inhibition of ethanolic extract (A), *n*-hexane (B), ethyl acetate (C), and *n*-butanol (D) fractions of *R. serpentina* root against AChE and BChE at 100 µg/mL.

**Table 1:** $IC_{50}$ values of extract and fractions of the root of *R. serpentina* against AChE.

| Samples | $IC_{50}$, µg/mL |
|---|---|
| Ethanol extract | 7.46 ± 0.28 |
| Ethyl acetate fraction | 23.62 ± 2.97 |
| *n*-Butanol fraction | 5.99 ± 0.86 |
| Galantamine | 0.63 ± 0.05 |

Data presented as mean ± standard deviation of three independent experiments, each done in triplicate.

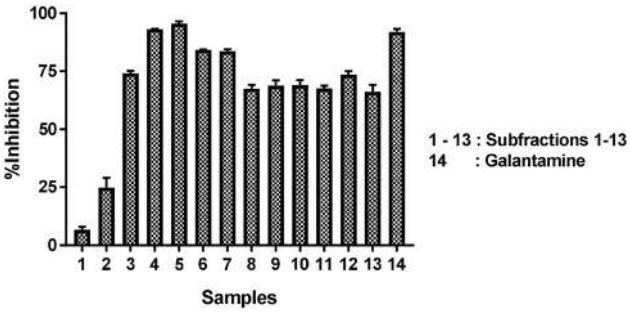


**Figure 2:** Inhibition of subfractions 1–13 of *R. serpentina* (100 µg/mL) and galantamine (100 µM) against AChE.

**Table 2:** $IC_{50}$ values of selected VLC subfractions of the root of *R. serpentina* against AChE.

| Samples | $IC_{50}$, µg/mL |
|---|---|
| Subfraction VLC 4 | 7.08 ± 0.53 |
| Subfraction VLC 5 | 4.87 ± 0.45 |
| Subfraction VLC 6 | 19.88 ± 1.58 |
| Subfraction VLC 7 | 47.22 ± 1.08 |

Data presented as mean ± standard deviation of three independent experiments, each done in triplicate.

These enzymes hydrolyze the ACh molecule into choline and ethanoic acid. In the healthy brain, AChE predominates, while BChE plays a minor role. However, in AD patients, the activity of BChE is increased progressively, while the activity of AChE remains unchanged [19]. The cholinergic strategy in the therapy for AD patients included stimulation of cholinergic receptors or increasing the availability of ACh by inhibiting AChE and BChE enzymes [8].

The current AChE inhibitors approved by FDA as medication for AD patients are donepezil, rivastigmine, and galantamine. Considering that all the three drugs are alkaloids that are synthetic or natural origins, searching on the new AChE inhibitor from natural sources has focused on the alkaloids, although other class of compounds, such terpenes, sterols, and flavonoids have also shown potency as AChE inhibitor [20].

Plant from the genus *Rauvolfia* has been known to contain alkaloids. The well-known compound reserpine and rescinnamine have been used as antihypertensive agents. In the present studies, we evaluated the anti-AD activity of the ethanolic extract as well as fractions of *R. serpentina* by measuring the inhibition against AChE and BChE. The extract as well as several fractions have shown strong inhibitions against both enzymes. The *n*-hexane fraction gave the lowest inhibition compared to the ethanolic extract, the ethyl acetate and the *n*-butanol fractions. These results indicated that the active compounds are semipolar to polar compounds. At the concentration of 100 μg/mL, the samples tested demonstrated higher inhibition against BChE compared to AChE enzymes. Analysis by using unpaired t-test, suggested that there were significant differences observed between the inhibition of extracts as well as *n*-hexane, ethyl acetate, and *n*-butanol fractions against AChE and BChE enzymes with p-value < 0.001 for *n*-hexane and ethyl acetate fractions and p-value = 0.003 and 0.004 for the ethanolic extract and *n*-butanol fraction, respectively.

Since the highest enzyme inhibition was given by the *n*-butanol fraction, further fractionation was conducted on this sample by using VLC. Thirteen subfractions were yielded and subjected to the AChE inhibitory assay. The results showed that subfractions 4–7 gave higher potency compared to other subfractions. These subfractions were eluted in a solvent combination of dichloromethane:methanol (85:15) to dichloromethane:methanol (60:40), which suggested that the active compounds are semipolar.

The phytochemical screening of subfractions 4–7 indicated the presence of alkaloid. *R. serpentina* has been known to contain more than 60 alkaloids in the root [11, 15]. Another plant from the same genus, *Rauvolfia reflexa*, has been reported to show inhibition against AChE enzyme. The methanol extract as well as the isolated indole alkaloids from *R. reflexa* demonstrated inhibition against both AChE and BChE enzymes [21]. Several indole alkaloids were found to be more selective against BChE compared to AChE [21, 22]. This suggested that indole alkaloids present in the root of *R. serpentina* may responsible for the cholinesterase inhibitory activity.

## Conclusions

The ethanolic extract as well as fractions from the root of *R. serpentina* inhibited cholinesterase enzymes. The alkaloid compounds in the extract and fractions may contributed to the AChE and BChE inhibition.

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Abdulloh Machin*, Imam Susilo and Djoko A. Purwanto

# Green tea and its active compound epigallocathechin-3-gallate (EGCG) inhibit neuronal apoptosis in a middle cerebral artery occlusion (MCAO) model

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**Abstract**

**Objectives:** To determine the effect of green tea with the active ingredient epigallocathechin-3-gallate (EGCG) on the inhibition of apoptosis in the middle cerebral artery occlusion (MCAO) model.

**Methods:** Four month old male *Rattus norvegicus* rats with a body weight of 200–275 g was used for the MCAO model and divided into five groups, and the treatment was carried out for 7 days. Before being sacrificed, the subject had 1 cc of blood drawn for high mobility group box 1 (HMGB-1) examination using enzyme-linked immunosorbent assay (ELISA), and after being sacrificed, the brain tissue specimen was taken to examine caspase-3 and B-cell lymphoma 3 (BCL-3) using immunohistochemistry methods.

**Results:** There was no significant difference in HMGB-1 results for the treatment group compared to the control group (P1: 384.20 ± 231.72 [p = 0.553]; P2: 379.11 ± 268.4 [p = 0.526]; P3: 284, 87 ± 276.19 [p = 0.140]; P4: 435.32 ± 279.95 [p = 0.912]). There is a significant increase in BCL-2 expression between the treatment group compared to the control group (P1: 2.58 ± 0.51 [p = 0.04]; P2: 3.36 ± 0.50 [p<0.001]; P3: 4.00 ± 0.42 [p<0.001]; P4: 3.60 ± 0.52 [p<0.001]). There was a significant difference in caspase-3 expression compared to the control group in the P3 group (P1: 4.33 ± 0.49 [p = 0.652]; P2: 4.09 ± 0.30 [p = 0.136]; P3: 3.58 ± 0.51 [p = 0.01]; P4: 3.89 ± 0.42 [p = 0.063]). There is no correlation between HMGB-1 and caspase-3 (r = –0.063; p = 0.613) or BCL-2 (r = –0.106; p = 0.396). There is significant negative correlation between caspase-3 and BCL-2 (r = –0.459; p = 0.000).

**Conclusions:** Green tea with the active ingredient EGCG can inhibit neuronal cell death through the apoptotic pathway and not through the activation of HMGB-1.

**Keywords:** BCL-2; Caspase-3; EGCG; green tea; HMGB-1; MCAO.

## Introduction

There is a decrease in blood flow during ischemic stroke that causes a decrease in adenosine triphosphate (ATP) production and causes lactic acidosis and homeostatic ion imbalance [1–3]. Homeostatic ion imbalance will cause calcium influx that will trigger phospholipase and protease and will damage cell membrane and protein [4, 5]. An increase in intracellular calcium will also trigger reactive oxygen species on mitochondria that will cause damage [6, 7].

High mobility group box 1 (HMGB-1) is a nuclear factor protein and a stress marker in the cells [8–10]. Some studies show that HMGB-1 has capability as a proinflammatory mediator that triggers inflammation during ischemia or sepsis [11–13]. HMGB-1 also increases during a pathologic condition and hours after ischemic injuries such as myocardial infarction or ischemic neuronal injury and cell death [13]. Some studies show that the HMGB-1 level increases after ischemic stroke, myocardial infarction, and hemorrhagic shock; thus, it plays an important role in the pathogenesis of ischemic stroke [8, 9, 14].

Ischemic injury can cause apoptotic cell death, and apoptotic cell death also occurs in stroke. The key mediator of apoptotic cell death is caspase-3 [15, 16]. Caspase-3 is the most well-characterized effector caspase and will trigger apoptosis when it is activated by other caspases [17]. Caspase-3 as an effector caspase plays a crucial role during neuronal development and under pathological conditions in the brain tissue such as cerebral ischemia, intracerebral hemorrhage, and brain injury. Caspase-3 is the most abundant cysteine

***Corresponding author: Abdulloh Machin**, Department Neurology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 813 3000 8306, E-mail: abdulloh.m@fk.unair.ac.id
**Imam Susilo,** Department Clinical Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Djoko A. Purwanto,** Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

aspartate expressed in adult rodent brains; thus, a change in its expression is easier to monitor [2]. Asahi et al. reported in his study that during 1 h after ischemic stroke there is upregulation of mRNA caspase-3 in the brain ischemic tissue that will increase caspase-3 expression. Namura et al. also detected caspase-3 and its product in mouse brain tissues during early reperfusion or 2 h after middle cerebral artery occlusion (MCAO) [16, 18, 19].

B-cell lymphoma 2 (BCL-2) was first identified in B-cell lymphoma, and it functions as a key regulator in the intrinsic apoptosis pathway in the mitochondria. BCL-2 also have function in the antiapoptotic pathway with B-cell lymphoma extra large (BCL-XL), myeloid cell leukemia 1 (MCL-1), and BCL-2-like protein 2 (BCL-2L2) with contour BCL-2 homology (BH) domain. BCL-2 is widely expressed during development in the brain including neuroepithelial cells and cerebral ventricle [20]. Study on primary neuronal cell culture and animal model shows that BCL-2 overexpression can protect neuronal damage and infarct size again N-methyl-D-aspartate (NMDA) induced excitotoxicity [21, 22]. Overexpression of BCL-2 also blocks apoptosis-inducing factor (AIF) and will improve cortical neuron survival after cerebral ischemia [22]. BCL-2 also acts as a key regulator of mitochondrial permeability and maintains mitochondria homeostasis. It is released in response to molecular-induced apoptosis and will prevent cell from apoptotic cell death. BCL-2 and BCL-XL protein are located in the mitochondria and endoplasmic reticulum. BCL-2 in the mitochondria maintains mitochondrial integrity and inhibits the release of proapoptotic molecule [20, 22, 23].

Green tea is the second most common drink in the world and has some beneficial effect because it has some polyphenol known as catechin and also has antioxidant property. Epigallocatechin-3-gallate (EGCG) is one of the green tea catechins and it makes up 63% of total catechin. EGCG is a potent antioxidant and a free-radical scavenger [24, 25]. Green tea can prevent neurodegenerative disease; a meta-analysis shows that people who drink more than three cups of tea a day have 21% less chance of having stroke than people who drink less than one cup a day [26]. Studies on animal models show that treatment using EGCG in an ischemic reperfusion brain tissue model decreases ischemic lesion [25, 27–29]. EGCG has free-radical scavenger function and can protect neuronal cell from oxidative damage induced by pro-oxidant agent [30]. Some animal studies show that EGCG increases mitochondrial function and decreases oxidative stress [31, 32]. Treatment with 30 mg/kg BW of EGCG can prevent isoproterenol-induced mitochondrial damage in the animal model [32].

Based on information above, green tea with its active compound EGCG may play an important role in cerebral neuroprotection because it has antioxidant and free-radical scavenger effect. It is important to conduct research to determine the effect of green tea treatment with its active compound EGCG on the level of HMGB-1 and expression of neuronal apoptosis marker in an MCAO model.

# Materials and methods

We performed an MCAO model using male *Rattus norvegicus* rats obtained from Gajah Mada University's breeding center that have been published on Eurasia J Biosci 14, 1813–1820 (2020). We evaluated the HMGB-1 level using enzyme-linked immunosorbent assay (ELISA) methods (LS Bio). On the 7$^{th}$ day, we obtained blood for serum ELISA examination.

### Immunohistochemistry examination

The brain tissue used is from a male *R. norvegicus* MCAO model and was obtained from the animal laboratory in the Faculty of Pharmacy, Universitas Airlangga. The tissue taken is the 1.5 cm area of the brain tissue in front of and behind the bregma and was then stored in paraffin blocks. The paraffin block was then cut as thick as 5 μm, and the slice was placed on the slide and heated at 65°C for 2 h so that the tissue can stick to the microscopic slide. The microscopic slides were dipped in xylene three times for 5 min each to remove paraffin. The tissue was rehydrated by dipping in 100% ethanol, then 95% ethanol, and then in 70% ethanol; then the microscopic slides were removed from 70% alcohol and then soaked in Tris buffer for 1 h. Peroxide activity is then removed by dipping in a 3% peroxide solution for 3 min. The microscopic slides were cleaned from Tris buffer for 3 min and given a conjugate enzyme after administration of the body and dissolved in Tris buffer saline with 1% bovine saline albumin and incubated at room temperature for 1 h, then give chromogen for 10 min at room temperature and rinse with running water for 5 min. The microscopic slide was read according to the guidelines of DC Allred, MD, et al. [33] by means of scoring consisting of proportion scores, with a score of 0 indicating no cells showed marker expression in all fields of view, a score of 1 when an expression of 0–1% was obtained in the field of view examined, a score of 2 when an expression of >1–10% was obtained in the field of view, a score of 3 when an expression of >10–33.3% was obtained, a score of 4 when an expression of >33.3–66.6% was obtained, and a score of 5 when a marker expression of >66.6–100% was obtained. Then, the intensity score is calculated, that is, a score of 0 if no restraint is obtained, a score of 1 if weakening is obtained, a score of 2 if moderate warming is obtained, and a score of 3 if strong warming is obtained. The final score is the proportion score added to the intensity score.

### Statistical analysis

All variables were tested descriptively, and then, normality was tested using the Kolmogorov–Smirnov test. Differences in expression

between the two groups were analyzed using the Kruskal–Wallis test, followed by the Mann–Whitney test. Furthermore, all variables were tested for correlation using the Pearson or Spearman test. Then, all variables were analyzed using path analysis.

## Results

We performed Immunohistochemical assessment for BCL-2 and caspase-3. We also performed ELISA for HMGB-1. There is a significant difference between all groups ($p > 0.01$), so we performed Kruskal–Wallis and Mann–Whitney tests to test the difference between groups (see Table 1).

Our data show that there is no significant difference between the control and intervention group with repect to the HMGB-1 level. According to our finding, neither EGCG nor green tea extract influenced the HMGB-1 level because of excessive release of HMGB-1 during acute ischemic stroke.

According to Table 2, the higher dose EGCG (30 mg/kg BW) and green tea extract (30 mg/kg BW) can suppress caspase-3 expression. This result shows that EGCG can inhibit apoptosis in the *R. norvegicus* MCAO model. There is a decrease in caspase-3 expression in the group treated with the lower dose of EGCG, but the decrese was not statistically significant. This result shows that inhibition of caspase-3 expression is dose dependent and significant inhibition of EGCG started at the dose of 30 mg/kg BW. Figure 1 shows expression of caspase-3 in neuronal cells after treatment with 30 mg/kg BW of EGCG.

According to Table 3, either EGCG or extract green tea can increase BCL-2 expression in the MCAO model. The intensity of expression of BCL-2 is dependent on dosage. Green tea extract also increases BCL-2 expression; the expression in the group treated with green tea extract is similar to that in the group treated with 30 mg/kg BW of EGCG. This result shows that either EGCG or green tea extract can increase antiapoptotic activity and that this activity depends on the dose of EGCG. Green tea extract also can decrease apoptotic activity, and this activity is similar to that of its active compound EGCG. Figure 2 shows expression of BCL-2 in neuronal cells after treatment using 30 mg/kg BW of EGCG.

According to Table 4, there is negative correlation between HMGB-1 and caspase-3, but is not statistically significant ($r = -0.063$; $p = 0.613$), and there is no statistically significant and negative correlation between HMGB-1 and caspase-3 ($r = -0.106$; $p = 0.396$). There is also significant negative correlation between BCL-2 and caspase-3 ($p = -0.459$; $p = 0.000$). These data show that the HMGB-1 level did not influence the expression of either caspase-3 or BCL-2. Correlation between caspase-3 and BCL-2 shows that EGCG can decrease caspase-3 expression and increase BCL-2 expression or in another

**Table 1:** Mean difference of HMGB-1 between the groups shows that there is no significant difference between intervention groups compared to the stroke control group.

| Group | Mean ± SD | p-Value |
|---|---|---|
| P0 (n = 10) | 1458.42 ± 329.11 | – |
| P1 (n = 12) | 384.20 ± 231.72 | 0.553 |
| P2 (n = 11) | 379.11 ± 268.47 | 0.526 |
| P3 (n = 12) | 284.87 ± 276.19 | 0.140 |
| P4(n = 10) | 435.32 ± 279.95 | 0.912 |

SD = standard deviation; HMGB-1 = high mobility group box 1; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.

**Table 2:** Mean difference of caspase-3 shows that there is the significant difference in caspase-3 expression in the group treated with 30 mg/kg BW of EGCG.

| Group | Mean ± SD | p-Value |
|---|---|---|
| P0 (n = 10) | 4.40 ± 0.70 | – |
| P1 (n = 12) | 4.33 ± 0.49 | 0.652 |
| P2 (n = 11) | 4.09 ± 0.30 | 0.136 |
| P3 (n = 12) | 3.58 ± 0.51 | 0.010 |
| P4(n = 10) | 3.89 ± 0.42 | 0.063 |

SD = standard deviation; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.This result shows that only the higher dose of EGCG can suppress caspase-3 expression.

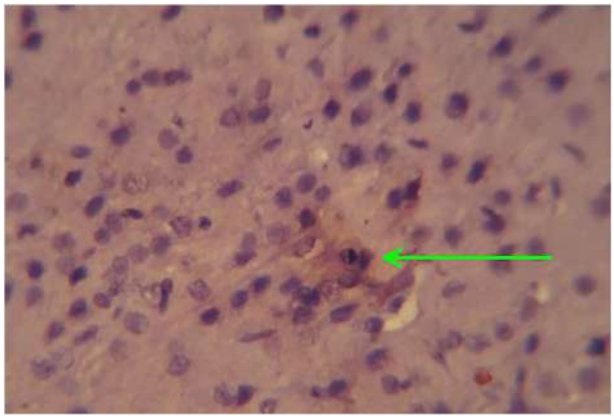

**Figure 1:** Caspase-3 expression in neuronal cell, shown in green arrow. There is increase in expression of caspase-3 expression (brown) in neuronal cells in the MCAO model. MCAO = middle cerebral artery occlusion.

**Table 3:** Mean difference of BCL-2 expression between the groups shows that there is increase in BCL-2 expression in intervention groups compared to the control group.

| Group | Mean ± SD | p-Value |
|---|---|---|
| P0 (n = 10) | 1.80 ± 1.03 | – |
| P1 (n = 12) | 2.58 ± 0.51 | 0.040 |
| P2 (n = 11) | 3.36 ± 0.50 | 0.001 |
| P3 (n = 12) | 4.00 ± 0.42 | 0.001 |
| P4(n = 10) | 3.60 ± 0.52 | 0.001 |

SD = standard deviation; BCL-2 = B-cell lymphoma 2; EGCG = epigallocathechin-3-gallate; P0 = control group; P1 = group treated with 10 mg/kg BW of EGCG; P2 = group treated with 20 mg/kg BW of EGCG; P3 = group treated with 30 mg/kg BW of EGCG; P4 = group treated with 30 mg/kg BW of green tea extract.There is significant difference between the control group and the sham group.

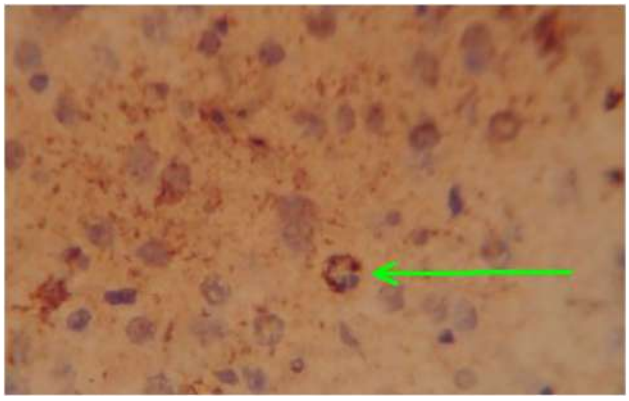

**Figure 2:** Expression of BCL-2 in neuronal cells is shown in green arrow. There is increase in BCL-2 expression in neuronal cells (brown). BCL-2 = B-cell lymphoma 2.

**Table 4:** Correlation between variables.

| Variable I | Variable 2 | r | p-Value |
|---|---|---|---|
| HMGB-1 | Caspase-3 | −0.063 | 0.613 |
| Caspase-3 | BCL-2 | −0.459 | 0.000 |
| BCL-2 | HMGB-1 | −0.106 | 0.396 |

BCL-2 = B-cell lymphoma 2; HMGB-1 = high mobility group box 1.There is significant negative correlation between expression of caspase-3 and BCL-2, but there is no significant correlation between HMGB-1 and caspase-3 expression or between BCL-2 and HMGB-1 expression.

word EGCG can protect neuronal cells from apoptosis through decreasing proapoptotic protein expression and increasing antiapoptotic protein expression.

## Discussion

Our study shows no difference in the HMGB-1 level between the intervention group and control group, and in another way, it shows that neither green tea extract nor EGCG, as its active compound, influenced the HMGB-1 level. Our finding is different from that of other studies that show that EGCG can decrease HMGB-1 expression [34, 35].

HMGB-1 is secreted actively and passively from cells, active release of HMGB-1 have function to maintain cell and tissue homeostasis and passive release is triggered with insult that threatens the cells. HMGB-1 is a marker of cell damage, and it is released in cells during stress as danger cell signals [8, 36–39]. In response to many stresses or injuries in cells, HMGB-1 is released passively as danger-associated molecular patterns that will trigger inflammation; thus, during acute ischemic stroke, HMGB-1 is released from neuronal cells or other cells in cerebral tissue, and this will accelerate cell injury in cerebral tissue [10, 40]. During acute ischemic stroke, there is increase infree radicals as a result of oxidative stress, and this will increase HMGB-1 levels [41–43]. EGCG as an active compound of green tea has strong antioxidant property and also acts as a free radical scavenger that can decrease the HMGB-1 level [25, 34, 35, 43, 44]. Our study shows that EGCG has no effect on the HMGB-1 level because during acute stroke, there is excessive cell damage not only from necrosis mechanism but also from other forms of cell death; this excessive cell death will let neither green tea extract nor its active compound EGCG to block the release of HMGB-1 from neuronal cells [45, 46]. Our study also found that neuroprotective mechanism of EGCG is not through HMGB-1 pathways [46].

Caspase-3 is a key regulator of apoptotic cell death because it involves in the final common pathway of apoptosis. Our study shows that the high dose of EGCG can inhibit caspase-3 expression, and this implies that the high dose of EGCG can prevent neuronal apoptotic cell death in the MCAO model throughout its inhibition [2, 47]. This result is similar to that of the study by Nan et al. [48] which shows that treatment using EGCG will diminish expression of caspase-3. Some studies also show that EGCG can inhibit apoptosis through its antioxidant activity and as a free radical scavenger [25, 32]. During cerebral ischemia, there is increase in excitatory amino acids, free radicals, NO, and inflammation that will trigger neuronal death including apoptosis. There is increase in mitochondrial function during acute ischemia, which will activate cytochrome c, caspase, and apoptotic bodies. EGCG that has free radical scavenger effect thus will decrease apoptotic cell death [48]. EGCG also stabilized mitochondria through its antioxidant and free radical scavenger properties [49]. Our intervention group treated with standardized green tea extract shows no significant difference compared with the control group; this may be because it needs a higher dose of

EGCG to inhibit apoptosis in the MCAO model because in the EGCG-treated group, significant caspase-3 inhibition is shown only in the group treated with the highest dose of EGCG (30 mg/kg BW).

BCL-2 is a main antiapoptosis protein; it regulates apoptosis through the mitochondrial complex that maintains the integrity of the outer mitochondrial membrane [20, 22]. Expression of BCL-2 is increased in our intervention study, which means that either green tea extract or its active compound EGCG can inhibit apoptosis through increasing BCL-2 expression [48, 50].

There is a different mechanism of EGCG in the context of stroke and malignancy with regard to the effect on apoptosis. Effect of EGCG in malignancy can induce apoptosis; however, in stroke, EGCG can prevent neuronal apoptosis, and this is different because of different expression of *silent information regulator* (SIRT3). EGCG will significantly reduce SIRT3 expression in malignancy, but not in normal cells; thus, EGCG can prevent apoptotic neuronal death [51].

There is no correlation between HMGB-1 and caspase-3 in our study. Zhang et al. [52] studied colorectal cancer and found that increase in HMGB-1 levels will increase activation of caspase-3. Another study also found that HMGB-1 release promotes apoptosis in cancer cells [53]. The role of HMGB-1 in stroke is different between the acute phase and recovery phase. HMGB-1 in the acute phase promotes necrosis and triggers inflammation, but in the recovery phase, HMGB-1 mediates plasticity and recovery [54]. Oxidation of EGCG will produce dimer EGCG (theasinensin) and cause aggregation of HMGB-1 and promote autophagia; in our study, EGCG treatment showed no effect because excessive release of HMGB-1 prevented EGCG from blocking HMGB-1 [46]. In our study, there were no significant correlation between HMGB-1 and caspase-3, this result shows that caspase-3 expression is not through the HMGB-1 pathway [46].

Our study found that antiapoptotic property of EGCG through inhibition of caspase-3 expression and increasing BCL-2 expression. This action may be because of antioxidant property and free radical scavenger effect of EGCG [25, 27, 55]. Neuroprotective effect of EGCG has been demonstrated in many stroke model studies [25, 27, 29, 55]. One of the mechanisms of neuroprotective EGCG is through antiapoptotic effect and properties that can stabilize the mitochondrial membrane [24, 32, 48, 49]. Mitochondria has been long known to play a role in apoptosis through oxidative stress that triggers cytochrome c which can stimulate apoptosis [32].

Our study is a true experimental study using either EGCG as the active compound or green tea extract, giving new insights into neuroprotective mechanism of EGCG in the stroke model that should continue in clinical trials. Our study is a true experimental trial that we can control other confounding factors that will affect this study.

The limitation of our study is that it is an animal model study that should be proven in clinical trials to know the usefulness in stroke patients. Our study using immunohistochemistry method, resulting in semiquantitative data. For more objective result and assessment we used Allred score.

## Conclusions

Green tea treatment with its active compound EGCG inhibits the expression of the neuronal apoptosis marker in the MCAO model and shows no effect on the level of HMGB-1.

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Mahardian Rahmadi*, Dian Suasana, Silvy Restuning Lailis, Dinda Monika Nusantara Ratri and Chrismawan Ardianto

# The effects of quercetin on nicotine-induced reward effects in mice

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**Abstract**

**Objectives:** Tobacco smoking remains the primary cause of preventable mortality and morbidity in the world. The complexity of the nicotine dependency process included the withdrawal effect that triggers recurrence being the main problem. Quercetin, known as an antioxidant, binds free radicals and modulates endogenous antioxidants through Nrf2 activations is expected as a potential agent to reduce the risk of nicotine dependence. This research aims to evaluate quercetin's effects on reducing the risk of nicotine addiction.

**Methods:** Conditioned Place Preference (CPP) with a biased design was used to evaluate nicotine's reward effects in male Balb/C mice. Preconditioning test was performed on day 1; conditioning test was done twice daily on day 2–4 by administering quercetin (i.p.) 50 mg/kg along with nicotine (s.c.) 0.5 mg/kg or Cigarette Smoke Extract (CSE) (s.c.) contained nicotine 0.5 mg/kg; and postconditioning test was performed on day 5 continue with extinction test on day 6, 8, 10, 12, and reinstatement test on day 13. The duration spent in each compartment was recorded and analyzed.

**Results:** Nicotine 0.5 mg/kg and CSE 0.5 mg/kg significantly induced reward effects ($p<0.05$). There was no decrease of reward effect during the extinction-reinstatement stage of the postconditioning phase ($p>0.05$), while quercetin 50 mg/kg both induced along with nicotine or CSE was able to inhibit the reward effect of nicotine ($p>0.05$).

**Conclusions:** Quercetin reduced the risk of nicotine dependence and has a potential effect to use as a therapy for nicotine dependence, especially as a preventive agent.

**Keywords:** cigarette smoke extract; conditioned place preference; nicotine dependence; quercetin; tobacco addiction.

## Introduction

Tobacco smoking remains the primary cause of preventable mortality and morbidity [1]. In the world, there were an estimated 6.4 million deaths in 2015 due to smoking, representing a 4.7% increase in smoking-related deaths since 2005. Smoking was a significant risk factor for chronic respiratory disease and was the second-leading risk factor for mortality after high-systolic blood pressure [2]. The prevalence of smokers in Indonesia was the highest in Southeast Asia [3] and the third worldwide after China and India [2]. RISKESDAS 2018 data showed that the prevalence of smokers aged 10–18 years in Indonesia increased from 7.2% in 2013 to 9.1% in 2018 [4]. Many treatments are available for a subject who has an addiction to nicotine or tobacco products, such as nicotine replacement therapy (NRT), varenicline, and bupropion [5]. However, smoking cessation based on clinical trials of these therapies still far from expectations. The therapeutic success rates observed in the clinical trials of NRT was 10–20%, bupropion was 15–25%, and varenicline was 23–40%. Based on a survey of 70% of smokers who wanted to quit smoking, only about 10% were successful [6]. Alterations and long-term adaptation in synaptic plasticity were considered the causes of the low smoking cessation rates [7]. In addition to smoking cessation, smoking initiation prevention also needs to reduce smoking prevalence to prevent the harmful effects of smoking on health [8].

Tobacco smoking addiction is a complex process, mainly associated with nicotine's addictive effect [5]. Nicotine binds to nicotinic acetylcholine receptor (nAChR) in the ventral tegmental area (VTA), which activates dopamine neurons in the nucleus accumbens (NAc) [9]. Neurotransmitters such as dopamine, norepinephrine, acetylcholine, glutamate, γ-aminobutyric acid (GABA),

***Corresponding author: Mahardian Rahmadi,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +62 81224656516, E-mail: mahardianr@ff.unair.ac.id
**Dian Suasana, Silvy Restuning Lailis, Dinda Monika Nusantara Ratri and Chrismawan Ardianto,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

and endorphin are released of the activation of these receptors [10]. Nicotine exposure results in greater and persistent activation of dopamine neurons, an action that enhances nicotine's effects. Structural changes occur during chronic nicotine exposure, such as the greater density of nAChR in many parts of the brain due to nAChR desensitization and upregulation [11]. The upregulation of nAChRs is thought to be associated with the development of physical dependence, including withdrawal symptoms, when nicotine exposure is stopped [10]. Increased dopamine receptor affinity in NAc, impaired neuronal homeostasis, altered epigenetic processes, and many other neuroadaptations are associated with chronic nicotine exposure [12].

Several recent studies reported that oxidative stress modulates the reinforcing effect of drug abuse [13, 14]. Increased production of ROS, known as a toxic mediator, can damage the structure and function of the dopaminergic system and contribute to the development of drug addiction [13]. Released ROS in various parts of the brain occurs due to long-term exposure to tobacco smoke. One micromolar concentration of nicotine significantly elevated ROS levels in rat mesencephalic cells [15]. In cocaine addiction, cocaine exposure evoked ROS production in the nucleus accumbens, prefrontal cortex, and striatum. Dopamine, which is released after nicotine exposure as a signal of pleasure, was reported to increase ROS levels. Specifically, there is the release of hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$) due to dopamine metabolism [16]. In response to increased ROS, the nucleus erythroid factor 2-related factor 2 (Nrf2) activated by the cell, moves to the nucleus to bind antioxidant response elements (ARE) as a part of the endogenous antioxidant defense process, and eventually increase the expression of various endogenous antioxidants such as superoxide dismutase, glutathione, and catalase [17–19].

Quercetin, from the Latin word "*Quercetum*," is a bioflavonoid known for its potent antioxidant effects. This substance is reported to have abundant beneficial effects such as anticancer effects, prevention of cardiovascular disease, protection of neurodegenerative disorders, antiulcer, antibacterial, and antiallergic [20]. The administration of quercetin effectively reduced oxidative damage and increased the neuroprotective effect of fish oil in a neurotoxicity rat model [21]. Quercetin inhibits the formation of ROS and provides neuroprotection in the brain. It suppresses nerve inflammation, stimulates nerve regeneration, and improves memory, learning, and cognitive function [20]. Quercetin acts directly as free radicals scavenger [22] and modulates endogenous antioxidants such as SOD and γ-glutamyl cysteine-synthetase (GCS), which are known as rate-limiting enzyme for glutathione (GCH) synthesis, through the activation of Nrf2 [22, 23]. While the effect of quercetin in tobacco or nicotine dependence is still not fully known.

Decreased ROS and increased endogenous antioxidants by quercetin may inhibit reward signals related to nicotine's reinforcing effect in the mesolimbic dopaminergic pathway. We evaluated this, in mice, by tested the quercetin administering along with nicotine or cigarette smoke extract (CSE) as an addictive agent, then we observed the developed reward effect.

# Materials and methods

## Animals

Male Balb/c mice were used in this study (aged 8–12 weeks; weighed 20–30 g). Their environmental conditions were maintained at normal temperature (25 °C ± 2 °C) and humidity (60 ± 10%) on a 12 h light/12 h dark cycle. Food and drink were provided *ad libitum*. All treatment protocols in this study were approved by the Research Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga (Animal Care and Use Committee [ACUC]) and performed at Animal Research Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya.

## Drug and treatments

Quercetin purchased from the Tokyo Chemical Industry (Japan) was dissolved in vehicles (5% tween 80). Quercetin is prepared no more than 30 min before injection. Nicotine purchased from Sigma–Aldrich (US) was dissolved in 0.9% salt solution from PT. Widatra Bakti Indonesia. Meanwhile, CSE was prepared following the protocol developed by Gellner et al. [24] through the apparatus by bubbling the smoke extract from a lit commercial filter cigarette into normal saline to obtain aqueous elements from cigarette smoke, including nicotine and minor alkaloids. Cigarettes were smoked for 2 s followed by 30 s intervals in 35 mL saline solution. CSE was prepared fresh daily for experimentation and stored in the refrigerator (2–8 °C) no more than 24 h. Before testing the effect of quercetin on the addictive effect of CSE and nicotine, we conducted preclinical test to determine the CSE and nicotine doses used. We tested nicotine 0.1 mg/kg, nicotine 0.5 mg/kg, CSE 0.1 mg/kg, and CSE 0.5 mg/kg for its addictive effects. Dose conversion is based on information on nicotine levels per cigarette used and previous studies [25]. Our preclinical validation results showed that the dose of nicotine 0.5 mg/kg and CSE 0.5 mg significantly induced addiction (conditioned place preference [CPP] score $p<0.01$) and in the extinction-reinstatement phase significantly showed drug-seeking behavior ($p<0.05$ for nicotine; $p<0.01$ for CSE). The dose of both nicotine and the CSE used in this study was 0.5 mg/kg administered subcutaneously [26], while the dose of quercetin used was 50 mg/kg administered intraperitoneally [27, 28].

All mice were randomly divided into five groups assigned to treatment conditions. The normal control group P1 received 1 mL/kg normal saline (s.c.), the test group P2 received 0.5 mg/kg nicotine (s.c),

the test group P3 received 0.5 mg/kg CSE (s.c), the test group P4 received 50 mg/kg quercetin (i.p) along with 0.5 mg/kg nicotine (s.c), and the test group P5 received 50 mg/kg quercetin (i.p) along with 0.5 mg/kg CSE (s.c). Quercetin was injected 60 min before nicotine or CSE injection, then 10 min after nicotine or CSE injection, mice were tested on CPP instrument. The CPP with a biased design using two compartments (white walls with a smooth floor surface and black walls with a rough floor surface) was used to evaluate the reward effect [29, 30]. The tests comprised three different stages, namely preconditioning, conditioning, and postconditioning continued with the extinction stage and the reinstatement stage [31].

### Preconditioning test (day 1)

This phase assessed mice's tendency to prefer one of the CPP compartments to determine a drug-paired chamber or vehicle paired chamber (biased design). The test was performed by placing the mice in the middle between the compartments then allowed them to access the compartments for 15 min [29]. Each subject's least preferred compartment was assigned to the drug, and the most preferred compartment was assigned to the drug vehicle [30].

### Conditioning test (day 2–4)

This phase represents the stage of intoxication in the initial cycle of addiction [29]. The conditioning test was carried out two times per day for 3 days in the selected CPP chamber with a closed partition. On day 2 in the morning (09.00 am), all mice were received 1 mL/kg normal saline then put them into the preferred chamber for 30 min, and in the afternoon (03.00 pm), all mice received treatments depending on the assigned test group (P1–P5) as previously described then put them into the nonpreferred chamber for 30 min. The next day, all mice received treatments with a switched schedule between morning and afternoon from the previous day.

### Postconditioning test (day 5)

This phase assessed the tendency of mice to prefer one of the CPP compartments due to the addiction. The procedures performed were the same as the procedures performed in the preconditioning test.

### Extinction training test (day 6, 8, 10, and 12)

In this stage, the duration of drug-seeking behavior (craving and relapse to an addictive agent) observed due to the withdrawal effect. The procedures performed were the same as the procedures performed in the postconditioning test.

### Reinstatement of place preference test (day 13)

This stage represents the preoccupation or anticipation stage of addiction-related to drug-seeking behavior returned after re-exposure of nicotine. Mice were re-injected with nicotine or CSE by giving quercetin 60 min in advance. After 10 min, mice were put into the drug-paired chamber for 15 min for observation.

### Data analyses

The duration both spent in the drug-paired chamber was compared between groups to assess the risk of addiction also the occurrence of relapse. Statistical analysis performed using SPSS 25.0 software for normality test and Graph-Pad Prism 6.0 software for one-way analysis of variance (ANOVA) test followed by the Tukey Posthoc test for multiple comparisons. p values less than 0.05 were considered statistically significant. All analyzed data were expressed as mean ± SEM.

## Results

### The effect of quercetin on reducing the reward effect of nicotine

We examined the tendency of mice to prefer one compartment as a sign of addiction due to the addictive reward effect on the preconditioning – postconditioning phase after administering nicotine or CSE, both with and without quercetin. The result showed the test group that administered by nicotine 0.5 mg/kg (222.20 ± 132.00; $p<0.05$) and CSE 0.05 mg/kg (255.00 ± 108.44; $p<0.01$) was significantly induced the reward effect compared to the control group (–160.00 ± 38.95; $p>0.05$). While quercetin, both were administering along with nicotine (–61.40 ± 44.75; $p>0.05$) and CSE (20.80 ± 92.38), was able

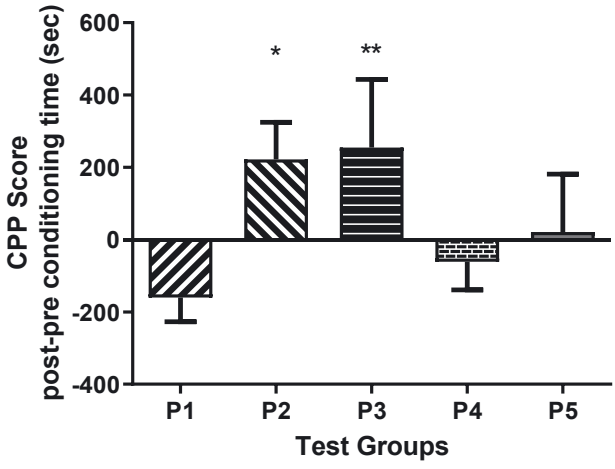


**Figure 1:** Quercetin inhibits the reward effect of nicotine. Conditioned place preference score (postconditioning – preconditioning) spent in the nonpreferred chamber of the P4 and P5 groups did not increase significantly compared to the P2 and P3 groups. Quercetin administered 60 min before nicotine or CSE in the conditioning test. Each column represents the mean ± SEM of five mice, *$p<0.05$; **$p<0.01$. CSE, cigarette smoked extract. P1: normal control, saline 1 mL/kg; P2: nicotine 0.5 mg/kg; P3: CSE contained nicotine 0.5 mg/kg; P4: Quercetin 50 mg/kg + nicotine 0.5 mg/kg; P5: Quercetin 50 mg/kg + CSE contained nicotine 0.5 mg/kg.

to inhibit the reward effect by reducing the preference time to the drug-paired chamber (Figure 1).

### Extinction of addiction and the effect of quercetin on inhibiting the relapse

We further observed the extinction stage to determine how long drug-seeking behavior lasts and the reinstatement stage to evaluate the occurrence of relapse (preoccupation) after re-exposure of nicotine or CSE. The result showed that there was an insignificant increase in the CPP score between the extinction stage and the postconditioning phase in the nicotine group (636.8 ± 129.02 vs. 495.8 ± 165.77; $p>0.05$) and an insignificant decrease of CPP score in the CSE group (184.6 ± 103.93 vs. 464.6 ± 104.21; $p>0.05$). These results show that both have persisted the reward effects until the end of the extinction training test, with the level of nicotine addiction being higher than CSE. Meanwhile, the results of the reinstatement phase showed that the CPP score at the reinstatement stage and extinction stage were not significant increase both in the nicotine test group (450.8 ± 200.51; $p>0.05$) and CSE test group (285.8 ± 183.99; $p>0.05$). These results suggested that quercetin slightly prevented the relapse, although it was not significant (Figure 2).

## Discussion

Administering mice with nicotine and CSE were a method to model smoking tobacco addiction. Most preclinical models of nicotine dependence use nicotine alone because nicotine is the primary psychoactive agent in tobacco. When investigated, this model was not in line with the potential for smoking addiction because more than 7,000 compounds in cigarettes and their contribution to smoking addiction have not been thoroughly studied. CSE is a model designed to obtain other compounds in cigarettes along with nicotine, particularly the aqueous constituents from cigarettes inhaled while smoking [24]. We used both CSE and nicotine to determine the potential for quercetin to reduce its addictive effects. We considered using commercial filter cigarettes for CSE preparation due to its compounds are not mixed with cloves as in kretek cigarettes. Besides, it is also related to the consumption of filter cigarettes are used by both men and women (women prefer filter cigarettes to kretek) [32]. Nicotine in cigarettes increases dopamine release in the brain, reinforcing brain stimulation and activating the rewards pathway that leads to compulsive drug-seeking and triggers physical addiction to nicotine [33]. Both nicotine and CSE have a similar effect on the burst activity of dopamine neurons in VTA [34], with CSE rated as more sensitive to stress than nicotine alone (a potential trigger for relapse in smokers) [35] and result in more significant upregulations of nAChR than nicotine [34]. Besides being a stimulant substance, previous studies suggested that nicotine-induced were increased ROS levels and contributed to drug addiction [13, 15].

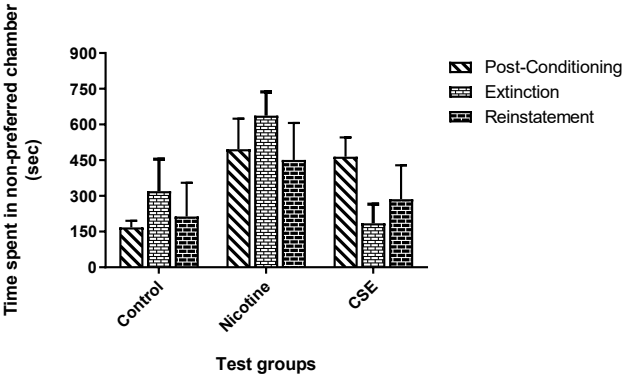


**Figure 2:** Assessment of drug-seeking behavior (craving and relapse to an addictive agent). The duration of the extinction training was insignificant compared to the postconditioning test. Quercetin administration in the reinstatement test prevented relapse, although it was not significant. Each column represents the mean ± SEM of five mice. CSE, cigarette smoked extract.

In this study, we examined the effect of quercetin on the reward effect of nicotine addiction. We administered the addictive agents nicotine and CSE subcutaneous considering this route is most commonly used to mimic the rapid drug distribution of nicotine to the brain via inhalation [36, 37]. Quercetin was administered intraperitoneally based on our previous study (with the same dose) [28], associated with low bioavailability of quercetin when administered orally [38]. Quercetin is thought to prevent ROS formation caused by nicotine and modulate endogenous antioxidants in the nucleus accumbens. Thus, we hypothesized that quercetin reduces the risk of nicotine dependence. Based on the preferred time of preconditioning and postconditioning tests, we found that quercetin inhibited both nicotine and CSE's reward effect, indicating that quercetin reduced the risk of nicotine addiction. The test group that received quercetin before CSE or nicotine administration showed insignificant CPP scores compared to the control group, and significantly lower compared to the test group that did not receive quercetin. The control group showed no preference for either of the two compartments indicate that the test group's reward effect was solely a result of the effect of the drug administered [29].

Our results regarding the significant addictive effect after nicotine and CSE administration assessed from the duration spent in the drug-paired chamber are in line with a previous study. More specifically, Kota et al. [39] reported that subcutaneous administration of 0.5 mg/kg nicotine in adult mice resulted in significant preference in the drug-paired chamber. The difference of CPP score in the CSE group was higher than the nicotine group. This probably because in CSE, in addition to nicotine, other constituents will increase the effect of addiction. As is well known, there are more than 7,000 chemical compounds in cigarette smoke contributed to addiction [40].

We further evaluated the results of the extinction test and the reinstatement test, which used to evaluate craving and relapse to smoking. The extinction training test was not performed for the quercetin groups (P4 and P5) because they did not show any reward effect based on the post-conditioning test's CPP score. Whereas in the reinstatement test, mice initially administered quercetin followed by nicotine or CSE in assessing quercetin's ability to prevent relapse. The effect of nicotine addiction and CSE lasted about 1 week assessed by the insignificant time preference for the extinction test compared to post-conditioning with a slight increase of preference time in the nicotine group and a slight decrease of preference time in the CSE groups. The reward effect is expressed as extinction if the duration spent in the drug-paired chamber is decreased compared to the postconditioning phase. Our results showed that the nicotine test group had a higher but not significant addiction rate than the CSE test group. Cross et al. [41] reported that the reinforced response of self-administration of nicotine was higher than CSE, while the reinstatement stimulus test showed that animals that had received CSE were more sensitive to reinstatement than had nicotine.

In the reinstatement test, we found that administering quercetin before re-exposure to addictive agents resulted in no significant change in the time preference for the drug-paired chamber. If observed from the data in pre and postconditioning CPP scores, CSE provided a higher reward effect than nicotine does, while quercetin prevented the occurrence of rewards at this stage. Likewise, in the reinstatement test, we found that quercetin could inhibit an increase in the reward effect due to CSE and nicotine administration, although it was not significant. Finally, when observed from the test's aspect by the time preference at all stages measured by CPP from the quercetin test groups compared to groups without quercetin, we conclude that administering quercetin reduced the risk of nicotine addiction by preventing the occurrence of reward effects.

Several studies suggested that antioxidants can be suitable candidates to reduce oxidative stress due to an addictive agent-induced [42]. Quercetin directly binds ROS because it has many effective structures capable of scavenging free radicals and indirectly acts to modulate an increased expression of endogenous antioxidants in the body [22]. Quercetin administration is predicted to reduce ROS production in the nucleus accumbens neurons and contribute to reducing the risk of nicotine dependence. In the previous study of other addictive agents, systemic ROS scavenger administration before cocaine administrations significantly reduced the rate of cocaine addiction [13]. Most previous studies have underlined the role of oxidative stress as an important mediator for nerve cell death in the brain's reward system [43]. Increased oxidative stress was found in the mice's brain reward system (nucleus accumbens and prefrontal cortex) that had received single or repeated cocaine injections [42]. Increased ROS due to nicotine exposure is thought to be involved in reward signaling in the mesolimbic dopaminergic pathway associated with its reinforcing effect. Based on our results, further research is needed to examine the molecular aspect of the quercetin in order to clarify information related to its effect, particularly the formation of ROS due to nicotine exposure and the effect of ROS scavenger from quercetin in inhibiting reward effect as an effort to reduce the risk of nicotine dependence.

## Conclusions

Our study demonstrated that quercetin has a potential effect on reducing the development of nicotine dependence. Quercetin has a future opportunity to be used as a preventive therapy in smoking cessation.

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Chrismawan Ardianto, Aniek Setiya Budiatin, I Nengah Budi Sumartha, Nurrahmi Nurrahmi, Mahardian Rahmadi and Junaidi Khotib*

# Resveratrol ameliorates physical and psychological stress-induced depressive-like behavior

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**Abstract**

**Objectives:** Depression is a mental disorder that profoundly affects all aspects of life, but currently, antidepressants have some problems with their effectiveness and side effects. Resveratrol is a compound that has the ability to regulate the hypothalamic-pituitary-adrenal axis. This study aimed to determine resveratrol's effect on physical and psychological stress-induced depressive-like behavior.

**Methods:** Mice were divided into control, physical stress, psychological stress groups. Treatment was conducted with fluvoxamine 20 mg/kg and resveratrol 20, 40, and 80 mg/kg for seven days. The depressive-like state was evaluated using a forced swim test (FST), tail suspension test (TST), and open field test (OFT).

**Results:** Physical stress and psychological stress induction increase the immobility time on FST and TST. Besides, there is an increase in time in central on OFT, which indicates an anxiety or mental illness-like behavior. However, the OFT examination on sniffing, rearing, grooming, and crossing behavior did not show a significant difference. Resveratrol 80 mg/kg and fluvoxamine 20 mg/kg were significantly reduced immobility time at TST compared to the physical stress group. While in psychological stress, resveratrol 80 mg/kg tended to decrease immobility time but not significant. A significant increase in time in central duration was seen in the resveratrol 40 mg/kg compared to the psychological stress. Stress induction causes increased amygdala corticotrophin-releasing factor (CRF) mRNA expression. However, neither resveratrol nor fluvoxamine affected amygdala CRF mRNA expression.

**Conclusions:** Resveratrol ameliorates depressive-like behavior induced by physical and psychological stress.

**Keywords:** depressive-like behavior; fluvoxamine; physical stress; psychological stress; resveratrol; mental illness.

***Corresponding author: Junaidi Khotib**, Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +6281331840710, E-mail: Junaidi-k@ff.unair.ac.id
**Chrismawan Ardianto, Aniek Setiya Budiatin, I Nengah Budi Sumartha, Nurrahmi Nurrahmi and Mahardian Rahmadi,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

## Introduction

Depression is a mental disorder that characterized by low self-confidence, hopelessness, worthlessness, insomnia, fatigue, reduced interest in sex and social interactions, and the onset of suicidal thoughts [1]. There are more than 264 million people affected by depression worldwide [2].

Stress is one of the predisposing factors for depression, anxiety, post-traumatic stress disorder. Both physical and psychological stress is known to cause depressive behavior. Apart from stress, abnormalities of the Hypothalamic-Pituitary-Adrenal (HPA) axis are also associated with depression. This situation begins when the peptide called corticotrophin-releasing factor (CRF) increases due to the stressor response and increases adrenocorticotropic hormone and cortisol in the blood. This peptide is known to affect the limbic system in several brain regions such as the hypothalamus and amygdala [3–5].

Nowadays, selective serotonin reuptake inhibitors are chosen for depression treatment. These antidepressants are known to affect serotonin and the HPA axis. Unfortunately, these antidepressants still have their therapeutic drawbacks. It has been reported that about 60% of patients experienced side effects in the form of insomnia, headaches, nausea, abdominal pain, sexual disturbances, hyponatremia, and serotonin syndrome [6, 7]. Besides, only about 50% of patients experienced an improvement after using antidepressants [8]. Therefore, it is necessary to look for antidepressants from natural herbs which are expected to show fewer side effects such as resveratrol [9].

Resveratrol is a polyphenol compound obtained from grapes, itadori plants, nuts, berries, and chocolate. Resveratrol has many benefits such as anti-inflammatory, neuronal protection effects, antioxidant, cardioprotective effects, preventing obesity and diabetes, and is used for diseases related to aging [10]. Resveratrol is preclinically known to reduce depressive-like behavior induced by lipopolysaccharide,

corticosterone, and chronic unpredictable mild stress in an animal model [7].

Nowadays, to understand treatment responses and biomolecular markers, an animal model is used. The communication box is an animal model used to study behavioral and physiological changes using physical stress and psychological stress induction. Physical stress induction is performed using footshock stress, while psychological stress induction responds to animals induced by physical stress [11].

In this experimental animal model, depressive behavior can be observed by looking at one of the main symptoms (despair behavior) and an additional symptom in the form of a change in locomotor activity. Besides, there are also conditions associated with depression, such as anxiety-like behavior. Despair behavior can be seen from the increased immobility time using the forced swim (FST) test and tail suspension test (TST). At the beginning of these two tests, the animal tries to get out of the test condition by making an active swimming motion or by trying to reach for its tail so that it is no longer positioned upside down. However, there will be a time when the animal will feel hopeless with the test condition because it has been arranged so that the animal does not can escape. As a result, these animals will show immobility behavior which is used as a reference for symptoms of depressive behavior. Meanwhile, behaviors such as anxiety-like behavior and changes in locomotor activity as symptoms of depressive behavior can be observed using an open field test (OFT). Anxiety-like behavior was observed with a decrease in the duration of the animal being in open space which was described as risk-taking behavior [11, 12].

In this study, we investigated resveratrol's effect on depressive-like behavior and CRF mRNA in the amygdala using FST, TST, OFT, and RT-PCR.

# Materials and methods

### Animal

The Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University has approved all experiment protocols. Handling of animals according to the Guidelines for the Care and Use of Laboratory Animals issued by the National Institutes of Health revised in 1985. Male mice weighing 20–30 g, aged 6–8 weeks, procured from Veterinary Farma, Surabaya, Indonesia. Animals are placed under a 12:12 h light/dark cycle in a polypropylene cage, facilitated food and water access, and were maintained at 22–25 °C. The animals were randomly subjected and had acclimatization for one week before the experiment.

### Experimental and treatment

Resveratrol (Res) (Tokyo Chemical Industry Co) dose 20, 40, 80 mg/kg and Fluvoxamine maleate (Flu) (Wako Pure Chemical Industries) dose 20 mg/kg were dissolved in 10% of tween 80. Mice were divided into 11 groups (5–6 mice in each group): control, physical stress (FS), psychological stress (PS), FS Flu 20, PS Flu 20, FS Res 20, PS Res 20, FS Res 40, PS Res 40, FS Res 80, and PS Res 80. The experimental method was carried out by modifying the model from Ikeda et al. [13] and Ge et al. [14]. The communication box was divided into 3 × 3 compartment separated with transparent acrylic (Figure 1). Physical stress exposure with communication box is done with 1 mA electrical footshock for 1 s with 9 s intervals for 5 min. To prevent electric shock, a plastic plate was placed on psychological stress grid floors. Before induction, mice were adapted for 1 h. Induction was carried out for 10 days and the administration of Res and Flu was carried out intraperitoneally from day four to day 10. A day after induction, behavior testing was carried out using OFT, TST, FST, and then sacrifice.

### Open field test

In this test, mice were placed in a box with a base measuring 40 × 40 and 30 cm in height. The box is divided into 16 squares measuring 10 × 10 cm marked with a line. Mice were placed on the corner face the wall and tested for 5 min. The behavior measured in this tool is the number of crossing, time in central, rearing, grooming, and sniffing. Mice were categorized as experiencing depression and accompanying symptoms in the form of anxiety if there was a decrease in the value of time in central, locomotor activity (crossing, rearing), grooming, and sniffing behavior toward control mice.

### Tail suspension test

The mice's tail was hung for 6 min on a hook that was placed 58 cm above the floor. In the last 4 min of the test, the immobility time is measured. The mice were separated from each other during testing. In this test, depressive behavior is described if the time immobility score is significantly higher than that of mice in the control group.

### Forced swim test

At FST, animals are placed in tubes filled with water conditioned so that the animals cannot escape. The behavioral cylinder was filled with water maintained at 24–25 °C. Mice get a single exposure session

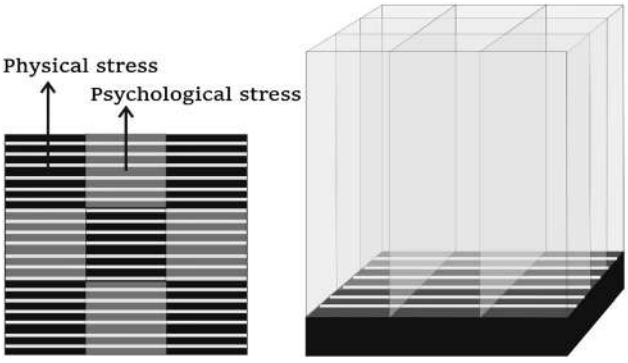


**Figure 1:** A communication box with electrical gird and transparent acrylic.

for 6 min. The first 2 min is used for the training time and the last 4 min is the measured period of immobility. The mice were placed into dry cages and dried with towels after the test session. In this test, depressive behavior is described if the time immobility score is significantly higher than that of mice in the control group.

### RT PCR

The amygdala was dissected, immediately frozen, and kept at −80 °C. Total RNA from amygdala isolated used PureLinkTM RNA Mini Kit (Life TechnologiesTM, USA). Reverse Transcription was performed used GoScript™ Reverse Transcription System (Promega, USA). PCR was conducted using GoTag® DNA Polymerase (Promega, USA). The following primers were used: CRF (Forward: 5′- GAAGAGAAAGGGGAAAGGCAAAGA-3′; Reverse: 5′- GCGGTGAGGGGCGTGGAGTT-3′) and β-actin (Forward: 5′-TGTTACCAACTGGGACGACA-3′; Reverse: 5′-AAGGAAGGCTGGAAAAGAGC-3′) PCR was performed on a thermal cycler as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturing for 40 s at 94 °C, annealing for 1 min at 55 °C, extension for 2 min at 72 °C and a final extension for 5 min at 72 °C. PCR products were analyzed using electrophoresis (Mupid-ex; Advance, Tokyo, Japan) with 2% agarose LE (Promega, USA) gels. Ethidium bromide (Sigma-Aldrich) was used to stained gel and photographed with UV transillumination. ImageJ (National Institutes of Health, USA) was used to determine the band intensity.

### Analytical statistic

All data are represented as mean ± SEM using Graph-Pad Prism version 6.0. One-way analysis of variance and the Bonferroni test were used to compare groups. $p<0.05$ and $p<0.01$ were considered significant.

# Results

## Model of depressive-like behavior

Model of depressive-like behavior in Figure 2 shows no significant difference between the control group compared with crossing (2A), sniffing (2B), rearing (2C), and grooming (2D) behavior, but the induction of psychological stress was able to reduce time in central (2E) significantly. Immobility time (2F) on TST significantly increased in the physical stress group only. Whereas at FST, both stressors increased immobility time (2G) significantly.

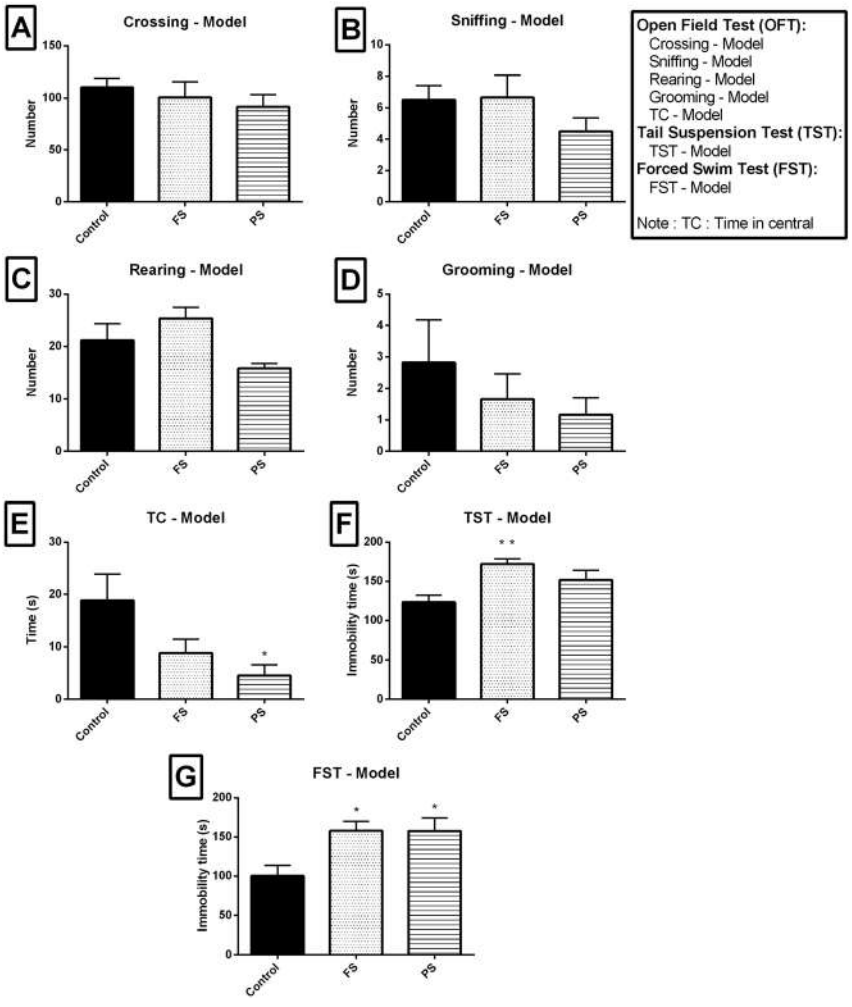


**Figure 2:** Effect of stress induction on the crossing (A), sniffing (B), rearing (C), grooming (D), time in central (E), tail suspension test (F), forced swim test (G). n=6 mice per group. FS=Physical stress, PS=Psychological stress *p<0.05 vs. control. **p<0.01 vs. control.

### Administration of resveratrol alleviated depressive-like behavior

The time in central parameters by administering 20 mg/kg of fluvoxamine or 20 and 80 mg/kg of resveratrol in the physical stress group did not provide a significant change, but this parameter tended to increase when giving resveratrol 40 mg/kg (3A) (Figure 3). Administration of 20 mg/kg of fluvoxamine or 20 and 80 mg/kg of resveratrol in psychological stress group tended of increasing time in central. A significant increase was seen in the resveratrol 40 mg/kg group compared to the other psychological stress group (3B).

In testing using TST, administration of resveratrol 20, 40 mg/kg in animals with physical stress induction gave a trend of decreasing immobility time. A significant decrease occurred in fluvoxamine 20 mg/kg or resveratrol 80 mg/kg group (3C). The parameter of immobility time in the psychological stress group given fluvoxamine or resveratrol tended to decrease but did not occur in the resveratrol 40 mg/kg group (3D).

At FST, there was a trend of decreasing immobility time in the physical stress group given 20 mg/kg of fluvoxamine or 20, 40, and 80 mg/kg of resveratrol. However, based on statistical analysis, this decrease was not significant (3E). The parameter of time of immobility in animals that were induced by psychological stress and then given fluvoxamine 20 mg/kg or resveratrol 80 mg/kg tended to decrease. However, it did not occur in the resveratrol 20 and 40 mg/kg groups (3F).

### Effect of resveratrol on CRF mRNA

The results showed that physical stress induction did not significantly affect CRF mRNA expression, whereas psychological stress induction increased amygdala CRF mRNA expression significantly (4A) (Figure 4). The administration of fluvoxamine or resveratrol did not significantly change the amygdala CRF mRNA expression in mice induced by physical stress (4B) and psychological stress group (4C).

## Discussion

Stress induction has been used in many experimental animals resulting in HPA axis dysregulation. In this study, the communication box model's induction shows that depressive-like behavior in mice induced by physical stress and psychological stress. This is indicated by an increase in immobility time on TST and FST. Besides, there is a decrease

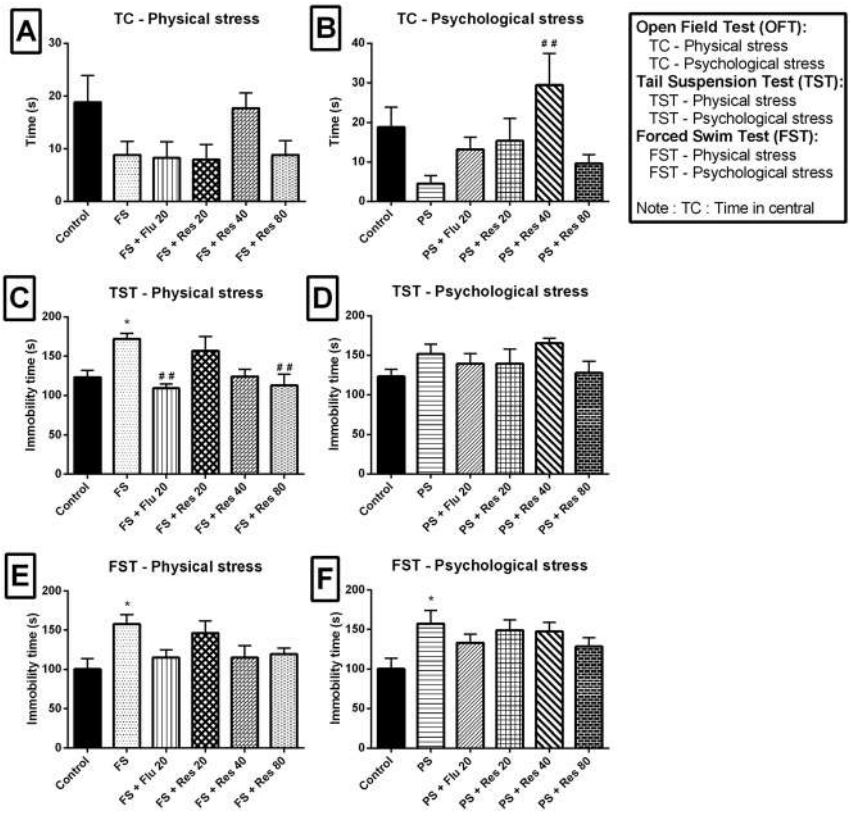


**Figure 3:** Time in central on physical stress (A) and psychological stress (B); TST immobility time on physical stress (C) and psychological stress (D); FST immobility time on physical stress (E) and psychological stress (F) responses by treatment of fluvoxamine (Flu) and resveratrol (Res). n=5–6 mice per group. *p<0.05 vs. control. **p<0.01 vs. stress group.

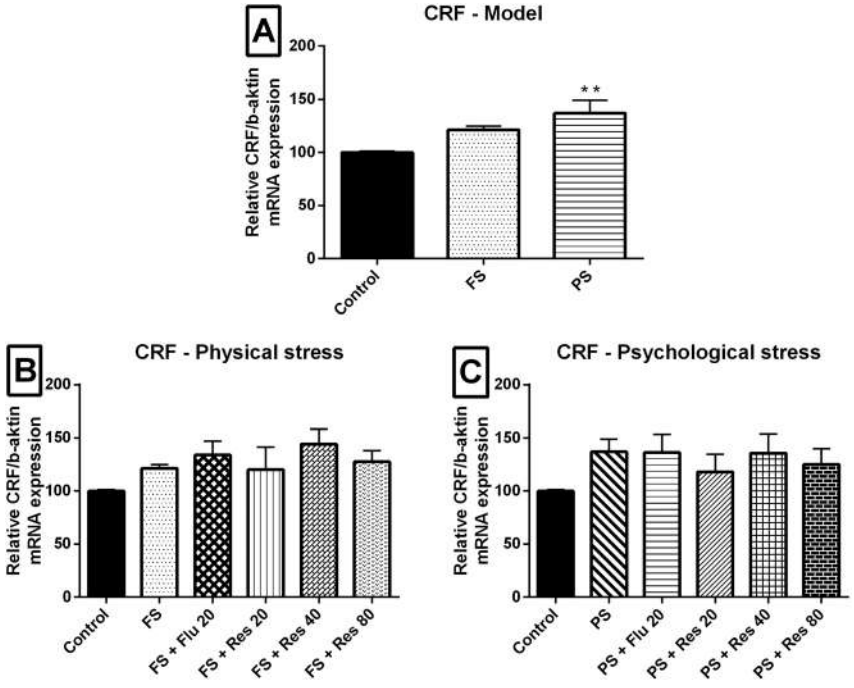


**Figure 4:** Physical (FS) and psychological stress (PS) effect on CRF mRNA expression (A). Effect of fluvoxamine (Flu) and resveratrol (Res) on CRF mRNA expression induced by physical stress (B) and psychological stress (C). n=3 per group. **p<0.01 vs. control.

in time in central, which indicates anxiety-like behavior. However, stress induction using the communication box model did not cause changes in the locomotor activity. The presence of depressive-like behavior is consistent with the previous study that uses social stress induction for psychological stress and physical stress induction with chronic unpredictable mild stress [15, 16].

Resveratrol 40 mg/kg decreased the time in central duration. It is indicated that resveratrol has the potential to treat anxiety-like behavior. This finding is consistent with the study in a post-traumatic stress disorder model [17]. The results of immobility time using FST and TST show that resveratrol has a similar antidepressant-like effect to fluvoxamine as behavioral therapy for depression in mice induced by physical and psychological stress. These results also show that the resveratrol antidepressant effect is more effective in physical stress than psychological stress. This can occur because the induction of physical stress and psychological stress can provide different molecular changes and metabolites associated with nerve development functions and cellular proliferation [18]. The differences were also observed in behavior patterns, corticosterone levels, and gene expression associated with plasticity [19].

Stress induction increased the amygdala CRF mRNA expression, consistent with the previous study [20]. Resveratrol 20, 40, 80 mg/kg and fluvoxamine 20 mg/kg did not show any changes in the amygdala CRF mRNA expression. This suggests that resveratrol and fluvoxamine do not act as antidepressants by affecting CRF projected from the amygdala. CRF mRNA from the amygdala is projected predominantly to the bed nucleus of stria terminalis, locus coeruleus, and minor projections to the ventral tegmental area and lateral hypothalamus [21].

There is evidence that 15 mg/kg of resveratrol for 16 days can eliminate depressive behavior by reducing despair behavior and reduce CRF mRNA expression in the hypothalamus [22]. Furthermore, resveratrol 40 mg/kg for 18 days can reduce CRF protein levels in the hippocampus, hypothalamus, and amygdala and reduce anxiety-like behavior [17]. However, a different effect was reported in another study, which showed that 20 mg/kg of resveratrol for seven days reduced depressive-like behavior and the amount of corticosterone but did not decrease hypothalamus CRF mRNA expression. The difference in duration of therapy using resveratrol may affect the results of the CRF biomarker test. This study improves the hypothesis that resveratrol reduces depressive behavior in experimental animals by more influencing the function of the HPA axis through the peripheral route than through the central route [14].

Besides, it is also possible that resveratrol influenced other depressive pathways such as antioxidant activities and increased amygdala and hippocampus brain-derived neurotrophic factor (BDNF) protein level to lead to antidepressant effect [14, 16].

## Conclusions

In conclusion, both physical and psychological stress induction increased depression-like behavior in mice.

Fluvoxamine as well as resveratrol reduced depression-like behavior but did not affect amygdala CRF mRNA expression.

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**Ethical approval:** All experiments were performed at the Laboratory of Animal Research, Faculty of Pharmacy, Airlangga University. The ethical committee of the Faculty of Veterinary, Airlangga University has approved the experimental protocol.

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Julaeha Julaeha, Umi Athiyah, Margarita Maria Maramis, Agus Sugianto and Andi Hermansyah*

# Translation and cross-cultural adaption of an instrument measuring patient's well-being under treatment for schizophrenia

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**Abstract**

**Objectives:** The Subjective Well-Being under Neuroleptic (SWN) Scale is a self-rating scale measuring the well-being of patients with schizophrenia under antipsychotic drug treatment. The instrument has been globally used, with issues regarding the well-being assessment scale across different cultures, patient characteristics, and country-setting remains a controversy. This study aimed to translate and culturally adapt the SWN scale into the Indonesian version (Indonesian Modified SWN or IM-SWN) and evaluate its validity and reliability.

**Methods:** The SWN instrument was translated and culturally adapted following internationally accepted procedures, including forward translation, expert panel review, backward-translation, pretesting and cognitive interviewing, and psychometric analysis for the final version of the scale. The translated instrument was tested on 108 schizophrenia patients. The instrument's validity and reliability were assessed using Pearson's correlation and Cronbach's Alpha coefficient. Additional analysis for the socio-demographic and psychometric properties of the patient was also conducted.

**Results:** The range of IM-SWN total score between 30 and 112. IM-SWN was found to have a high-reliability coefficient (0.897), and the internal consistency values of each question item ranged between 0.885 and 0.910. The results also showed a high correlation between five order factors (Physical functioning, mental functioning, self-control, emotional regulation, and social integration), with a total score of between 0.768 and 0.885.

**Conclusions:** This study highlighted that the IM-SWN is a valid and reliable instrument for measuring well-being among the Indonesian population with schizophrenia.

**Keywords:** antipsychotics; mental health; schizophrenia; subjective well-being; translation.

## Introduction

Within the past 10 years, there has been shifting focus on measuring patients' quality of life towards evaluating a complex set of Economic, Clinical and Humanistic Outcome (ECHO) based on patients' subjective experiences [1]. The World Health Organization (WHO) described the quality of life as "individual perceptions of their position in life in the context of the culture and value system in which they live, and in relation to their goals, expectation, standards, and concerns" [3]. The definition highlights the need to maintain quality of life in the longer term, which might be an issue for patients who received long-term therapy, such as schizophrenia.

Poor patient compliance, service disengagement, and comorbid disorder are some features attributed to the low quality of life in a patient with schizophrenia [2]. Moreover, the patient's condition might even be worsened with disabilities, severe mental illness, and a plethora of disruption both socially and individually to the patient's life [4]. Therefore, it is not surprising that treatment for patients with schizophrenia may comprise understanding the patient's autonomy, right, and opinion as an adjunct to pharmacological treatment [4]. The long-term goal for patients with schizophrenia is improved initial response of therapy, decreased level of severity, and improved social

***Corresponding author: Andi Hermansyah**, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62315933150, E-mail: andi-h@ff.unair.ac.id. https://orcid.org/0000-0002-9716-3126

**Julaeha Julaeha,** Faculty of Pharmacy, Universitas 17 Agustus 1945 Jakarta, Jakarta, Indonesia; and Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. https://orcid.org/0000-0002-8807-5175

**Umi Athiyah,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

**Margarita Maria Maramis,** Dr. Soetomo Academic Hospital, Surabaya, Indonesia; and Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

**Agus Sugianto,** Center for Public Mental Health, Universitas Gadjah Mada, Yogyakarta, Indonesia

functioning and life quality. This is why measuring the quality of life in such patients is challenging.

A number of published studies focused on evaluating the quality of life from the physician perspective; for instance, the Quality of Life Scale (QLS) [6–13]. However, this might be insufficient as patients with schizophrenia generally receive antipsychotic medication, which has not been included in such measurement. Therefore, compliance towards antipsychotic treatment is essential to be included within the full spectrum of measuring patients' quality of life [5]. Recently, there has been a change of interest in measuring the patient's well-being, such as the Subjective Well-being under neuroleptics (SWN) scale. The Subjective Well-being under neuroleptics (SWN) scale is an example of the questionnaire to assess the patient's quality of life [14, 15]. This questionnaire has been utilized in various current studies [16–19]. The SWN is translated into more than 40 languages [20–27]. However, there is no available scale developed in the Indonesian language despite the significant population of Indonesians suffering from schizophrenia. The presence of such scale may demonstrate its significance to the treatment in Indonesia. The objective of this study is to measure the validity and reliability of the Indonesian version SWN questionnaire as part of the translation and adaptation of the instrument.

# Materials and methods

### Study design

Ethics approval was obtained from the Research Ethics Committee of Menur Mental Hospital, Surabaya, East Java (No. 070/7556/305/2019) which was also the site for this study. From the electronic mail correspondence on 24 December 2019, the research team gained official permission and confirmation from the SWN scale developer to develop the scale into Indonesian. The study was a cross-sectional design with participants, which were purposively sampled.

### Participants

Outpatient schizophrenia patients were selected for this study. The inclusion criteria are patients with schizophrenia, aged 18 or older, consented to participate in the study and a patient who has no vision problems. The exclusion criteria are patients who suffered from other psychiatric illness and patients diagnosed with brain dysfunction or cognitive impairment. Informed consent was acquired from all participants prior to beginning the study. Participants were involved only after they signed informed consent. All researchers ensured participant data confidentiality and compliance with the Declaration of Helsinki. The total participants were 108 schizophrenia patients who completed the study; they either participated in online or offline interviews.

### Instrument

The original subjective well-being under neuroleptic treatment scale (SWN) consisted of 38 statements and later modified by the author in a shorter form consisted of 20 statements, each consists 10 positive statements and 10 negative statements, respectively [14, 15]. The patients filled out this questionnaire based on their understanding of health status, symptoms of psychosis, the effect on the antipsychotic, and nonmedical aspect through the preceding 7 days [15].

This study applied 6-point Likert scale from SWN short form (1–6). The total score varies from 20 to 120 points, and the higher score indicates greater well-being. There are five domains of SWN: physical function (PF), mental function (MF), self-control (SC), emotional regulation (ER), and social integration (SI) with each domain consisted of four statements. The score ranges for each domain from 4 points (worst) to 24 points (best) [15].

### Translation, cross-cultural adaption and SWN validation in Indonesian language

This study followed the Principles of Good Practice for the Translation and Culture Adaption Process to adapt the SWN short form into Indonesian version [28]. The original questionnaire was translated into Indonesian by a sworn translator and reversed back into English translation by a different sworn translator blindfolded to the original version.

Both versions have been analyzed and reviewed to be as accurate as possible to the original English version by three authors (JJ, UA, and AH), who are competent and fluent in Indonesian and English. The final Indonesian-language version was achieved through consensus among authors. The comprehensiveness of every part and items in this form was then examined by an expert panel involving one psychiatrist, two mental health pharmacists, and one schizophrenia caregiver from Indonesian Community Care for Schizophrenia (Figure 1).

### Data analysis

We used the IBM SPSS for windows version 24.0 for data analysis, and a $p<0.05$ was considered statistically significant. Descriptive analysis was presented for characteristics of participants and psychometric properties of SWN Indonesian version. For reliability analysis, the coefficient of internal consistency (Cronbach's Alpha) which is calculated based on the variance of each item, was utilized. The Pearson correlation coefficient was calculated to test the construct validity. Analysis Moment of Structural (AMOS) program was used for confirmatory factor analysis (CFA).

# Results

Table 1 reveals that male patients were major respondents (57%), the age ranged from 31–49 years were dominant

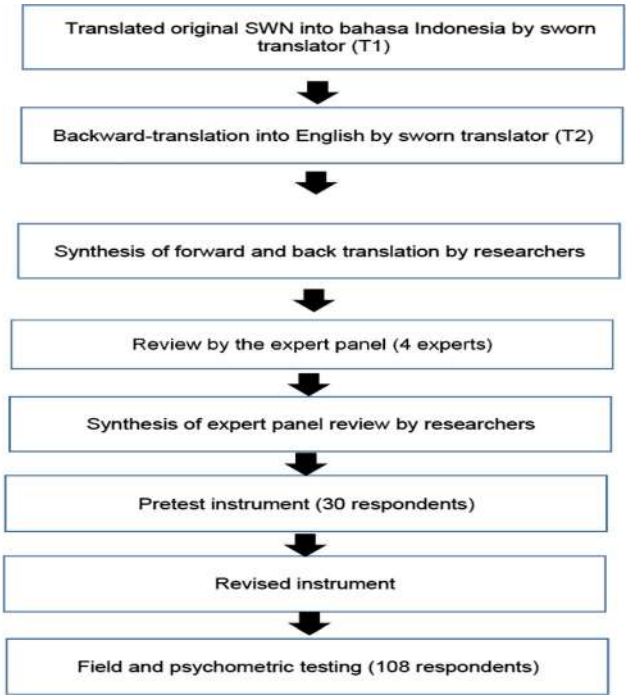


**Figure 1:** Flowchart of adaption of SWN into Indonesian version.

(65%) and most of the patients were single (60%), 37% have secondary education, and half of them were not a worker. The prescription frequency for antipsychotic as monotherapy was low (22%), with the majority of patients being on antipsychotics polypharmacy. Table 2 shows the lowest total score of SWN was 30, the highest total score of SWN was 112, and the mean of SWN scores were 82.88 (SD=16.745). The mean scores of self-control were highest (17.83; SD=3.266), followed by emotional-regulation (17.13; SD=4.501), social-integration (16.75; SD=4.752), mental function (15.95; SD=3.933), and physical function (15.21; SD=3.671).

The internal consistency among the Indonesian version items, as shown by Cronbach's coefficient alpha was high (0.897). This result also showed high internal consistency values of the items, which varied between 0.885 and 0.910 (Table 3). The construct validity of the scale was measured using Pearson correlations analysis. The construct validity for each domain and its total score between 0.768 and 0.885 (Table 4). A confirmatory factor analysis was conducted demonstrated comparative fit analysis index (CFI), the goodness of fit analysis index (GFI), root mean square of approximation (RMSEA) were 0.872 and 0.787, also root mean square error of approximation (RMSEA) were 0.79, respectively (Figure 2).

**Table 1:** Characteristic of respondent (n=108).

| Characteristics | n | % |
|---|---|---|
| **Gender** | | |
| Male | 62 | 57 |
| Female | 46 | 43 |
| **Age, year** | | |
| 18–30 | 25 | 23 |
| 31–49 | 70 | 65 |
| 50–65 | 11 | 10 |
| >65 | 2 | 2 |
| **Marital status** | | |
| Single | 65 | 60 |
| Married | 32 | 30 |
| Divorced | 11 | 10 |
| **Regional** | | |
| East Java and Bali | 57 | 53 |
| Yogyakarta | 7 | 6 |
| Central Java | 22 | 20 |
| West Java and Banten | 11 | 10 |
| Jakarta | 6 | 6 |
| Sumatra and Borneo | 5 | 5 |
| **Educational level** | | |
| Elementary school | 6 | 6 |
| Junior high school | 12 | 11 |
| Senior high school | 40 | 37 |
| Diploma | 15 | 14 |
| Undergraduate or higher | 35 | 32 |
| **Occupation** | | |
| Full time | 31 | 29 |
| Part time | 30 | 28 |
| Not worker | 47 | 43 |
| **Duration of treatment, year** | | |
| <1 | 11 | 10 |
| 1–5 | 39 | 36 |
| 6–10 | 24 | 22 |
| >10 | 34 | 32 |
| **Number of antipsychotics** | | |
| Monotherapy | 24 | 22 |
| 2 antipsychotics | 46 | 43 |
| ≥3 antipsychotics | 38 | 35 |

**Table 2:** Psychometric properties of the Indonesian version scale (n=108).

| | Minimum | Maximum | Mean | SD |
|---|---|---|---|---|
| Total score | 30 | 112 | 82.88 | 16.745 |
| Physical function | 4 | 24 | 15.21 | 3.671 |
| Mental function | 4 | 24 | 15.95 | 3.933 |
| Self-control | 8 | 24 | 17.83 | 3.266 |
| Emotional regulation | 4 | 24 | 17.13 | 4.501 |
| Social integration | 4 | 24 | 16.75 | 4.752 |

**Table 3:** Cronbach's alpha values of reliability tests (n=108).

| Item | Minimum | Maximum | Mean | SD | Cronbach's α if item deleted |
|---|---|---|---|---|---|
| Q1 | 1 | 6 | 4.18 | 1.420 | 0.893 |
| Q2 | 1 | 6 | 4.47 | 1.300 | 0.889 |
| Q3 | 1 | 6 | 4.48 | 1.308 | 0.889 |
| Q4 | 1 | 6 | 4.06 | 1.693 | 0.885 |
| Q5 | 1 | 6 | 3.63 | 1.754 | 0.910 |
| Q6 | 1 | 6 | 3.82 | 1.668 | 0.885 |
| Q7 | 1 | 6 | 4.25 | 1.340 | 0.902 |
| Q8 | 1 | 6 | 4.53 | 1.329 | 0.889 |
| Q9 | 1 | 6 | 3.31 | 1.412 | 0.891 |
| Q10 | 1 | 6 | 4.21 | 1.565 | 0.887 |
| Q11 | 1 | 6 | 3.56 | 1.474 | 0.890 |
| Q12 | 1 | 6 | 3.99 | 1.556 | 0.890 |
| Q13 | 1 | 6 | 4.07 | 1.477 | 0.889 |
| Q14 | 1 | 6 | 4.32 | 1.509 | 0.889 |
| Q15 | 1 | 6 | 4.94 | 1.035 | 0.895 |
| Q16 | 1 | 6 | 3.80 | 1.605 | 0.889 |
| Q17 | 1 | 6 | 3.66 | 1.542 | 0.889 |
| Q18 | 1 | 6 | 4.28 | 1.310 | 0.897 |
| Q19 | 1 | 6 | 4.73 | 0.943 | 0.898 |
| Q20 | 1 | 6 | 4.57 | 1.320 | 0.886 |

**Table 4:** Pearson correlation for each domain of the Indonesian version scale (n=108).

| | Pearson correlations | Sig. (2-tailed) |
|---|---|---|
| Physical function | 0.798** | 0.000 |
| Mental function | 0.794** | 0.000 |
| Self-control | 0.768** | 0.000 |
| Emotional regulation | 0.885** | 0.000 |
| Social integration | 0.884** | 0.000 |

Pearson product moment correlation coefficients: small (0.10–0.29), medium (0.30–0.49), and large (>0.50); **p<0.01.

# Discussion

Disease-specific quality of life and well-being instruments are more sensitive to treatment effects measure than generic instruments [29–31]. Patient report measurements may provide the most direct access to the individual's perceptions domain. The Indonesian version of SWN scale was created as an instrument for research and clinical practice to assess the subjective well-being in different dimensions of patients suffering from schizophrenia disorder medicated with antipsychotics.

The findings of this study showed acceptable internal consistency evidence, as well as construct validity for the modified scale. The modified scale's internal consistency was found not significantly differ from the original version (Cronbach's alpha=0.92), and the subscale reliabilities ranged from 0.818–0.852 [15]. It could, therefore, be concluded that the Indonesian version of SWN scale is internally consistent. Additionally, the principal component analysis results indicated that the Indonesian version is relatively similar to the original version [15]. In addition, the finding shows the correlation score is higher than SWN Turkish version (0.52–0.63) and Estonian version (0.55–0.68) [23, 24].

Recovery condition or functional remission in schizophrenia was determined as the attainment of three criteria: i) the ability to gain a job or voluntary work or to be an active student or head of a family with an engaged partner; (ii) independent life, single or with groups or spouse; and (iii) social connection with more than two contacts in the last 4 weeks or possessing a partner or spouse [32, 33]. Adequate subjective well-being can be used for an early outcome prediction and treatment planning [34]. The criterion of adequate subjective well-being was shown by SWN total score ≥80 points [32]. This study's results indicated more than half of patients with schizophrenia in functional remission condition based on these criteria.

This study shows no difference in the SWN score among participants based on different types and number of antipsychotics. Despite the controversy related to the impact of antipsychotics treatment on subjective well-being [14], this study offers the potential use of the Indonesian version as a scale to measure the subjective well-being of schizophrenia patients.

A cautious interpretation of the result of this study is required due to several methodological limitations. Firstly, this study sample size might not reflect and represent patients' overall condition with schizophrenia in Indonesia, particularly when the respondents in this study were recruited from outpatient settings. This is why further research in the inpatient setting is required, with larger-scale testing is necessary for the future. Secondly, this study did not examine a long-term period of the patient's condition, which this way may ignore any changes during the therapy. Therefore, a longitudinal study is recommended to observe the instrument's effectiveness when dealing with changes over time, including changes in age, social characteristics, and cognitive development. Thirdly, further study is needed to evaluate the Indonesian version's criterion validity, which was not part of this study analysis.

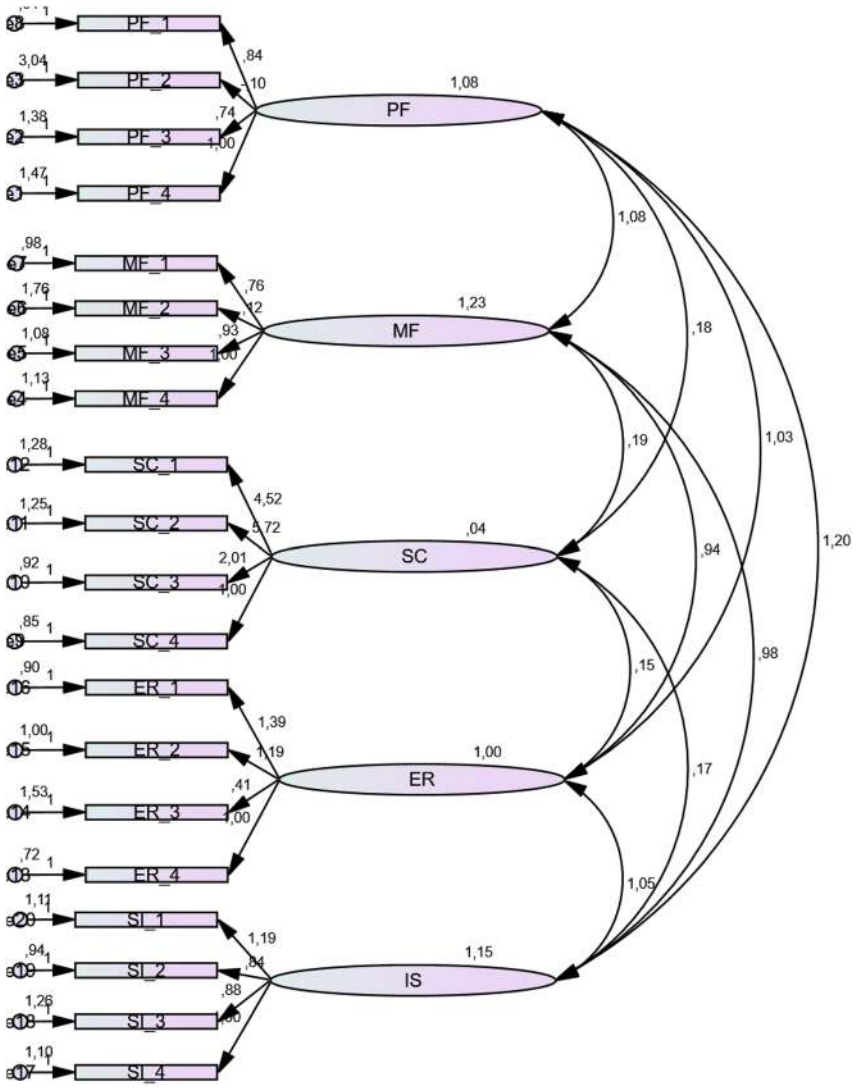


**Figure 2:** Confirmatory factor analysis of IM-SWN (n = 108). *PF=physical function; MF=mental function; SC=self-control; ER=emotion regulation; IS=integration social. CFI=0.871; GFI=0.787; RMSEA=0.79

## Conclusions

This study highlighted that the IM-SWN is a valid and reliable instrument for measuring well-being among the Indonesian population with schizophrenia under neuroleptic treatment.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.

**Ethical approval:** Ethical approval was obtained from the Research Ethics Committee of Menur Mental Hospital Surabaya, East Java, Indonesia with number 070/7556/305/2019.

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Tuhfatul Ulya, Chrismawan Ardianto*, Putri Anggreini, Aniek Setiya Budiatin, Dwi Setyawan and Junaidi Khotib

# Quercetin promotes behavioral recovery and biomolecular changes of melanocortin-4 receptor in mice with ischemic stroke

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**Abstract**

**Objectives:** Ischemic stroke is known as a common causes of disability, lower psychological well-being as well as preventable death. The pathogenesis of ischemic stroke process becomes worse immediately after oxidative stress occurs. One of the flavonoids with antioxidant abilities is quercetin. This study was aimed to investigate quercetin administration on the behavioral functions (motor and sensory) and expression of melanocortin-4 receptor (MC4R) in mice with ischemic stroke.

**Methods:** Male ICR mice were divided into sham, stroke, stroke with quercetin 100, 150, and 200 mg/kg. The stroke model was performed by blocking the left common carotid artery for 2 h. Quercetin was intraperitoneally administered daily for seven days. Evaluation was conducted during two weeks after induction using ladder rung walking test and narrow beam test for motoric function and adhesive removal tape test for sensory function. On day-14 mice were sacrificed, MC4R expression in the dorsal striatum was determined using RT-PCR.

**Results:** Stroke decreased the motor, sensory function and MC4R mRNA expression in dorsal striatum. Quercetin improved motor and sensory function, and upregulated expression of MC4R.

**Conclusions:** Quercetin administration after ischemic stroke improves behavioral function, possibly through the upregulation of MC4R in the brain.

***Corresponding author: Chrismawan Ardianto**, Faculty of Pharmacy, Department of Clinical Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 822 3320 9135, E-mail: chrismawan-a@ff.unair.ac.id

**Tuhfatul Ulya, Putri Anggreini, Aniek Setiya Budiatin and Junaidi Khotib,** Department of Clinical Pharmacy, Airlangga University, Surabaya, Indonesia

**Dwi Setyawan,** Department of Pharmaceutics, Airlangga University, Surabaya, Indonesia

**Keywords:** dorsal striatum; ischemic stroke; melanocortin-4 receptor; motor; preventable death; quercetin.

## Introduction

Ischemic stroke is a major risk factor for disability and the third leading cause of death all worldwide [1]. The rapid oxidation of protein, nucleic acid, and lipid is very worrying in the brain. The pathogenesis process triggers signals for interrelated metabolic processes, specifically decreased ATP production, increased intracellular calcium, and the formation of free radicals end up with neuronal cell death [2]. The condition becomes worse immediately after oxidative stress occurs, followed by the production of surplus reactive oxygen species (ROS) [3].

Oxidative stress is defined as an imbalance between ROS and their quenching by an antioxidant system, due to the overproduction of ROS and lack of antidotes [4]. Lots of evidence has shown that oxidative stress is one of the pathophysiology of early and primary cell death in the pathogenesis of ischemic stroke [2]. Several antioxidants were examined to enhance the defense system against nerve cell degeneration in order to prevent the progression of the harmful mechanisms involved in ischemia [5, 6].

Flavonoids are plant secondary metabolites that have been widely studied. The flavonoid group has a chemical structure that plays a role in its activity as antioxidants [7]. Quercetin is a flavonoid with antioxidant activity due to the presence of catechol groups and OH groups, the antioxidant pharmacophores [8–10]. In cerebral ischemia, the melanocortin system has neuroprotective effects likely mediated by the melanocortin-4 receptor (MC4R). Stimulation of MC4R affords neuroprotection by counteracting inflammation process and apoptosis [11]. However, there is no evidence showing the quercetin effect on the ischemic stroke-induced motor and sensory impairment from day to day, and its correlation with MC4R expression in the dorsal striatum.

The dorsal striatum is known as an important brain area in motor regulation. Previous study showed that injury to the striatum affects the recovery of gait in stroke

patients [12, 13]. Therefore, this study aimed to investigate the effects of quercetin administration as an antioxidant and neuroprotective agent on improving motor and sensory function and biomolecular change (MC4R expression) in dorsal striatum mice with ischemic stroke. This study used a left unilateral common carotid artery occlusion model to induce stroke in mice resulting in hypoxia on the brain tissue [14].

# Materials and methods

## Animals

Male ICR mice, weighing between 25 and 30 g were used. All mice were treated at a temperature of 25 °C ± 2 °C with a humidity of 60 ± 10%, free access to food and water was also provided. All experiments were carried out at the Animal Research Laboratory of the Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. The experimental protocol was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga.

## Drug and stroke surgery

Quercetin (Tokyo Chemical Industry, Tokyo, Japan) was dispersed into a mixture of carboxymethyl cellulose and tween 80 (1:1), with preparation less than 30 min before injection.

After acclimatization, the mice were randomly divided into five groups (n=8 in each group): (1) Sham group, (2) stroke group, (3) stroke with quercetin 100 mg/kg, (4) 150 mg/kg, (5) 200 mg/kg. Xylazine and ketamine were administered for anesthesia. The animals are positioned supine on the surgical table. Around 1 cm incision in the neck midline was made. The left common carotid artery was exposed and blocked by surgical silk for 2 h and then released for reperfusion. The Sham group was subjected to the same procedure without a carotid block. Quercetin-treated groups received a intraperitoneal injection of quercetin 30 min after stroke surgery, another group received an intraperitoneal injection of tween 0.5%. Quercetin was administered once a day for seven days intraperitoneally.

## Ladder rung walking test

The protocol, shown in Figure 1, was performed in accordance to the previous study [15] by using the ladder rung walking test apparatus shown in Figure 1, on day 0 (before stroke surgery), 1, 4, 7, 10, 14 after stroke induction. All mice were allowed to walk through the cylindrical stairs arranged with varying distances in 1-m course. Movement of the right mice hind limb in each step was observed. The camera was used for record it. Mice that slip show a decrease or impaired motor function. A seven-category scale was assessed according to the position of the foot placement on the rungs and the shape of error [16]. All animals were trained and tested five times per-session. Then, the average error score was analyzed. Error score data was presented as a percent of 100%.

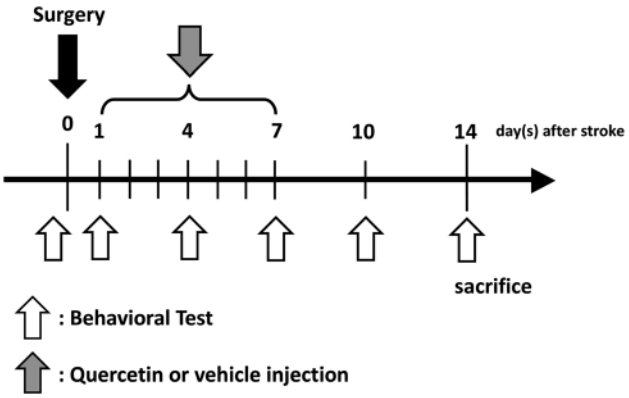


**Figure 1:** Schematic illustration of experimental design for examining the effect of quercetin on behavioral and biomolecular changes after ischemic stroke.

## Narrow beam test

The protocol was performed following the previous study [16]. Mice were placed to cross the narrow beam apparatus. Mice were placed in the initial zone, and the stopwatch started immediately after the animal was released. The time was recorded when the animal starts walking past the start line and get a slip. The time taken by animals to reach the final platform at the end of the beam was also recorded, with a maximum time of 15 s. Both the time until mice are found to slip or fall, as well as the total time taken to cross the beam, were measured for five trials. The tests performed on day 0 (before stroke surgery), 1, 4, 7, 10, 14 (after stroke surgery).

## Adhesive removal tape test

The protocol was performed following the previous study [17] with a few modifications. In this test, adhesive tapes of the same size (1 × 1 cm) is placed on the right forelimb of each animal with equal pressure. Each group was randomized to the order of adhesive placement (right or left). The initial contact time by left forelimb called time to touch, and the time required for the mice to remove the tape called time to remove in a maximum of 120 s was recorded. This test was performed regularly on day 0 (before stroke surgery) and days 1, 4, 7, 10, 14 (after stroke surgery).

## Reverse Transcription - Polymerase Chain Reaction (RT-PCR) Analysis

On day 14, the brain was extracted, the dorsal striatum was dissected and liquid nitrogen was used to immediately freeze the sample. Storage was carried out at −80 °C until use. RT-PCR was performed according to Ardianto et al. [18]. The PureLink® RNA mini kit (Invitrogen, Cat No. 12183018A) was used to isolate the total RNA. GoScript ™ RT (Promega, Cat No. A5000) was used for reverse transcription. The following primers were used: MC4R (forward: 5′-TTA ATA CCT GCT AGA CAA CTC A-3′, reverse: 5′-ATG TAT ACT TCC CTC CAC CTC TG-3′), and β-actin (forward: 5′-ACCCACACTGTGCCCATCTAA-3′, reverse: 5′-GCCACAGGATTCCATTA CCCAA-3′). Amplification was conducted using thermal cycler (Applied

Biosystems, USA) for RT-PCR with total cycle of 33. The denaturation cycle was carried out at 94 °C for 30 s, followed by the annealing at 58 °C for 1 min, and the extension at 72 °C for 1 min. Electrophoresis (MSMIDI Duo, USA) was used to analyze PCR products. Ethidium bromide (Sigma-Aldrich) was used to dye 2% Agarose gel. The band was captured with the aid of UV transillumination. ImageJ software (NIH, MD, USA) was used for image analysis.

### Statistical analysis

Data were presented as mean values ± SEM. Behavioral test and body weight data were analyzed using a two-way ANOVA followed by the Bonferroni post-hoc test. Relative expression of MC4R was analyzed using a one-way ANOVA followed by the Bonferroni post-hoc test. The difference was considered significant if $p<0.05$ (95%).

# Results

## The effect of quercetin on body weight

Animal body weight was measured to determine the effect of quercetin administration and stroke induction on nutritional deficits and metabolic disorders that might occur. The results of weight measurement showed that there were no significant differences between the study groups (Figure 2). In addition, there were no significant changes between giving quercetin at various doses to animal body weight.

## The effect of quercetin on ladder rung walking test

Stroke group with vehicle administration showed a significant decrease in motor function compared to the Sham group (two way ANOVA, stroke, $F_{1,14}=38.59$, $p<0.001$ vs. Sham) starting from day 1 through 14. In contrast, the stroke group treated with quercetin at doses of 100, 150, and 200 mg/kg showed a significant improvement in motor function when compared to the stroke group with vehicles, starting from day 1 and gradually increasing on days 4 through 14 (Figures 3 and 4).

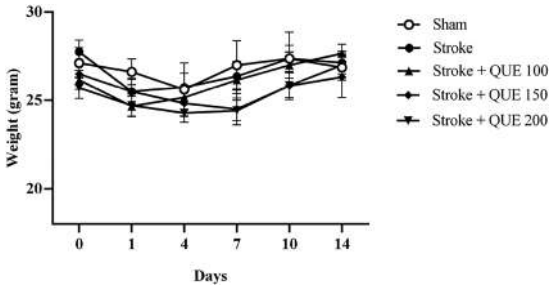


**Figure 2:** The effect of quercetin on body weight of mice induced ischemic stroke (mean ± SEM) of 6–8 mice. Administration of quercetin were conducted repeatedly day 1–7. QUE, quercetin.

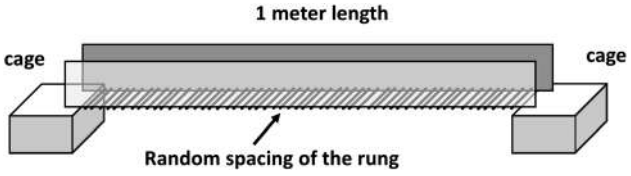


**Figure 3:** Ladder rung walking test apparatus for ladder rung walking test measurement.

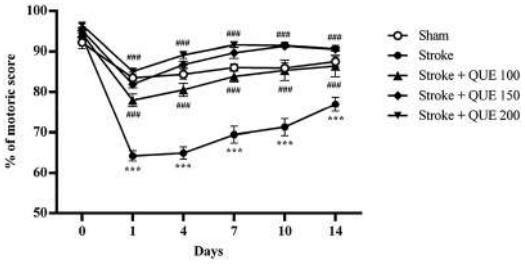


**Figure 4:** The motor function of ischemic stroke mice measured by ladder rung walking test (mean ± SEM) of 6–8 mice. ***$p<0.001$ vs. Sham group. $^{###}p<0.001$ vs. stroke group. QUE, quercetin.

## The effect of quercetin on narrow beam test

In addition, we also examined the effect of quercetin on stroke induction on motor function improvement using the narrow beam test. The results of the latency to slip showed that the stroke group had a significantly lower motor function compared to the Sham group on day 4 (Figure 5A). This was evidenced by mice that slips faster than the Sham group. Furthermore, the stroke group showed a decrease in motor function by spent more time to reach the finish, the difference was significant compared to the Sham group on day 4 (Figure 5B).

In contrast, the stroke group with quercetin administration (200 mg/kg) showed significantly improved motor function by increased time latency to slip on day 4. Then, the stroke group with quercetin at a dose of 150 and 200 mg/kg significantly suppressed time to reach the finish compared with the stroke group starting on day 1 (Figure 5).

## The effect of quercetin on adhesive removal tape test

The effects of stroke induction and quercetin administration on the sensory functions of mice showed the stroke

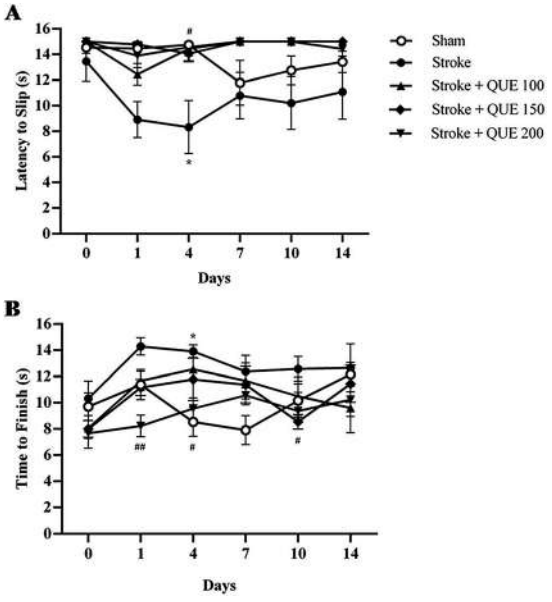


**Figure 5:** The effect of quercetin on motor function of ischemic stroke mice. (A) Latency to slip and (B) time to finish the beam were measured by narrow beam test (mean ± SEM) of 6–8 mice. *p<0.05 vs. Sham group. #p<0.05, ##p<0.01 vs. stroke group. QUE, quercetin.

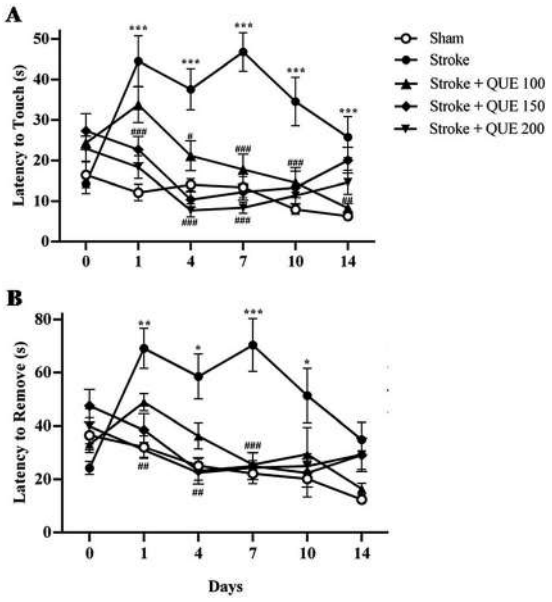


**Figure 6:** The effect of quercetin on sensory functions of mice induced by ischemic stroke. (A) Latency to touch the tape and (B) latency to remove the tape were measured by adhesive removal tape test (mean ± SEM) of 6–8 mice. *p<0.05, **p<0.01, ***p<0.001 vs. Sham group. #p<0.05, ##p<0.01, ###p<0.001 vs. stroke group. QUE, quercetin.

group had a significantly lower function than the Sham group on the latency to touch (Figure 6A), and latency to remove (Figure 6B) values starting on day 1, followed by day 4 through 14.

Furthermore, administration of quercetin (150 and 200 mg/kg) was significantly improves sensory function compared to the stroke group in the latency to touch from day 1, whereas doses 100 mg/kg from day 4 through 14. Then, administration of quercetin doses of 150 and 200 mg/kg significantly suppressed the latency to remove time compared to the stroke group starting on day 1, whereas doses 100 mg/kg from day 7 (Figure 6).

## The effect of quercetin on melanocortin-4 receptors

The results of examination MC4R expression in the dorsal striatum of the brain showed a significantly lower MC4R expression on stroke group compared to the Sham group (one way ANOVA, stroke, $F_{2,6}$=357.6, p<0.01 vs. Sham). In contrast, the stroke group with 100 mg/kg quercetin administration significantly increased MC4R expression compared to the stroke group (p<0.001 vs. stroke) on days 14 (Figure 7).

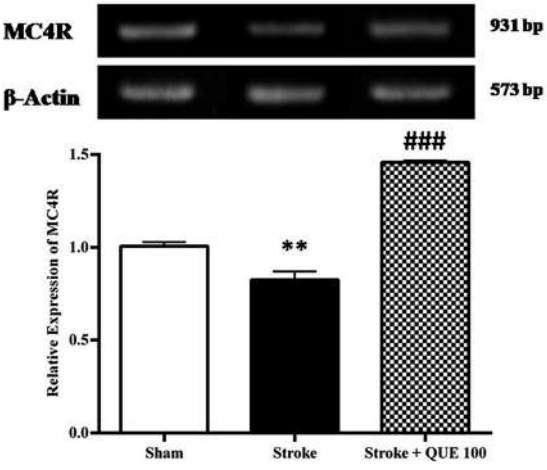


**Figure 7:** The effect of quercetin on relative expression of MC4R in dorsal striatum of mice induced by ischemic stroke (mean ± SEM) of three mice. **p<0.01 vs. Sham group. ###p<0.001 vs. stroke group. Bp, base pair; MC4R, melanocortin-4 receptor; QUE, quercetin.

# Discussion

This study was designed to determine changes of behavioral function (motor and sensory) and biomolecular changes in MC4R expression in the brain of mice after

stroke induced by the left unilateral common carotid artery occlusion method, and the improvements due to quercetin treatment. This stroke induction method is known to cause impairment of motor and sensory function and lead to neuronal cell death in the brain [14].

Quercetin administration did not affect mice's body weight from day 1 to day 14. This result is in agreement with studies from Barrenexte et al. [19] that dietary foods containing 0.5% quercetin for 28 days did not affect the body weight of mice compared to the control group. Similar results were shown by a high-sucrose diet containing 0.07, 0.2, and 0.02% quercetin for four weeks, which does not affect the body weight of rats [20].

The motor function examination results using the ladder rung walking test method showed a significant attenuation in motor function impairment after quercetin administration in days 1, 4, 7, 10, and 14 compared to the stroke group. Giving quercetin at a dose of 100, 150, and 200 mg/kg improves motor function in ischemic stroke conditions. Similarly, the results of the motor function test using the narrow beam test method also showed notable improvement with quercetin administration as compared to the stroke group. The result showed a dose-dependent trend of the effect of quercetin to improve motor function. A previous study reported that the post-injury administration of quercetin (50 mg/kg) improves the motor function between day 1 until 5 after trauma compared with the traumatic brain injury group [21]. Together, these results suggest that quercetin attenuates the stroke-induced motor function deficits.

Furthermore, our present data show stroke-induced sensory impairment that did not recover in a two-week examination period. Moreover, the present study revealed that quercetin (100, 150, and 200 mg/kg) decreased the time to touch and to remove the adhesive tape in ischemic stroke mice, starting from day 1 after stroke induction. A study from Chen et al. [22] showed that quercetin administration accelerates full sensory recovery in 18 days in mice with sciatic nerve-crush injury compared to the untreated mice. Together with our result, it is suggested that quercetin attenuates the sensory function impairment after an ischemic stroke attack. Furthermore, we found that all doses (100, 150, and 200 mg/kg) effectively improve stroke related-behaviors. Each dose has a certain level of speed in improving motor and sensory function. Although higher doses showed faster improvement, the three doses were not significantly different, meaning they were equally effective in improving motor and sensory function due to stroke in a relatively fast time.

It is well known that melanocortin system activity is involved in responding to the neuroinflammatory process in the brain. The activation, as well as the upregulation of MC4R, has been the biomolecular marker representing the anti-inflammatory process [23]. MC4R is reported expressed in the dorsal striatum, one of the critical brain regions that regulate motor function [24]. Therefore, we investigate the expression of MC4R in the dorsal striatum and effect of quercetin. Since we already found that 100, 150 and 200 mg/kg dose of quercetin are effective in ameliorates the stroke related-behaviors, for MC4R expression analysis we used representative yet effective dose of quercetin which is 100 mg/kg. Our present study revealed that the MC4R mRNA level in the dorsal striatum decreased in stroke. Furthermore, we found that treatment with quercetin ameliorated the decrease MC4R mRNA level in the dorsal striatum. A previous study showing that MC4R mRNA was upregulated in the striatum after severe hypoxic-ischemic brain injury compared with control [25]. The present result suggests that the upregulated MC4R mRNA in the dorsal striatum may reflect the brain capability to reduce the inflammatory process and restore the deficit in brain function affected by an ischemic stroke.

Previous study showed that MC4R stimulation prevents mitochondrial stress and oxidative damage due to ethanol through the activation of the Nrf-2 pathway in neuron culture [26]. In addition, quercetin has been reported to modulate the activation of the Nrf-2-ARE pathway [27]. Nrf2-ARE pathway activation increases the expression of detoxification enzymes such as glutamate-cysteine ligase and synthesis of glutathione, an endogenous antioxidant [28]. In conclusion, our present results indicate that quercetin attenuates the motor and sensory deficits induced by an ischemic stroke in mice. Additionally, it is suggested that quercetin reverse the functional impairment through the upregulation of MC4R in the dorsal striatum. Further study is needed to clarify the direct evidence explaining how the quercetin affects the expression of MC4R.

## Conclusions

This study shows that quercetin ameliorates motor and sensory impairment due to ischemic stroke conditions. Further, it is suggested that quercetin may promotes functional recovery from ischemic stroke condition by the MC4R upregulation.

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**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical approval:** All experiments were performed at the Animal Research Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia in accordance with the Guide for care and use of laboratory animal issued by National Institution of Health revised in 1985. The experimental protocol was approved with letter No: 2.KE.111.07.2019 by the Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga.

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Desak Ketut Ernawati*, Ida Ayu Alit Widhiartini and Endang Budiarti

# Knowledge and attitudes of healthcare professionals on prescribing errors

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**Abstract**

**Objectives:** This study aimed to evaluate the knowledge and attitudes of healthcare professionals on prescribing errors.

**Methods:** This was a cross-sectional study employing a questionnaire that consisted of 12 items on knowledge and 10 items on healthcare professionals' attitudes toward errors in prescribing process. The participants responded to the questionnaire with a 5-Likert scale of agreement. The domains assessed in the questionnaire were respondents' knowledge and attitudes on prescribing errors, professionals responsible for the errors, and professionals' competence on drug dose adjustment. Additionally, the questionnaire had two case scenarios to further assess the healthcare professionals' knowledge of prescribing errors. There were 300 questionnaires administered to physicians, nurses, and pharmacists who attended conferences in Denpasar from July to October 2019.

**Results:** There were 30 physicians, 58 nurses, and 69 pharmacists who responded to the survey. A response rate of 52.3% (157/300) was obtained. All healthcare professionals agreed that errors may occur in prescribing, dispensing, and administration process. All healthcare professionals understood that physician is responsible for ensuring drug safety in prescribing process and also supported a standardized form on drugs which may need drug dose personalization. Concerning item on the importance of collaboration in drug dose adjustment, although the healthcare professionals agreed on the statement, they had significant differences on the level agreement on the statement (p=0.029). The healthcare professionals also supported having regular training on drug dose adjustment based on individual patients' regimentation. The healthcare professionals' responses showed that the significant differences found on the statement of healthcare professionals should have competency on personalized dose calculation (p<0.001). All healthcare professionals agreed that physicians should have competency on drug dose adjustment, yet physicians showed less agreement that other health professionals should have the competency.

**Conclusions:** All healthcare professionals understood that medication errors may occur during the prescribing process but showed different attitudes on professionals who had competence in drug dose calculation. They emphasize the need to have a standardized prescription format for medication with dose changes. The respondents also recommend having regular training on medication safety for healthcare professionals.

**Keywords:** attitudes; healthcare professionals; knowledge; prescribing errors.

## Introduction

Patient safety is the standard of quality in healthcare services. In the context of patient safety, the safe use of medication is prominent. There is no clear definition of medication safety [1], in the current study, the safe use of medication will be focused on prescribing errors. A study involving over a thousand hospitals in the United States found that medication errors occurred every 22.7 h in each hospital [2]. The errors impacted adverse outcomes of 0.25% of the patients. A systematic review of medication error studies in South East Asia showed that dosing error was one of the frequent errors mostly reported in prescribing stage [3].

In the current hospital practice, the process of medication administration begins when the physician prescribes for the patients. The medication is prepared at the pharmacy and the hospital setting, the nurse administers the medication to the patients. The prescription is a piece of paper on which a doctor writes an order for medicine and which you give to a chemist or pharmacist to get the medicine [4]. The drug regimen on the prescription states the drug dose that applies to the general patient population. In its

***Corresponding author: Desak Ketut Ernawati,** Department of Pharmacology and Therapy, Universitas Udayana, Denpasar, Indonesia, Phone: +62 361 222510, E-mail: ketuternawati@unud.ac.id. https://orcid.org/0000-0003-0668-5429
**Ida Ayu Alit Widhiartini,** Department of Pharmacology and Therapy, Universitas Udayana, Denpasar, Indonesia
**Endang Budiarti,** Bethesda Hospital, Yogyakarta, Indonesia

development, a fixed drug dose cannot be applied to all patients. However, most hospitalized patients may need a more complex medication regimentation. Some patients may require dose adjustment based on their clinical status. For instance, the dose of intravenous dobutamine may be given based on the patient's blood pressure, heart rate, and volume of distribution. The dose adjustment is constantly changed thus healthcare professionals involved in the medication process need to accurately calculate the drug dosage based on the patient's response.

Prescribing error is one of the errors which may occur during the medication process [5]. There are different types of prescribing errors which range from an allergic reaction, wrong patient, wrong dose, wrong dosage forms, route of administration, and calculation errors. Studies showed that prescribing errors occurred in 1–14% on medication orders [6, 7]. The wrong dose was the most frequent errors identified in prescribing process in an Indonesian hospital setting [8]. While little is known on medication errors in primary care or community setting, potential serious outcomes were more likely to occur in parenteral administration, particularly cardiovascular and endocrine medication [9]. It has been also emphasized that some sources of errors may result in medication errors. The sources were working environment, high workload, communication failure, and lack of knowledge may contribute to the errors [6, 10]. Thus, this study aimed to assess the level of knowledge and attitudes of healthcare professionals in Denpasar on medication errors particularly on prescribing errors.

## Materials and methods

This was a cross-sectional study involving questionnaire administration to physicians, nurses, and pharmacists who attended seminars in Denpasar from July to October 2019. These healthcare professionals were selected because they are the professionals who are involved in the medication administration process. Permissions were obtained from the seminar committees before questionnaire administrations. Inclusion criteria were physicians, nurses, and pharmacists who attended conferences and responded to the questionnaire. Exclusion criteria were health professionals who refused to participate and incomplete responses. Ethical clearance was granted from the Faculty of Medicine, Udayana University, Ethical Committee Boards with ethical clearance number 1879/UN 14.2.2.VII.14/LP/2019. The questionnaire was developed to assess the level of knowledge and attitudes of the healthcare professionals on prescribing errors. Face and content validity were obtained by administering the questionnaire to 5 academics and 5 practitioners. Some amendments were made based on comments received during validation such as the sequence of statements of the questionnaires and rewording of items for clarity. The questionnaire consisted of 12 items on knowledge and 10 items on attitudes toward prescribing errors. Statements to assess knowledge on prescribing errors included where errors may occur during the medication process, professionals who responsible for prescribing errors, professionals' competence on drug dose adjustment, and knowledge on the needs of a standard format for prescription and on collaboration. Statements to evaluate attitudes included agreement on having a standard format of prescription and guideline for patients required dose adjustment, agreement on professionals who have the competencies in drug dose adjustment, and support on training, evaluation, and reporting on prescribing errors. Each item had 5 Likert scales in which 1 showed strong disagreement to 5 for strong agreement to the statement. Responses of healthcare professionals on the items were analyzed using Analysis of Variance (ANOVA). Two case studies on prescribing errors were also employed to assess healthcare professionals' understanding of the clarity of a prescription, drug dose preparation, administration, and recommendations from lessons learned from the cases.

## Results

Of the 300 questionnaires administered to seminars attended by doctors, nurses, and pharmacists, 157 questionnaires were returned. This gave a 52.3% of response rate. Demographic information of respondents can be seen in Table 1.

Table 1 shows the majority of healthcare professionals across the professions were female and worked in the hospital. The healthcare professionals indicated that they spent around 4–11 h per week activities on continuing professional development. The respondents have been working for more than 5 years. The majority of respondents were working at the hospitals.

Table 2 shows that although all healthcare professionals had strong agreements that medication errors may occur in any stage of the medication process (Items 1–3), pharmacists had higher scores than other health professions on the items. Pharmacists had also higher score on the statement that physician has the responsibility in prescribing errors.

**Table 1:** Participants demographic.

| | Physician (n=30) | Nurse (n=58) | Pharmacist (n=69) |
|---|---|---|---|
| Sex, % male/female | 43/57 | 26/74 | 18/82 |
| Time spent on CPD, hours/week | 3.9 ± 4.7 | 4.4 ± 5.1 | 11.2 ± 15 |
| Means of working experiences, years | 5.5 ± 7.9 | 8.5 ± 9.3 | 7.1 ± 5.9 |
| Place of work, % | | | |
| Hospital | 47 | 40 | 92 |
| Clinic | 3 | 2 | 0 |
| Public health centre | 7 | 12 | 2 |
| Community pharmacy | 17 | 11 | 3 |
| Others | 26 | 35 | 3 |

Statement on health professionals who have the competence in ensuring the safe use of medication during the medication process (Items 7–9) showed that the health professions involved in the medication process realized that the respective professions have the responsibilities in ensuring the safe use of medication. For instance, on Item 7 (only the physician answered the statement), the physician had the highest agreement. Meanwhile, on Item 8, the pharmacist showed the highest score of agreement on the statement. Similarly, on Item 9, the nurse group showed agreement on the statement that they are capable of administering a medication that needs dose adjustment. This means that the healthcare professionals understood errors that may occur in the medication process and knew their roles during the process

Table 3 shows that all healthcare professionals agreed that a standard format of prescription and a guideline for dose adjustment is required for the patient in need. Although all healthcare professionals also agreed that physicians should be able to calculate the dose adjustment based on the patient's clinical status, some disagreement was found as to whether pharmacists and nurses should be able to calculate the dose. Meanwhile, all healthcare professionals had a similar agreement on having regular training on drug dose adjustment. Both pharmacist and nurse groups agreed that they need to be able to calculate the dose, while other professions showed fewer agreements.

There were two cases of prescribing errors employed. One case was on Nicardipin to control the patient's blood pressure post-stroke hemorrhage. The patient blood pressure was 200/130 mmHg with targeted blood pressure at 150/100 mmHg. In the case scenario, it was written that the Nicardipin dose was as required to reach the targeted blood pressure. The other case was on Nitroglycerin which was given to a patient with a blood pressure of 190/110 mmHg. In the case scenario, Nitroglycerin injection was given at 6 mL/h, and the dose increases every 0.5 mL/h until the targeted blood pressure reached 120 mmHg. For both cases, participants responded on dose clarity in the prescription, medication preparation, problems related to their competence, and recommendation as well as lessons learned from the cases. It was found that physicians, nurses, and pharmacists were able to identify the problems based on their professionals' competencies. They also were able to identify the unclear dose, duration, dosage forms as well as route of administration in the cases. Interestingly, although they identified incomplete information on drug dosing, the majority of the respondents suggested preparing the medication as written in the cases. A small proportion of respondents indicated asking for clarification from the prescriber. The respondents mentioned that clarity on drug dose, duration, frequency, drug dosage forms, and route of administration as lessons learned in ensuring the safe use of medication. The respondents also

**Table 2:** Means comparison of items on knowledge on prescribing errors.

| No | Items | Physician | Nurse | Pharmacist | p-Value |
|---|---|---|---|---|---|
| 1 | Medication errors may occur in prescribing process | 4.2 ± 0.8 | 4.1 ± 0.8 | 4.5 ± 0.9 | 0.037* |
| 2 | Medication errors may occur in transcribing process | 4.2 ± 0.5 | 4.1 ± 0.8 | 4.5 ± 0.8 | 0.010* |
| 3 | Medication errors may occur in the administration process | 3.9 ± 0.7 | 3.9 ± 0.8 | 4.3 ± 0.8 | 0.012* |
| 4 | Physician is responsible for prescribing errors | 4.1 ± 0.7 | 4.0 ± 0.8 | 4.5 ± 0.8 | 0.010* |
| 5 | Pharmacist is responsible for prescribing errors | 3.7 ± 0.9 | 3.6 ± 1.0 | 3.4 ± 1.3 | 0.498 |
| 6 | Nurse is responsible for prescribing errors | 3.3 ± 1.1 | 2.8 ± 1.5 | 2.8 ± 1.3 | 0.128 |
| 7 | I am capable of prescribing for the patient who needs dose adjustment (for physician) | 4.2 ± 0.6 | – | – | – |
| 8 | I am capable of dispensing a prescription for the patient who needs dose adjustment (for pharmacist) | – | – | 4.1 ± 0.7 | – |
| 9 | I am capable of administering medications for the patient who needs dose adjustment (for nurse) | – | 3.5 ± 1.0 | – | – |
| 10 | Health professionals need a standard prescription format for patient who need dose adjustment | 4.2 ± 0.5 | 4.2 ± 0.8 | 4.3 ± 0.8 | 0.587 |
| 11 | A careful drug dose calculation as an intra-vascular route of administration could reduce dosing errors | 4.0 ± 0.9 | 4.2 ± 0.7 | 4.4 ± 0.8 | 0.099 |
| 12 | Physician, nurses, and pharmacist need to work in collaboration to ensure an accurate drug dose calculation for patient in need | 4.4 ± 0.5 | 4.2 ± 0.7 | 4.6 ± 0.8 | 0.029* |

*$p<0.05$ showed a statistically significant difference in each group.

**Table 3:** Means comparisons of items on attitudes toward prescribing errors.

| No | Item | Physicians | Nurses | Pharmacists | p-Value |
|---|---|---|---|---|---|
| 1 | Standard format of prescription is required in patients who need dose adjustment based on clinical status | 4.2 ± 0.5 | 4.2 ± 0.8 | 4.3 ± 0.8 | 0.409 |
| 2 | A guideline on dose adjustment based on patient's clinical status is required to ensure the safe use of medication | 4.3 ± 0.4 | 4.3 ± 0.5 | 4.4 ± 0.8 | 0.713 |
| 3 | Physician should be able to calculate drug dose adjustment based on patient's clinical status | 4.3 ± 0.5 | 4.3 ± 0.5 | 4.1 ± 1.0 | 0.075 |
| 4 | Pharmacist should be able to calculate drug dose adjustment based on patient's clinical status | 3.6 ± 1.4 | 4.1 ± 0.8 | 4.6 ± 0.8 | 0.001* |
| 5 | Nurse should be able to calculate drug dose adjustment based on patient's clinical status | 3.4 ± 1.5 | 4.1 ± 0.7 | 2.8 ± 1.2 | 0.001* |
| 6 | Regular training on prescribing of drug dose adjustment based on patients' clinical status is required | 4.2 ± 0.6 | 4.4 ± 0.6 | 4.5 ± 0.8 | 0.258 |
| 7 | Regular evaluation of healthcare professionals' competence on ensuring the safe use of medication is required | 4.3 ± 0.5 | 4.4 ± 0.5 | 4.5 ± 0.8 | 0.641 |
| 8 | Healthcare professionals' motivation may influence working performance to ensure patient safety | 4.2 ± 0.5 | 4.4 ± 0.5 | 4.4 ± 0.8 | 0.362 |
| 9 | Prescribing errors should be reported to improve healthcare service | 4.1 ± 0.5 | 4.4 ± 0.5 | 4.5 ± 0.8 | 0.005* |
| 10 | All healthcare professionals have the obligation to report prescribing errors | 4.1 ± 0.4 | 4.3 ± 0.5 | 4.5 ± 0.8 | 0.057 |

*p<0.05 showed a statistically significant difference in each group.

recommended that a standard format for prescription and collaboration between physicians, nurses, and pharmacists are important in medication safety. Collaboration in terms of communication for confirmation of drug dose clarity before drug administration.

## Discussion

Findings of this study suggested that healthcare professionals who responded in the current study understood that medication errors may occur during the medication administration process. The professionals have to perform their responsibility in ensuring the safe use of medication. Healthcare professionals need to be aware of their contribution to the errors thus they could prevent the errors proportionally based on their knowledge and competence. Healthcare professionals need to be aware of their contribution to the errors thus they could prevent the errors from occurring. Drug dose knowledge was one of the knowledge gaps identified amongst ICU nurses [10]. The authors of the study also found that nurses in ICU have a lack of knowledge on the common medication used and some errors identified in the setting. This highlights the importance of drug knowledge in reducing medication errors.

This study found that although healthcare professionals agreed on the statement on the importance of collaboration is required in ensuring safe drug dosing, the p-value on Item 12 showed a significant difference. The posthoc test indicated that the different score was significant between nurse and pharmacist (p=0.008). This suggests that physicians and pharmacists had more agreement than nurses on the importance of collaboration to ensure the accurate drug dose calculation for the patient in need. Although mixed results were found in the literature on the impact of collaboration, the importance of collaboration in ensuring medication safety was highlighted [11]. Manias highlighted opportunities for collaboration in enhancing medication safety in the future particularly across transitional care. The results in Table 3 indicate that healthcare professionals' understanding of the potential contribution of nurses and pharmacists in errors on drug calculation is lacking. This may be a reflection of the role of nurses on medication based on the physician's instruction [12]. However, nurses need to be able to calculate the correct dose of the drug to minimize the risk of dosing errors because the nurses administer the medication to the patients. Thus, training for nurses on drug dose calculation may be needed.

In terms of reporting prescribing error, although all healthcare professionals agreed that reporting is their obligation, yet physician group showed less agreement that the reporting will improve healthcare service. These suggest the need of having training or workshop on medication errors is required although the results of the current questionnaire identified the healthcare professionals knew medication errors. This study also indicated that physicians, nurses, and pharmacists agreed that regular training on prescribing for the drug with dose adjustment is required as well as a regular evaluation on competencies of ensuring the safe use of

medication. Some methods are found in the literature on a model of training on interprofessional collaboration [13–16]. One of the approaches identified was employing a behavioral science to improve nurses training on drug dose calculation [14]. This model of training which is conducted with a partnership with other healthcare professionals may facilitate improvement toward knowledge of medication safety.

The two case scenarios employed in the current study showed similar results to the questionnaire on prescribing errors. Incongruent, the healthcare professionals also recommended collaboration amongst healthcare professionals and a standardized format of prescription in ensuring medication safety. A protocol and guideline were indicated as an area for a communication tool in interdisciplinary in medication errors [11]. The standardized format of prescription for medication that required dose adjustment should facilitate a well-written communication between healthcare professionals. Further, bridging a relation and negotiation between tasks and roles of healthcare professionals are considered ways to collaborate.

Despite the findings of this study, it has some limitations. Firstly, the response rate was around 50%. This was a reflection of the nature of the survey type of study design. Secondly, there may be a selection bias during data collection, because the respondents were administered during conferences attended by the healthcare professionals. The questionnaire was administered at three international and national conferences conducted in Denpasar in 2019. Topics of the conferences covered interdisciplinary approaches to healthy aging, medication safety in hospital pharmacy, and international conference on health science. Lastly, Findings on knowledge and attitudes of healthcare professionals in the current study were unable to be analyzed using factorial analysis because the limited number of respondents participated in the questionnaire.

## Conclusion

This study found that healthcare professionals knew errors may occur in the medication process particularly in prescribing errors. However, the professionals had different attitudes toward professionals who had competence in drug dose calculation. The healthcare professionals agreed toward having a standardized format of prescription in the patient who needs various drug dose regimentation. They also supported having regular training on medication safety particularly on prescribing errors to ensure the safe use of medication.

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Aguslina Kirtishanti, Siswandono Siswodihardjo*, I Ketut Sudiana, Desak G. A. Suprabawati and Aristika Dinaryanti

# Inhibition of Ras and STAT3 activity of 4-(*tert*-butyl)-*N*-carbamoylbenzamide as antiproliferative agent in HER2-expressing breast cancer cells

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**Abstract**

**Objectives:** Human epidermal growth factor receptor type 2 (HER2)-expressing breast cancer patients indicate poor prognosis in disease progression. HER2 overexpression can increase activities of Ras-mitogen activated protein kinase (Ras-MAPK) pathway and Janus Kinase (JAK)-STAT3, increasing breast cancer cell proliferation as demonstrated by marker Ki67. Therapeutic options for HER2-expressing breast cancer are limited and have major side effects, so anticancer development as an antiproliferative is needed. From previous research, synthetic chemical 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound has cytotoxic activity *in vitro* on HER2-expressing breast cancer cells. This study wanted to determine the mechanism 4TBCB compound in inhibiting HER2 signaling through Rat Sarcoma (Ras) and signal transducer and activator of transcription 3 (STAT3) pathway in HER2-expressing breast cancer cells.

**Methods:** Breast cancer cells were isolated from the biopsy tissue of breast cancer patients. The isolated cells were cultured and given 4TBCB test compound with three concentrations (0.305, 0.61, and 1.22 mM) and lapatinib 0.05 mM as a comparison compound. Cancer cell cultures were stained with monoclonal antibodies phosphorylated HER2 (pHER2), phosphorylated Ras (pRas), phosphorylated STAT3 (pSTAT3), and Ki67. The expression of pHER2, pRas, pSTAT3, and Ki67 proteins was observed using the immunofluorescence method and the results were compared with control cells, namely cancer cells that were not given 4TBCB and lapatinib but stained with monoclonal antibodies.

**Results:** 4TBCB compounds (0.61 and 1.22 mM) and lapatinib can reduce pHER2, pRas, pSTAT3, and Ki67 expressions compared to control cells.

**Conclusions:** 4TBCB compounds (0.61 and 1.22 mM) can reduce pHER2, pRas, pSTAT3, Ki67 expressions and predicted to inhibit HER2 signaling through the Ras and STAT3 pathways in HER2-expressing breast cancer cells.

**Keywords:** antiproliferative; breast cancer cells; HER2; Ras; STAT3; 4-(*tert*-butyl)-*N*-carbamoylbenzamide.

## Introduction

Breast cancer is the second most malignant disease of all types of cancers in the world in 2018 and becomes an alarming disease for women around the world [1]. Twenty-five to thirty percent of patients with breast cancer are expressed with human epidermal growth factor receptor type 2 (HER2) excessively and this correlates with the increase of aggressiveness, poor prognosis, and shorter survival periods [2, 3]. HER2 is a family of human epidermal growth factor receptor (HER) that has a tyrosine kinase activity. HER2 receptor dimerization causes autophosphorylation of tyrosine residues in the cytoplasmic intracellular domain, thus, it activates HER2 signaling pathways, including Ras-mitogen activated protein kinase (Ras-MAPK) pathway, signal transducer and activator of transcription 3 (STAT3) and phosphoinositide-3-kinase-Akt (PI3K-Akt). Activating the HER2 signaling pathway leads to cell proliferation, cell differentiation, adhesion, migration, as well as cell survival [4–6]. HER2 activation enhances the level of Rat Sarcoma (Ras). In a study conducted by Eckert, five out of nine breast cancer cell lines exhibit an increase of the active Ras-GTP level due to HER-2 activation [7]. The active Ras phosphorylates and

***Corresponding author: Siswandono Siswodihardjo**, Department of Medicinal Chemistry, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +62 8123206328, E-mail: prof.sis@ff.unair.ac.id
**Aguslina Kirtishanti,** Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia
**I Ketut Sudiana,** Department of Pathology, Faculty of Medicine, University of Airlangga, Surabaya, Indonesia
**Desak G. A. Suprabawati,** Department of Oncology Surgery, Dr. Soetomo General Hospital, Surabaya, Indonesia
**Aristika Dinaryanti,** Stem Cell Research and Development Center, University of Airlangga, Surabaya, Indonesia

activates the next Raf, and subsequently activates Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase (MEK) and Erk1 and Erk2 [5, 7]. Ras-MAPK pathway involves a protein kinase cascade which contributes to regulate cell proliferation and cell survival [8, 9]. The activation of the STAT3 pathway on HER2 overexpression has been studied by Chung in breast cancer cell culture. His research stated that the STAT3 mRNA expression increased 3.62 times in Michigan Cancer Foundation (MCF)-7/HER2 compared to MCF-7-cell culture [10].

Ki67 is a marker related to cell proliferation assessing the growth of malignant cells. Ki67 is expressed throughout the cell cycle and the peaks during mitosis and at a lesser extent in normal breast tissue from estrogen receptor negative cells than in breast cancer tissue. Therefore, Ki67 can be used as an additional factor for decision making in developing treatment strategies for breast cancer patients [11].

HER2 positive breast cancer therapy is limited, with major side effects. One anticancer used for HER2 positive breast cancer is lapatinib, a tyrosine kinase inhibitor that inhibits the phosphorylation of tyrosine residues in the intracellular domain of the HER2 and EGFR cytoplasmic receptors, which causes inhibition of intracellular signaling pathways that reduce cell proliferation and induce apoptosis [12]. Currently, lapatinib is known to build resistance in a number of breast cancer patients [12, 13]. This is a significant underlying cause for developing new anticancer drugs with maximum therapeutic effect in inhibiting HER2 signaling in the Ras and STAT3 pathways.

Urea-derivative compounds have been extensively developed due to its cytotoxic activity against breast cancer cell lines. The synthesis of *N*-(phenylcarbamothioyl)-benzamide derivatives against MCF-7 cell lines has been reported to have better $IC_{50}$ value compared to hydroxyurea, the least complex derivative of urea [14]. Li et al., synthesized and investigated the structure–activity relationship of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas derivative compound and thiourea derivatives and found them to potentially inhibit EGFR and HER2 kinase, as well as inhibit MCF-7 cell proliferation [2]. The docking, synthesis, and cytotoxic test against T47D cell lines on *N*-(allylcarbamothioyl) benzamide compounds, yielded better outcome on $IC_{50}$ (56.50 μg/mL) compared to 5-fluorouracil (5FU=132.37 μg/mL) [15].

Another way to develop anticancer drugs is by structure modification. The 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound is derived from structure modification of a parent compound *N*-carbamoylbenzamide aiming to enhance its cytotoxic activity. From previous research, it has known that 4TBCB cytotoxic activity of HER2-expressing breast cancer cells using the MTT (Microculture Tetrazolium) method gave an $IC_{50}$ value of 0.61 mM and hydroxyurea with an $IC_{50}$ value of 11.61 mM. This suggests that 4TBCB compounds have better cytotoxic activity to choose compared to hydroxyurea [16]. Therefore, 4TBCB can be used as anticancer candidates for HER2-expressing breast cancer. Meanwhile, this study aims to investigate the mechanism of the 4TBCB as an inhibitor of HER2 signaling on Ras and STAT3 pathway against HER2-expressing breast cancer cells.

# Materials and methods

### Isolation of breast cancer cells

This research is under the approval of the Health and Research, Ethical Commission of RSUD Dr. Soetomo Surabaya with ethical clearance number 1456/KEPK/VIII/2019. Breast cancer cells were isolated from the biopsy tissue of breast cancer patient, washed with NaCl solution, chopped and given 0.075% collagenase type 1 (Worthington, USA) for 45 min at 37 °C, then filtered with cell strainer. The supernatant obtained was centrifuged at 3,000 rpm for 5 min. Cell pellets were washed with PBS and centrifuged again for 5 min. Cell pellets were cultured in alpha Minimal Essential Medium (MEM) (Gibco, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Gibco, USA), 1% penicillin–streptomycin (Gibco, USA), and 1% amphotericin B (Sigma-Aldric, USA), then incubated in a $CO_2$ incubator at 37 °C. The medium was replaced every 3 days and after the cells reached 90%, confluent cell passage was carried out [17]. After isolation, cells were identified against cell surface markers (CD24, CD44, and CD90) by flow cytometry to ensure that the isolated cells were breast cancer cells. The cells were identified using HER2 immunofluorescence monoclonal antibody.

### Expressions of pHER2, pRas, pSTAT3, and Ki67 protein using immunofluorescence assay

The 4TBCB was used as test compound and lapatinib as compared. The 4TBCB was made in three concentrations of 0.305 mM ($0.5 \times IC_{50}$), 0.61 mM ($1 \times IC_{50}$), and 1.22 mM ($2 \times IC_{50}$), while lapatinib 0.05 mM ($1 \times IC_{50}$) was based on *in vitro* cytotoxicity tests using the MTT method. From the cytotoxicity test, the $IC_{50}$ values of 4TBCB and lapatinib were 0.61 mM ($1 \times IC_{50}$) and 0.05 mM ($1 \times IC_{50}$), respectively [16]. The protein expressions observed were pHER2, pRas , pSTAT3 and Ki67. Each protein expression was observed in five groups of cancer cells consisting of three groups of cancer cells given 4TBCB compounds with concentrations of 0.305, 0.61, and 1.22 mM. Meanwhile, one group of cancer cells was given 0.05 mM lapatinib, and one control group of cancer cells was not given 4TBCB and lapatinib, but only given monoclonal antibodies (pHER2, pRas, pSTAT3, and Ki67). Each group of cells was made on five replications.

Breast cancer cells were planted on 24 well plates totaling $10^5$ cells/1,000 μL/well, then incubated in a $CO_2$ incubator at 37 °C until reaching 90% confluent. Cell cultures that have reached 90% confluent were given 4TBCB and lapatinib according to the prepared concentrations, then incubated again for 24 h, followed by cell culture fixation using methanol for 15 min, then washed twice with PBS (Sigma Aldrich, USA) – tween 0.2%. The cells were then given

TritonX100 (Sigma Aldrich, USA) 0.5% for 5 min to cell permeabilization and washed with 0.2% PBS tween three times for 1 min. Furthermore, the cells were given a blocking solution of 1% BSA (Sigma Aldrich, USA) for 30 min, then washed with 0.2% PBS tween and stained with monoclonal antibodies (Biolegend). The monoclonal antibodies were diluted in a ratio of 1:100 in BSA. Cells that have been stained with monoclonal antibodies were then incubated overnight in a container wrapped in aluminum foil. Furthermore, the cells were given secondary antibody labeled FITC and incubated for 1 h. The results were observed using a 100× fluorescence microscope (Automated Fluorescence Microscope, BX63, Olympus, USA) magnification [17]. If the cells express the pHER2, pRas, pSTAT3, and Ki67 proteins, the cells will fluoresce green. Fluorescence microscopy was observed over 10 large viewing areas for each cell replication. In the immunofluorescence method, negative control cells were needed to validate the method. Negative control cells were cancer cells that were not given 4TBCB, lapatinib, and monoclonal antibodies. Negative control cells must not provide fluorescence to certify that the immunofluorescence method is valid, these cells were not included in the study cell group, but only to validate the method.

### Statistical analysis

Immunofluorescence visualization images were transferred into the ImageJ program to obtain quantitative data in numerical form, but there is no unit for data [18, 19]. The numerical data obtained were analyzed statistically using one way ANOVA or Kruskal Wallis depending on the normality and homogeneity of the data, and then continued with the post hoc test with the Tukey or Mann-Whitney test. The statistical analysis was performed using the SPSS version 25 program and the data was said to be statistically significant if the p value was ≤0.05.

To examine the relationship between pHER2, pRas, pSTAT3, and Ki67 variables in predicting the mechanism of action of 4TBCB, path analysis was used. From the path analysis, the $\beta$ coefficients and p value will be obtained. The coefficient $\beta$ shows the magnitude of the direct effect on a variable and p value shows the significance of the effect on that variable.

## Results

Identification of breast cancer cells by flow cytometry and immunofluorescence is shown in Figures 1 and 2.

Figure 1 shows the number of cancer cells in the right upper quadrant, which express a combination of cell surface markers. Cells that express CD24, CD44, and CD90 are indicated as breast cancer cells. CD24, CD44, and CD90 are cell surface markers commonly found in solid tumor, one of which is breast cancer [20, 21].

Figure 2 shows that breast cancer cells fluoresce green, meaning that they express the HER2 protein. The immunofluorescence visualization of breast cancer cells expressing pHER2, pRas, pSTAT3, and Ki67 proteins and mean graph of target protein expression are shown in Figures 3–6.

Statistical analysis of pHER2 expression tested using Kruskal Wallis showed significant difference in the 95% confidence level between groups of breast cancer cells, and Mann Whitney test showed no significant difference ($p>0.05$) between the control group and the 4TBCB 0.305 mM group. This shows that 4TBCB 0.305 mM compound does not inhibit HER2 phosphorylation, so that it does not decrease pHER2 expression in breast cancer cells. The 4TBCB 0.61 mM group, 4TBCB 1.22 mM group, and the lapatinib group were significantly different from the control cell group. This suggests that 4TBCB 0.61, 1.22 mM, and lapatinib can inhibit HER2 phosphorylation thus reducing pHER2 expression. The decrease in pHER2 expression was in line with the increase in the concentration of 4TBCB (Figure 3F).

The pRas expression analyzed using one way ANOVA showed a significant difference in the 95% confidence level between cell groups, followed by the Tukey test showing significant difference ($p\leq0.05$) between all the 4TBCB compound groups and the control cell group, as well as the lapatinib group was significantly different from the control cell group. This suggests that 4TBCB compounds ranging at 0.305–1.22 mM can inhibit intracellular signals that inhibit Ras phosphorylation thus reducing pRas expression. Lapatinib can also decrease pRas expression in breast cancer cells. The decrease in pRas expression along with an increase in the concentration of 4TBCB is shown in Figure 4F.

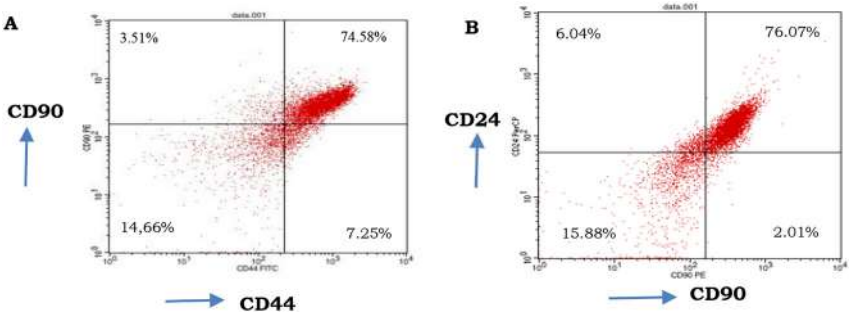


**Figure 1:** Identification of breast cancer cells by flow cytometry. Breast cancer cells expressing CD44/CD90 (A). Breast cancer cells expressing CD90/CD24 (B).

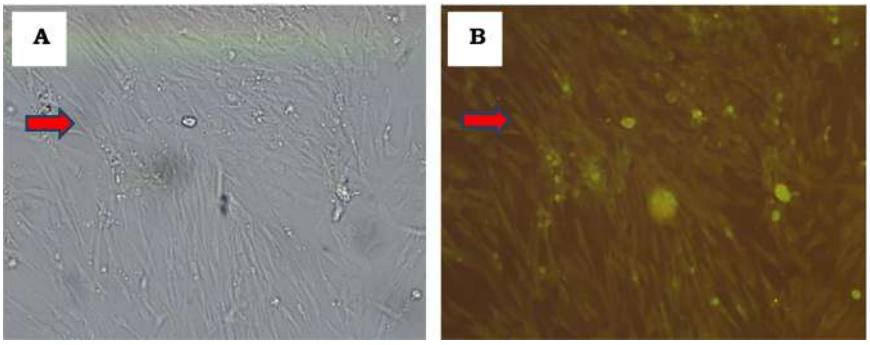


**Figure 2:** Identification of breast cancer cells expressing human epidermal growth factor receptor type 2 (HER2) by immunofluorescence. Phase-contrast (A) and immunofluorescence visualization (B) of HER2-expressing cells.

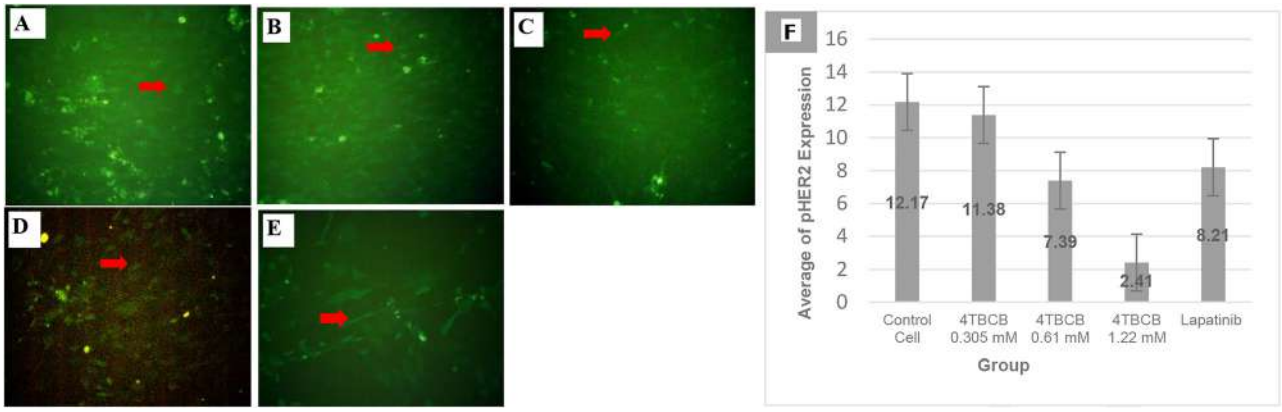


**Figure 3:** Visualization of immunofluorescence of breast cancer cells expressing phosphorylated human epidermal growth factor receptor type 2 (pHER2) and mean graph pHER2 expression. The red arrow shows breast cancer cells that express pHER2. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pHER2 protein expression in breast cancer cells (F).

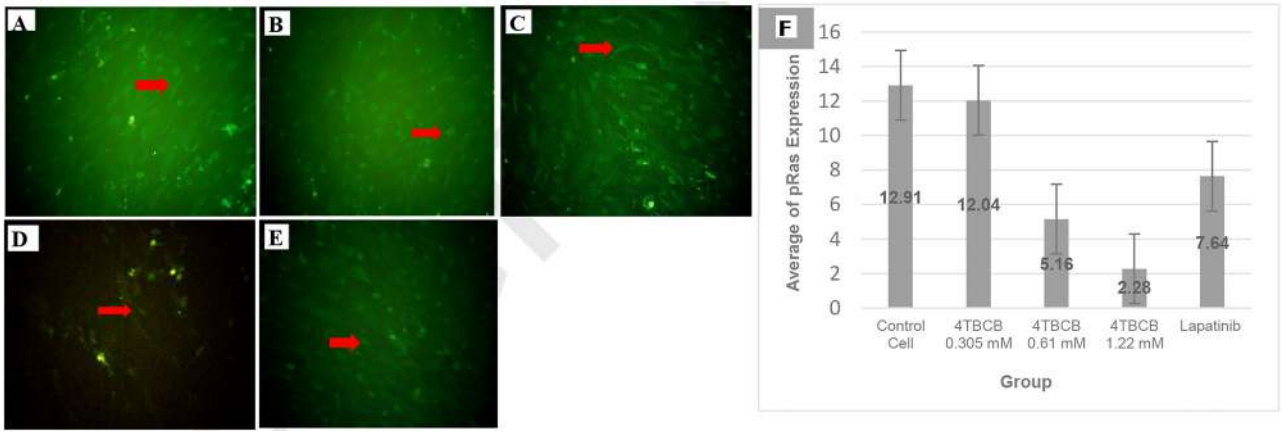


**Figure 4:** Visualization of immunofluorescence of breast cancer cells expressing phosphorylated Ras (pRas) and mean graph pRas expression. The red arrows indicate breast cancer cells that express pRas. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pRas protein expression in breast cancer cells (F).

Statistical calculation of pSTAT3 expression tested using one way ANOVA showed a significant difference in the 95% confidence level between groups of breast cancer cells, followed by Tukey test showing no significant difference between the 4TBCB 0.305 mM group and the control cell group. This indicates that 4TBCB

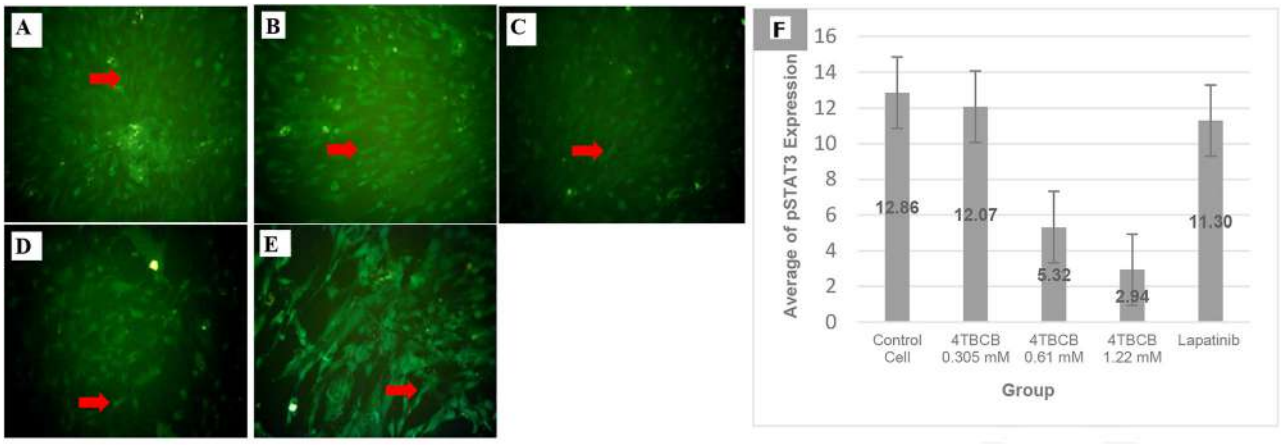


**Figure 5:** Visualization of immunofluorescence of breast cancer cells expressing phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and mean graph pSTAT3 expression. The red arrows indicate breast cancer cells that express pSTAT3. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pSTAT3 protein expression in breast cancer cells (F).

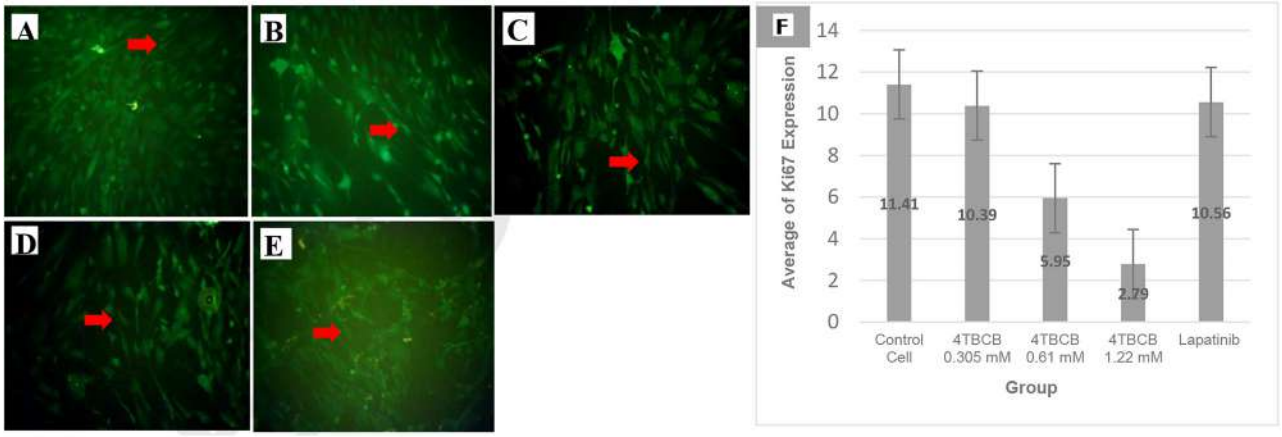


**Figure 6:** Visualization of immunofluorescence of breast cancer cells expressing Ki67 and mean graph Ki67 expression. The red arrows indicate breast cancer cells that express Ki67. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of Ki67 protein expression in breast cancer cells (F).

0.305 mM compound does not inhibit intracellular signaling so that it does not inhibit STAT3 phosphorylation; as a result pSTAT3 expression does not decrease in breast cancer cells. The 4TBCB 0.61 mM, 4TBCB 1.22 mM, and lapatinib groups were significantly different from the control cell groups, so, it can be said that the compound 4TBCB 0.61 mM, 4TBCB 1.22 mM, and lapatinib can reduce the pSTAT3 expression in breast cancer cells (Figure 5F).

Statistical analysis using Kruskal Wallis on Ki67 expression showed a significant difference in the 95% confidence level between groups of breast cancer cells, followed by the Mann Whitney test showing no significant difference between the 4TBCB 0.305 mM and lapatinib and the control cell group. This means that 4TBCB 0.305 mM compound cannot reduce the Ki67 expression as well as lapatinib. The 4TBCB 0.61 mM and 4TBCB 1.22 mM groups were significantly different from the control cell group, meaning that the two groups of 4TBCB compounds could reduce Ki67 expression and decrease Ki67 expression along with the increase in the concentration of 4TBCB compounds (Figure 6F).

The relationship between variables was analyzed using path analysis to determine the mechanism of action of the 4TBCB compound. The path analysis chart is shown in Figure 7. Prediction of HER2 signaling inhibition on the Ras and STAT3 pathway due to 4TBCB administration is shown in Figure 8.

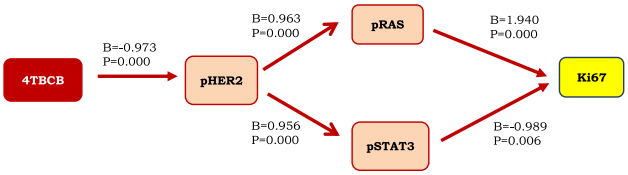


**Figure 7:** The path analysis of 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound.

Based on Figure 7, the 4TBCB compound has a negative $\beta$ coefficient value (−0.973) and is significant to the pHER2 expression (p = 0.000). This means that 4TBCB has a direct effect on the pHER2 expression by reducing the pHER2 by −0.973. The decrease in pHER2 expression also has a direct effect on the decrease in praise and pSTAT3 expressions by 0.963 and 0.956, respectively. The decrease in pRas and pSTAT3 expressions directly affects the decrease in Ki67 expression.

Figure 8 is a prediction of the mechanism of action of 4TBCB in inhibiting HER2 signaling through the Ras and STAT3 pathways in breast cancer cells. By inhibiting HER2 protein phosphorylation, it is predicted that there will be inhibition of intracellular signal through the Ras and STAT3 pathways so that a decrease in cancer cell proliferation is indicated by a decrease in Ki67 expression.

## Discussion

Figure 3F proves that 4TBCB 0.61 and 1.22 mM compounds work by inhibiting HER2 phosphorylation in the intracellular domain so that there is a decrease in pHER2 expression. The parameter of physical–chemical properties that plays the most important role in the distribution process of drugs in the body is the lipophilic parameter. Lipophilic parameters often used are the logarithm of the partition coefficient (log P) and the Hansh–Fujita $\pi$ constant. The log P prediction of 4TBCB using the ChemDraw program was 2.26, while the *N*-carbamoylbenzamide parent compound had a predictive log P 0.56. Based on the value of the substituted constant used in aromatic substitution according to the Topliss approach model, the 4-t-butyl group has lipophilic properties with a value of $\pi$ = 1.98 [22]. The presence of this group increased the lipophilic properties of the 4TBCB. Therefore, log P and $\pi$ constant predicted that 4TBCB has good lipophilic properties to penetrate cell membranes well. The decreased expression of pHER2 protein causes an intracellular signal to phosphorylate Ras and STAT3 to be inhibited, thereby inhibiting DNA replication, which results in decrease in proliferation of HER2-expressing breast cancer cells.

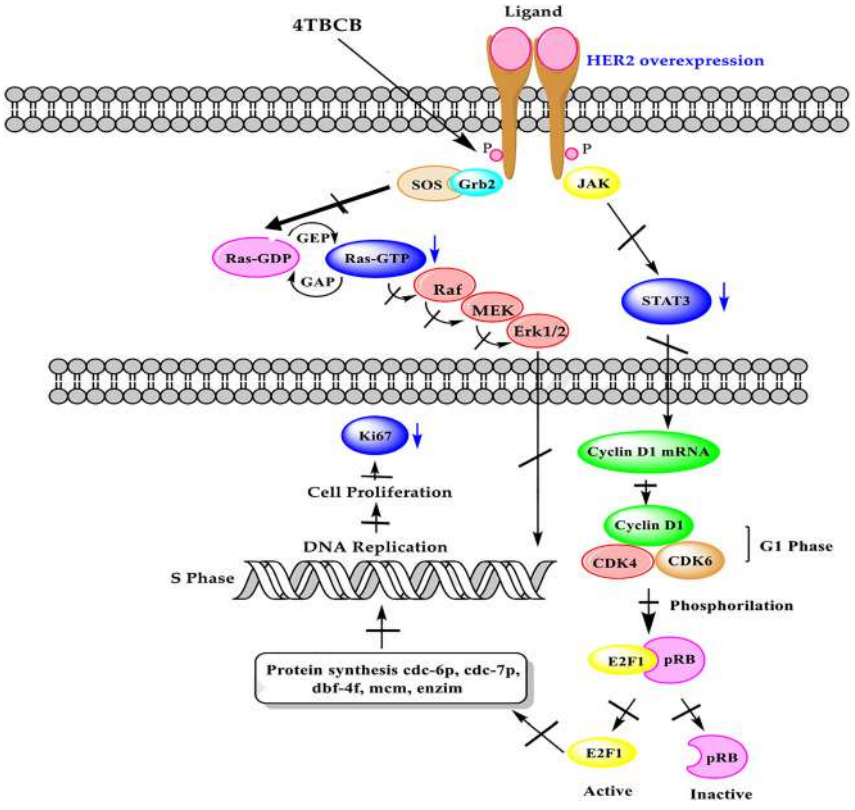


**Figure 8:** Prediction of HER2 signaling inhibition on the Ras and signal transducer and activator of transcription 3 (STAT3) pathway due to 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) administration.

The 4TBCB ranging from 0.305 to 1.22 mM can decrease the pRas protein expression. The decrease in pRas protein expression is in line with the increase in the concentration of 4TBCB compound (Figure 4F). Ras genes encode Ras protein that play a role in intracellular signal transduction to trigger cell multiplication. This protein displays different conformational forms, Ras-GDP (Ras-Guanine Diphosphate) when inactive, and Ras-GTP (Ras-Guanine Triphosphate) while active. The balance between Ras-GDP and Ras-GTP is regulated by guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). Ras-GDP (in the "off" state) is transformed into Ras-GTP (in the "on" state) by the GEF enzyme. On the contrary, the "on" to "off" position is regulated by the enzyme GAP [23–25]. In breast cancer, Ras is activated by overexpressing HER2 and activating signal transduction to the nucleus by simultaneously activating the target effectors (RAF, MEK, and Extracellular Signal-Regulated Kinase [Erk]). The continuous activation of Ras-MAPK triggers an increase in the number of DNA replications and cell proliferation [5, 7]. Therefore, 4TBCB was proven to inhibit HER2 phosphorylation, which inhibits the activation of Ras pathway and causes the pRas expression to decrease.

The pSTAT3 expression analysis proved that 4TBCB at 0.61 and 1.22 mM inhibited HER2 intracellular signals which resulted in inhibition of STAT3 phosphorylation and decreased pSTAT3 expression (Figure 5F). STAT3 is a transcription factor that regulates transcription and gene expression in cellular processes. STAT3 can be activated in all breast cancer subtypes. Various tyrosine kinase receptors (EGFR, HER2, and VEGF) can phosphorylate STAT3 in breast cancer [11, 26].

The Ki67 expression analysis showed a decrease in Ki67 expression in line with the increase in concentration of the 4TBCB compound (0.61 and 1.22 mM) (Figure 6F). Ki67 is a core protein associated with cell proliferation, expressed in the cell cycle in the S, G1, G2, and M phases in the nucleus. Ki67 expression differs from phase-to-phase and peaks during mitosis. Therefore, Ki67 is used as a marker to evaluate cell proliferation to determine specific therapy for each patient [11, 27]. The classification in molecular subtypes of breast cancer states that the luminal B and HER2 overexpression had the highest Ki67 index in accordance with a study conducted by Hashmi and Ragab on patients with breast cancer in hospitals [11, 27]. This research suggested that 4TBCB lowered Ki67 expression led to lower proliferation of HER2-expressing breast cancer cells.

Lapatinib is clinically used as anticancer for HER2-positive breast cancer patients. Lapatinib is a tyrosine kinase inhibitor that selectively inhibits HER2 and EGFR [28]. The analysis results shown in Figures 3F–5F prove that lapatinib can reduce the expression of pHER2, pRas, and pSTAT3. This study has shown that lapatinib did not decrease Ki67 expression, possibly because there are other intracellular signaling pathways other than the Ras and STAT3 pathways that can activate cell proliferation so that Ki67 expression remains high with lapatinib administration. In this study, 4TBCB was compared with lapatinib which is clinically used as anticancer, so it can be said that 4TBCB has a potential to be an anticancer. However, 4TBCB required a greater concentration in reducing pHER2, pRas, pSTAT3, and Ki67, namely 0.61 mM ($1 \times IC_{50}$) compared to lapatinib at 0.05 mM ($1 \times IC_{50}$).

The mechanism of action of the 4TBCB can be explained using path analysis between variables. Based on the results of path analysis, 4TBCB is predicted to work through inhibition of HER2 signaling on the Ras and STAT3 pathways in HER2-expressing breast cancer cells.

The 4TBCB compound as a whole is predicted to inhibit HER2 signaling in the Ras and STAT3 pathways shown in the graph in Figure 8. Inhibition of HER2 phosphorylation inhibits Ras protein activation thus Raf effectors are not recruited and subsequently do not activate Mek1/2 and Erk1/2. Erk1/2 is a transcription factor that plays a role in the synthesis of proteins required for DNA replication in cancer cells. Inhibited Erk1/2 activation will decrease DNA replication, thus decreasing cell proliferation. The inhibition of HER2 phosphorylation also inhibits Janus Kinase (JAK) activation, thereby inhibiting STAT3 phosphorylation. STAT3 phosphorylation increases cyclin D1 mRNA expression, causing the formation of complex cyclin D1 bonds with CDK4 and CDK6 [29, 30]. These complex bonds cause pRB phosphorylation and then activate transcription factor E2F1, which mediate the synthesis of cdc 6p protein needed to trigger the formation of O replication bubbles that DNA replication occurs [25]. The inhibition of STAT3 activation due to HER2 phosphorylation inhibition causes DNA replication to decrease and cancer cell proliferation will also decrease. Therefore, 4TBCB compound can be said to be a candidate for antiproliferative agent for HER2-expressing breast cancer cells.

## Conclusions

4TBCB compounds at concentrations of 0.61 and 1.22 mM have been shown to reduce the expression of pHER2, pRas,

pSTAT3, and Ki67 proteins and are thought to have an inhibitory mechanism of HER2 signaling through the Ras and STAT3 pathways on HER2-expressing breast cancer cells.

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**Informed consent**: Informed consent was obtained from all individuals included in this study.
**Ethical approval**: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by Ethical Commission of General Hospital in Surabaya, Indonesia.

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Maria Apriliani Gani, Ahmad Dzulfikri Nurhan, Aniek Setiya Budiatin, Siswandono Siswodihardjo and Junaidi Khotib*

# Predicting the molecular mechanism of glucosamine in accelerating bone defect repair by stimulating osteogenic proteins

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**Abstract**

**Objectives:** Bone defect is serious condition that is usually caused by traffic accident. Chitosan is a polymer developed as a scaffold to treat bone defect. However, the mechanism by which chitosan can accelerate bone growth in defect area is still unclear. This study aims to identify proteins which are crucial to the osteogenic properties of chitosan monomer using an *in silico* study.

**Methods:** Molecular docking was carried out on chitosan monomer, which are D-glucosamine and glucosamine 6-phosphate units against bone morphogenetic protein 2 (BMP-2), fibronectin, fibroblast growth factor (Fgf), and phosphate transporter (PiT) using AutoDock Vina. Ligand preparation was carried out using Chem3D version 15.0.0.106, while protein preparation was performed using AutoDockTools version 1.5.6.

**Results:** The results showed that glucosamine 6-phosphate had the best binding affinity with fibronectin and PiT, which was –5.7 kcal $mol^{-1}$ on both proteins, while D-glucosamine had the best binding affinity with PiT (–5.2 kcal $mol^{-1}$).

**Conclusions:** This study suggests that the osteogenic properties of chitosan may be due to the presence of bonds between glucosamine units and fibronectin and/or PiT. However, *in vitro* studies need to be done to prove this.

***Corresponding author: Junaidi Khotib**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 813 318 40710, E-mail: junaidi-k@ff.unair.ac.id
**Maria Apriliani Gani, Ahmad Dzulfikri Nurhan and Aniek Setiya Budiatin,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Siswandono Siswodihardjo,** Department of Chemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia

**Keywords:** bone defect; chitosan; glucosamine; molecular docking; osteogenic; traffic accident.

## Introduction

Bone defect is a serious condition caused by pathological circumstances (such as osteoporosis and osteopenia) or local trauma [1, 2]. In Indonesia, it is estimated that 41.8% of men and 90% of women have osteopenia. Even in 2050, it is predicted that there will be pelvic bone defects in 50% of total Asian population [3]. As tissue regenerates, bone defect healing can occur on its own, especially over small gaps. However, this requires a process that tends to be long, even in some conditions it causes delay and failure of bone healing. This complication is known to cost up to AUD $ 4.9 million [4, 5].

Bone graft is a procedure used to accelerate bone growth in defect area. One of the materials used as bone graft biomaterial is chitosan. Chitosan is a polysaccharide composed of *N*-acetylglucosamine and D-glucosamine units [6]. In bone defect condition, increased proliferation and differentiation of osteoblasts is desirable. Previous studies showed that chitosan-based scaffold increased markers of osteoblast differentiation *in vitro* [7–9]. Furthermore, this scaffold also increases bone volume in the defect area *in vivo* [7]. Not only chitosan, glucosamine, a unit of chitosan, has anti-inflammatory and anti-catabolic activity which makes it commonly used to treat osteoarthritis [10]. A meta-analysis conducted by Zhu et al. found that glucosamine improved joint stiffness and increased chondrogenesis by providing a substrate for cartilage matrix synthesis *in vitro* [10–12].

Several molecular mechanisms of glucosamine related to chondrogenesis have been reported, one of which is by inhibiting the activation of NF-kappaB [10, 13]. However, the mechanism of glucosamine/chitosan in bone regeneration and osteogenesis remains unclear. In a number of studies several proteins have been reported to have osteogenic properties. These proteins include bone morphogenetic protein 2 (BMP-2) which can increase osteoblast differentiation, fibronectin which plays a role in material

osteoconductivity, and fibroblast growth factor (Fgf) as well as phosphate transporter (PiT) which were previously reported to play a role in the osteogenic properties of hydroxyapatite (HA) [14–16].

These reports indicate the possibility that BMP-2, fibronectin, Fgf and PiT contribute to the osteogenic properties of chitosan. Therefore, this study conducted an *in silico* molecular docking study on chitosan monomers, the D-glucosamine and glucosamine 6-phosphate, on those four proteins. The results of this study may predict the osteogenic mechanism of action of both glucosamine and chitosan which will be the basis for investigating the molecular mechanism of biomaterials in repairing bone defects.

# Materials and methods

### Ligand preparation and virtual protein elucidation

The ligands used in this study were glucosamine 6-phosphate and D-glucosamine whose chemical structure was obtained by searching on PubChem (http://pubchem.ncbi.nlm.nih.gov/). The ligand structure was formed using ChemDraw version 15.0.0.106 and optimized using MMFF94 Minimization in Chem3D version 15.0.0.106. Furthermore, the ligands were saved in MOL2 format. On the other hand, the preparation of the four docking target proteins was started by downloading the proteins from Protein Data Bank (https://www.rcsb.org/). The four proteins were human BMP-2 (PDB ID: 3BMP), fibroblast growth factor 4 (Fgf4, PDB ID: 1IJT), human fibronectin (PDB ID: 1FNF), and PiT (PDB ID: 4J05). Protein preparation was carried out using AutoDockTools version 1.5.6. by removing water molecules and certain solvent residues, adding a Kolman charge, and repairing the missing atoms in those proteins. The prepared proteins were then stored in PDBQT format.

### Molecular docking

Molecular docking was run using an Inter (R) Celeron (R) 2955U @ 1.40 GHZ processor, RAM 2.00 GB system type 64 bit Operating System. The docking techniques used were blind docking and targeted docking. Targeted docking was performed on predicted protein binding pockets using DoGSiteScorer (https://proteinsplus.zbh.uni-hamburg.de/#dogsite). The binding pocket used was one with the highest drug score for Fgf, fibronectin, and PiT. On the other hand, the docking method used on the BMP-2 was blind docking. Blind docking was used because the binding pocket with the highest drug score on this protein almost covered all sides of the protein. The grid boxes used for each protein were $52 \times 58 \times 46$ Å center at −9.377, 27.13, 11.25 for BMP-2, $48 \times 40 \times 48$ Å center at 13.983, −3.854, 21.563 for Fgf, $48 \times 48 \times 48$ Å center at 6.459, 0.028, 0.028 for fibronectin, $66 \times 66 \times 80$ Å center at 87.748, −16.016, 0.0 for PiT. Docking results were considered valid if the compound did not change location at least three times after docking. The protein and ligand interactions were further visualized using Discovery Studio Visualizer v17.2.016349.

# Results

*In silico* molecular docking studies of glucosamine on several osteogenic receptors have been carried out. Based on the results, glucosamine 6-phosphate has binding affinity of −5.2, −5.2, −5.7, and −5.7 kcal $mol^{-1}$ respectively on BMP-2, Fgf, fibronectin, and PiT (Table 1). Furthermore, based on the interaction of glucosamine 6-phosphate on osteogenic protein binding pockets (Figure 1), this compound has hydrogen (classical), electrostatic (charge), and unfavorable (acceptor/donor clash) bonds on BMP-2. Whereas in Fgf, glucosamine 6-phosphate has hydrogen (classical, nonclassical) and unfavorable (charge repulsion) bonds. Besides, in fibronectin, glucosamine 6-phosphate forms hydrogen (classical) and unfavorable (acceptor/donor clash) bonds. The chemical bonds formed with PiT are hydrogen (classical, nonclassical) and electrostatic (charge, Pi-charge) bonds.

Moreover, based on the results of molecular docking, the binding affinity of D-glucosamine with osteogenic protein is lower than that of glucosamine 6-phosphate. D-glucosamine has binding affinity of −4.5, −4.4, −4.9, and −5.2 kcal $mol^{-1}$ on BMP-2, Fgf, fibronectin, and PiT, respectively (Table 1). The interaction of D-glucosamine with osteogenic protein is shown in Figure 2. D-glucosamine forms hydrogen bonds (classical) in the binding pocket of BMP-2. Whereas in Fgf and fibronectin, the bonds formed between D-glucosamine and proteins are hydrogen (classical) and unfavorable (acceptor/donor clash) bonds. As for PiT, D-glucosamine forms hydrogen (classical, nonclassical) and unfavorable (acceptor/donor clash) bounds.

# Discussion

D-glucosamine is one of the units that forms chitosan along with *N*-acetylglucosamine which is bound through beta1, 4-glycoside bonds [17], while glucosamine 6-phosphate is a

**Table 1:** Binding energy of glucosamine 6-phosphate and D-glucosamine in their specific binding pocket of the osteogenic proteins.

| Ligand | PDB ID, Kcal $mol^{-1}$ | | | |
|---|---|---|---|---|
| | 3BMP[a] | 1IJT[b] | 1FNF[c] | 4J05[d] |
| Glucosamine 6-phosphate | −5.2 | −5.2 | −5.7 | −5.7 |
| D-glucosamine | −4.5 | −4.4 | −4.9 | −5.2 |

[a]PDB ID for human bone morphogenetic protein 2. [b]PDB ID for fibroblast growth factor 4. [c]PDB ID for human fibronectin. [d]PDB ID for phosphate transporter.

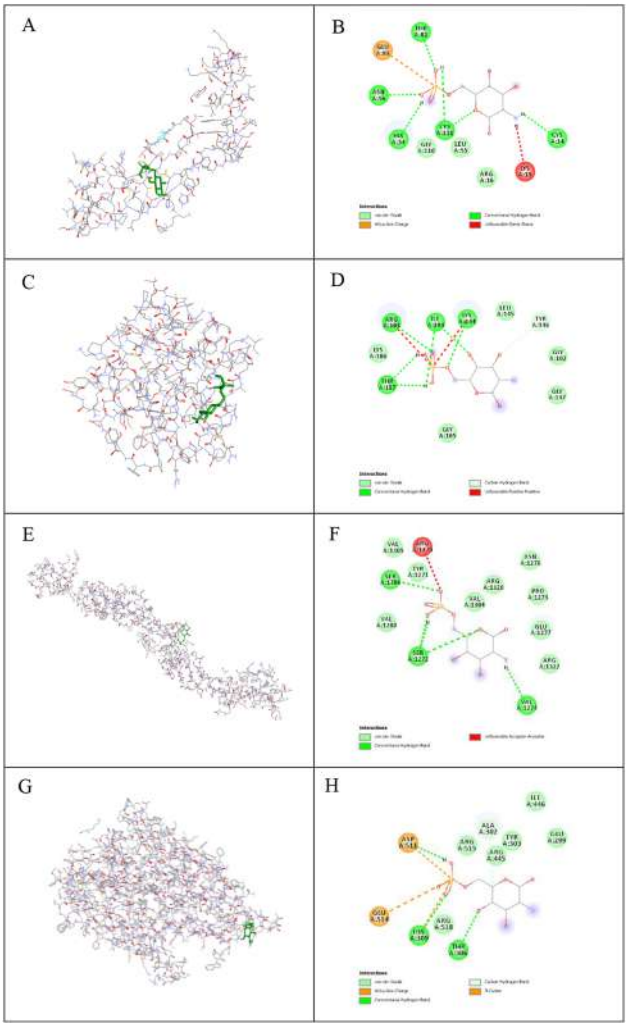


**Figure 1:** Interaction of glucosamine 6-phosphate in specific site of bone morphogenetic protein 2 (BMP-2) (A–B), fibroblast growth factor (Fgf) receptor (C–D), fibronectin (E–F), phosphate transporter (PiT) (G–H). Panel A, C, D and G illustrate the binding location of glucosamine 6-phosphate on the proteins (green color is glucosamine 6-phosphate). Panel B, D, F and H show the interaction glucosamine 6-phosphate on their specific binding pocket of each amino acid residue.

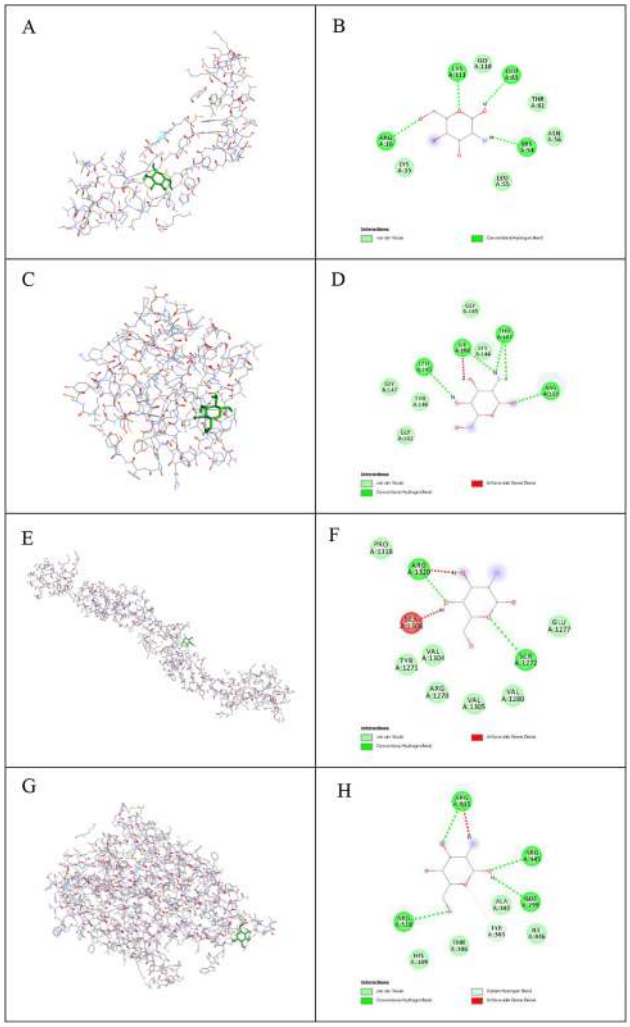


**Figure 2:** Interaction of D-glucosamine to the specific site of BMP-2 (A–B), Fgf receptor (C–D), fibronectin (E–F), PiT (G–H). A, C, E and G illustrate the location binding D-glucosamine on the proteins (green color is D-glucosamine). Panel B, D, F and H show the interaction of D-glucosamine in their specific binding pocket of amino acid residue.

natural form of glucosamine produced in hexosamine biosynthesis pathway [17, 18]. Molecular docking showed that glucosamine 6-phosphate and D-glucosamine had great binding affinity on BMP-2. BMP-2 is a protein that belongs to the transforming growth factor-beta (TGF-beta) family and is known to increase osteoblast differentiation by Smad4 and mTORC1 pathways [15]. Previous studies reported that there was an increase in BMP-2 expression caused by chitosan compared to the group without chitosan [19]. Based on the present study, it was possible that the increased expression of BMP-2 was due to the interaction of glucosamine units on this protein. This may occur due to the interaction of biomaterials with BMP-2 to promote biomaterials' biocompatibility [20]. This interaction initiates further molecular responses, such as increased in Runt related transcription factor 2 (Runx2), type 1 collagen, bone sialoprotein (BSP), osteocalcin, alkaline phosphatase genes expression [21, 22], as well as an increased in BMP-2 expression [19]. However, this should be explored in *in vitro* studies.

Osteoconductivity of a biomaterial is highly dependent on the interaction of the material with cells involved in bone regeneration. However, before bone cell adhesion occurs, proteins present in the extracellular matrix must be absorbed into the implanted material. Fibronectin is a protein present in the extracellular matrix. This protein connects the interaction between the extracellular matrix

and osteogenic cells by binding to integrin receptors on cell surface [16]. Chitosan polymer is known to have a high affinity for protein. Asghari Sana et al. reported that human dental pulp stem cells cultured with chitosan were able to cause immobilization of fibronectin [23]. The present study have shown that fibronectin immobilization may be due to the binding of glucosamine to the fibronectin protein. In the present study, the protein site used as a docking target was selected based on the highest drug score of fibronectin protein. Drug score is the tendency for a ligand to occupy a binding pocket (in a ratio of 0–1, the higher the drug score, the greater the tendency). In general, when a ligand binds to a protein, functional changes occur in the associated protein [24]. Based on the binding energy present in Table 1 and chemical bonds formed between glucosamine 6-phosphate or D-glucosamine with amino acids in the binding pocket (Figure 1E, F and Figure 2E, F), the ligands are predicted to bind to the fibronectin protein and cause functional changes, which may cause immobilization of fibronectin. However, this needs to be explored in further studies.

The role of biomaterials in bone defect repair is unclear. However Ha et al. proved that the osteogenic properties of one of the biomaterials (nano hydroxyapatite) depend on two membrane proteins, the Fgf and PiT. Researchers also found that Frs2 and Erk1/2 were activated proteins due to the interaction of nano hydroxyapatite with those two receptors [14]. This pathway causes changes in the expression of osteoblast markers which then make osteoblasts differentiate into osteocytes [14]. The Fgf used as the docking target in this study was Fgf4. Fgf4 is reported to stimulate stem cell proliferation which in turn leads to osteogenic differentiation [25]. The present study found that glucosamine 6-phosphate and D-glucosamine are potentially inhibiting Fgf and PiT based on the binding affinity value. Therefore, the osteogenic properties of chitosan may be due to the binding of glucosamine units with the Fgf and PiT receptors. However, this also needs to be explored in *in vitro* studies.

Glucosamine 6-phosphate has the best binding affinity value in fibronectin and PiT, while D-glucosamine has the best binding affinity value in PiT alone. Therefore, it is possible that fibronectin and PiT are the proteins that have the most influence on osteogenic properties of both glucosamine and chitosan. However, given the complexity of the extracellular biomaterial-cell-matrix interaction, further studies are needed to prove it. This study has several limitations. One was the use of glucosamine units to describe the chitosan polymer, which, in fact, was not necessarily degraded completely when implanted into bone.

# Conclusions

In conclusion, chitosan is a polymer used as a scaffold to accelerate bone growth in defect area. As with other biomaterials, the molecular mechanism of chitosan in relation to its osteogenic properties is unclear. This study shows that the chitosan units, the glucosamine 6-phosphate and D-glucosamine, have good binding affinity on BMP-2, fibronectin, Fgf, and PiT. Glucosamine 6-phosphate has the best binding affinity value on fibronectin and PiT, while D-glucosamine on PiT. Therefore, these two proteins may have the most crucial role in the osteogenic properties of glucosamine or chitosan. However, further research on this needs to be done.

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Salamun, Fatimah, Ahmad Fauzi, Seling N. Praduwana and Ni'matuzahroh*

# Larvicidal toxicity and parasporal inclusion of native *Bacillus thuringiensis* BK5.2 against *Aedes aegypti*

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**Abstract**

**Objectives:** Native *Bacillus thuringiensis* BK5.2, isolated from soil of Baluran National Park, East Java, Indonesia, has been shown to be toxic against *Aedes aegypti* larvae. This study aims to determine the strength and the speed of the toxicity of *B. thuringiensis* BK5.2 against *A. aegypti* larvae in lethal concentration (LC) and lethal time (LT), as well as detection of toxin structure and parasporal inclusion.

**Methods:** LC values were determined by the mortality of *A. aegypti* third instar larvae after 24 and 48 h exposure to five various concentrations of *B. thuringiensis* BK5.2, while LT values were determined based on the mortality of *A. aegypti* third instar larvae due to exposure to $LC_{90}$ concentration at 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 h. Larvicidal toxicity was determined based on value of $LC_{50}$ and $LC_{90}$ (CFU/mL), as well as $LT_{50}$ and $LT_{90}$ (hours) analysed with Probit analysis. Parasporal inclusion was detected using transmission electron microscope (TEM) and scanning electron microscope (SEM).

**Results:** Based on bioassay, $LC_{50}$ and $LC_{90}$ values were $11.6 \times 10^6$ and $22.7 \times 10^6$ CFU/mL, respectively, at 24 h exposure, as well as $8.3 \times 10^6$ and $15.4 \times 10^6$ CFU/mL, respectively, at 48 h exposure, while the value of $LT_{50}$ and $LT_{90}$ were 19.0 and 26.6 h, respectively. Morphological observation of the dead larvae showed there was damage on abdomen and thorax region. Detection by TEM and SEM showed there was cuboidal parasporal inclusion.

**Conclusions:** Native *B. thuringiensis* BK5.2 has high toxicity against *A. aegypti* larvae and detected flatcuboidal toxin in parasporal inclusion.

**Keywords:** *Aedes aegypti*; *Bacillus thuringiensis* BK5.2; larvicidal toxicity; parasporal inclusion.

## Introduction

The *Aedes aegypti* mosquito is a vector, which has transmitted various diseases such as dengue hemorrhagic fever (DHF), yellow fever, encephalitis virus, filariasis, and Zika [1]. The diseases still a serious public health problem in the world, including Indonesia [2, 3]. Efforts to overcome the problem of vector borne diseases are controlling the vector population to below the control threshold. Chemical control had been implemented in endemic areas, but it had proven to have many negative impacts in the form of target insect resistance, killing non target insects, and negative impacts on the environment. Biological control experts suggested using bioinsecticides and continue to look for biological controllers as an alternative for biological control of disease vectors [4].

One of the biological control agents developed as a bioinsecticide is an entomopathogenic bacteria from the *Bacillus*. El-kersh et al. [5], had isolated and characterized a local strain of *B. thuringiensis* from Saudi Arabia and a number of isolates had larvicidal toxicity to the *Anopheles gambiens*, the vector of malaria. Suryadi et al. [6] had isolated and characterized *Bacillus sphaericus* which could be developed as a bioinsecticide for biological control against the *Anopheles aconitus* malaria mosquito from Lombok Island. Biological control experts hope that the biological control community will pay attention to the perspectives and opportunities for the role of biological control agents in the integrated control of diseases transmitted by vectors [4].

Salamun et al. [7] have isolated and carried out isolation and characterization of local entomopathogens that have the potential to kill *A. aegypti* larvae, from the soil in the Baluran National Park area of East Java, Indonesia. One of the potential local isolates with isolate code BK5.2 has

***Corresponding author: Ni'matuzahroh**, Faculty of Science and Technology, Faculty of Advanced Technology and Multidiscipline, University -CoE- Research Center for Bio-Molecule Engineering, Universitas Airlangga, Surabaya, Indonesia, Phone: (+6231) 5936501, E-mail: nimatuzahroh@fst.unair.ac.id
**Salamun and Fatimah,** Faculty of Science and Technology, University -CoE- Research Center for Bio-Molecule Engineering, Universitas Airlangga, Surabaya, Indonesia
**Ahmad Fauzi and Seling N. Praduwana,** Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia

been identified as *B. thuringiensis* [7]. This paper reports the results of bioassay to determine the strength of the toxicity of local entomopathogenic bacteria to *A. aegypti* larvae, observing the location of organ damage in the test larvae, and detection of toxin structures of parasporal inclusion in the sporulation phase of *B. thuringiensis* BK5.2.

# Materials and methods

This research was conducted at the Laboratory of Entomology, Institute of Tropical Diseases and the Laboratory of Microbiology, Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia. The two laboratories served as a breeding ground for the test larvae of *A. aegypti* and as a place to prepare test bacteria and test the toxicity of bacteria against *A. aegypti* larvae.

### Preparation of bacterial isolate and *A. aegypti*

The bacterial isolate used in this study was *Bacillus thuringiensis* BK5.2 isolated from the soil of Baluran National Park, East Java, Indonesia. While the *A. aegypti* used was the third instar larvae. Before being used for bioassay, bacterial isolate was rejuvenated.

### Bioassay

The determination of lethal concentration (LC) to determine the strength of *B. thuringiensis* BK5.2 toxicity against *A. aegypti* larvae was carried out by growing rejuvenated bacteria in NYSM liquid media for 72 h, with agitation 130 rpm, at 30 °C. Then, bacterial densities were measured at $OD_{600}$. The final culture concentrations were adjusted to be $2 \times 10^7$; $3.4 \times 10^7$; $7.2 \times 10^7$; $1.38 \times 10^8$; $2.68 \times 10^8$ in 10% (v/v) NYSM media, so that five different concentrations were obtained as treatments. A total of 20 larvae of *A. aegypti* were inserted into each bacterial culture with various concentrations. Each treatment had three replications. Larval mortality was observed during the 24 and 48 h exposure time.

Determination of the lethal time (LT) of *B. thuringiensis* BK5.2 against *A. aegypti* larvae to determine the speed of toxicity was carried out on $LC_{90}$ concentration. A total of 20 larvae were exposed to *B. thuringiensis* BK5.2 with $LC_{90}$ concentration. Each treatment was carried out in triplicates. Larval mortality was observed for 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 h of exposure time.

In the determination of LC and LT in the bioassay, a negative control was used in the form of NYSM media (10% v/v) without an inoculum. In this study, there was no positive control, because this was a preliminary study to determine toxicity, the presence of parasporal bodies, and it had not yet reached the determination of the dosage. The results of the observations on the bioassay in the form of larval mortality are served in percent, and used to determine $LC_{50}$ and $LC_{90}$, as well as $LT_{50}$ and $LT_{90}$ from *B. thuringiensis* BK5.2 against *A. aegypti* larvae. The results of the toxicity of *B. thuringiensis* BK5.2 against *A. aegypti* were analyzed by Probit analysis using the Minitab 17 application [8]. The dead larvae exposed to *B. thuringiensis* BK5.2 and normal larvae were observed using light microscope (Nikon Light Microscope, 10× magnification) to observe different morphology caused by *B. thuringiensis* BK5.2.

### Detection of endospore structure and parasporal inclusion

Ultrastructural detection of cells and parasporal inclusions of *B. thuringiensis* BK5.2 were started by rejuvenating, purifying, and multiplying the isolate with liquid culture on NYSM media, grown in a shaker incubator at 30 °C, 130 rpm. After obtaining a pure liquid culture of *B. thuringiensis* BK5.2, then it was sent for examination of the transmission electron microscope (TEM) on TEM and Histology Laboratory, Eijkman Institute for Molecular Biology, Jakarta, using TEM JEOL1010, 80.0 kV, 10,000× magnification. The scanning electron microscope (SEM) examination was sent to LPPT Gadjah Mada University, Yogyakarta, using SEI 10 kV WD10 mm SS30 10,000× magnification.

# Results

## Bioassay

Bioassay results of *B. thuringiensis* BK5.2 bacterial isolate against *A. aegypti* throughout 24 and 48 h exposure were shown in Table 1 and Figure 1. In Table 1, the data was used to determine the value of $LC_{50}$ and $LC_{90}$, while in Figure 1, the data was used to determine the value of $LT_{50}$ and $LT_{90}$. In Figure 1, the value of $R^2$ was relatively low because the result on exposure time showed a slow larval mortality rate (%) at 4–8 h, then increased at 10–24 h and got faster at the end of the bioassay.

The comparison results between $LC_{50}$ and $LC_{90}$ values of *B. thuringiensis* BK5.2 at the 24 and 48 h exposure are presented in Figure 2.

Value of $LC_{50}$ and $LC_{90}$ as well as $LT_{50}$ and $LT_{90}$ were carried out to determine the concentration required by *B. thuringiensis* BK5.2 to kill 50 and 90% and the length of time required to kill 50 and 90% of the larvae population of *A. aegypti*. Based on the percentage of larval mortality in the treatment, *B. thuringiensis* BK5.2 was included in the

**Table 1:** The concentration series (CFU/mL) of *Bacillus thuringiensis* BK5.2 on mortality of third instar larvae (%) of *Aedes aegypti*, 24 and 48 h exposure.

| Concentration, cells density | CFU/mL | 24 h exposure, larval mortality = % | 48 h exposure, larval mortality = % |
|---|---|---|---|
| $C_1$ | $2 \times 10^7$ | 3.3 ± 5.8 | 3.3 ± 5.8 |
| $C_2$ | $3.4 \times 10^7$ | 20 ± 10 | 26.6 ± 5.8 |
| $C_3$ | $7.2 \times 10^7$ | 40 ± 10 | 50 ± 0 |
| $C_4$ | $1.38 \times 10^8$ | 63.3 ± 5.8 | 80 ± 10 |
| $C_5$ | $2.68 \times 10^8$ | 93.3 ± 5.8 | 100 ± 0 |

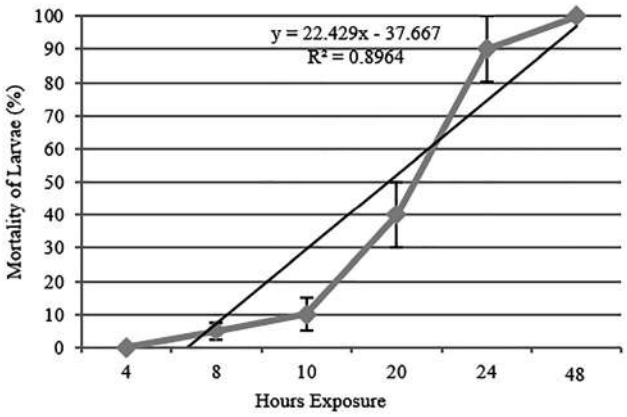


**Figure 1:** The toxicity test of the *Bacillus thuringiensis* BK5.2 to kill the third instar larvae (%) of *Aedes aegypti* in the observation time series; 0; 4; 8; 10; 20; 24; and 48 h.

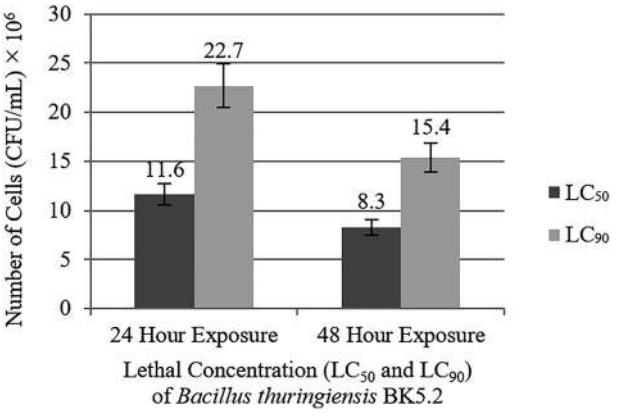


**Figure 2:** Value of $LC_{50}$ and $LC_{90}$ (CFU/mL) *Bacillus thuringiensis* BK5.2 against third instar larvae of *Aedes aegypti* at the 24 and 48 h exposure.

high toxicity potential category. In this study, the dead larvae showed the abdomen and thorax damage, as shown in Figure 3.

The speed of toxicity in Lethal Time (LT) of *B. thuringiensis* BK5.2 on $LC_{90}$ concentration against third instar larvae of *A. aegypti* was shown from larval mortality at 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 h of exposure time. The value of $LT_{50}$ and $LT_{90}$ of *B. thuringiensis* BK5.2 at observation 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 h, are presented in Figure 4.

## Detection of endospore structure and parasporal inclusion

The detection of endospore structures and parasporal inclusion using transmission electron microscope (TEM)

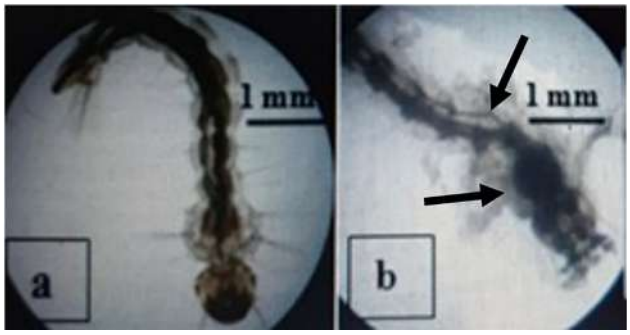


**Figure 3:** Light microscopy images of *Aedes aegypti* larvae. (a) Normal larvae; (b) larvae after exposed to *B. thuringiensis* BK5.2 showed the abdomen and thorax damage (black arrows) (personal photo result, 2018), by Nikon light microscope, 10× magnification.

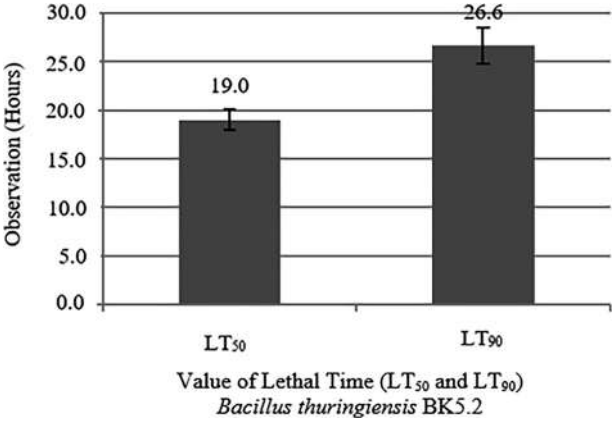


**Figure 4:** The value of $LT_{50}$ and $LT_{90}$ (hours) *Bacillus thuringiensis* BK5.2 (on $LC_{90}$ concentration = $15.4 \times 10^6$) against third instar larvae of *Aedes aegypti* calculated based on probit analysis between larval mortality (%) with the time series of observation 0; 0.5; 1; 2; 4; 8; 10; 20; 24; and 48 h.

and scanning electron microscope (SEM) of *B. thuringiensis* BK5.2 are presented in Figures 5 and 6.

# Discussion

Some bacteria in the spore-forming bacilli group produced protein crystals that are entomopathogenic. *B. thuringiensis* has high toxicity to *Aedes* larvae because the bacteria are able to synthesize parasporal crystal inclusions during sporulation and produce a protein δ-endotoxin. Protein crystals consist of one or more insecticidal crystal protein (ICP) encoded with the crystal (Cry) gene and the cytolytic (Cyt) gene [9, 10]. Cry protein has been proven to be very effective in controlling the dengue fever and malaria vectors

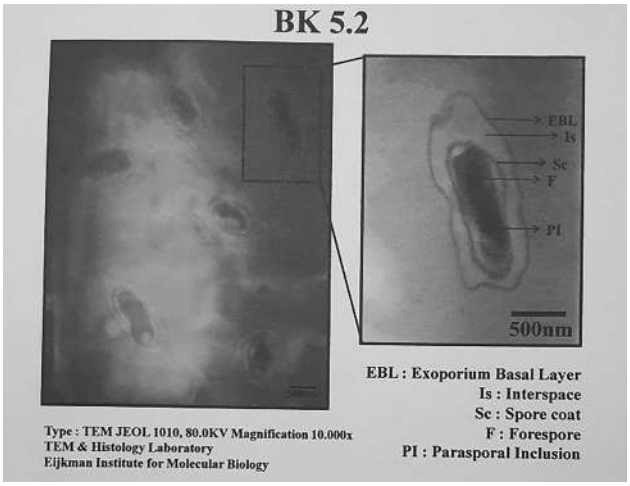


**Figure 5:** Transmission electron microscopy (TEM) image shows *Bacillus thuringiensis* BK5.2 endospore, with the organelles. EBL = exosporium basal layer, Is = interspace, Sc = spore coat, F = forespore, PI = parasporal inclusion, by TEM JEOL1010 80.0 kV, 10,000× magnification.

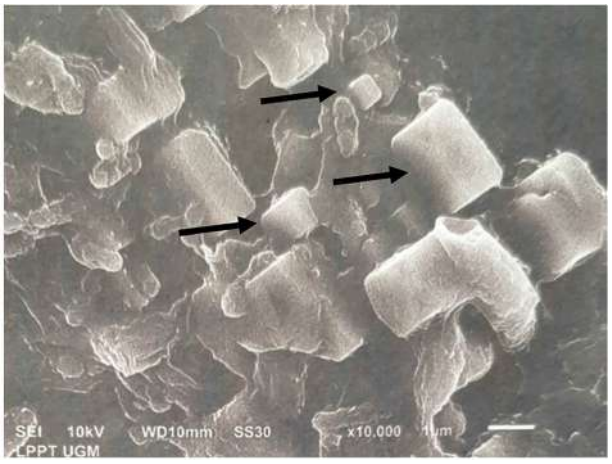


**Figure 6:** Scanning electron microscopy (SEM) image shows local entomopathogen *Bacillus thuringiensis* BK5.2 with parasporal inclusion (PI) of flatcuboidal shape (black arrows) by SEI 10 kV WD10 mm SS30, 10,000× magnification.

[10]. According to Ben-Dov [11], the toxin proteins that are thought to be insecticidal are Cry15, Cry23, Cry25, Cry30, and Cry35 and specific to the Diptera group of insects are Cry4B and Cry4C [11].

The test larvae death occurred due to the action of the toxin produced by *B. thuringiensis*. Bacterial endospores are eaten by larvae and lysis because conditions are not suitable for the bacterial life conditions, so that protein crystals and spores are separated from the bacteria. The protein crystal toxin (δ-endotoxin) produced by bacteria in the form of inactive pro-toxin is converted into active toxin, if it is in an alkaline pH condition between 10 and 12 which corresponds to the conditions in the insect digestive tract. The larvae's digestive tract also contains the protease enzyme which also activates the toxin. The active toxin then binds to a specific receptor on the intestinal membrane of larvae, resulting in the formation of pores and causing lysis of epithelial cells of the larvae intestine. This condition can interfere with fluid permeability so that cells become swollen and then burst and result in larval death [9, 10, 12]. The characteristics of larvae infected with *B. thuringiensis*, larvae are black in color and experience intestinal paralysis [13]. Another indication can be seen from the activity of eating larvae which decreases and can even stop so that the larvae become weak [14]. The larval instar level affects the sensitivity of *A. aegypti* larvae against toxins produced by *B. thuringiensis* bacteria [15]. In this study, the dead larvae showed the abdomen and thorax damage (Figure 3).

The $LC_{50}$ and $LC_{90}$ values of *B. thuringiensis* BK5.2 (Figure 2), were high potential when compared with previous reports such as the research report where the isolate *B. thuringiensis* indigenous Malang city, PWR4 32, had a 72 h $LC_{50}$ value of $22.79 \times 10^7$ cells/mL [16]. Also reported that *B. thuringiensis* indigenous W.Swh.S.K2, had a 48-h $LC_{50}$ value of $3.53 \times 10^7$ cells/mL [17].

Based on the probit analysis, showed that the $LT_{50}$ value of *B. thuringiensis* BK5.2 against larvae *A. aegypti* was 19 h. The mortality rate of test larvae is influenced by the number of spores eaten by the larvae. This has to do with the activity of bacteria in the digestive tract which includes the formation of spores and crystals. As reported by Gama et al. [16] the more spores formed, the more protein crystals were released by *B. thuringiensis* to kill *A. aegypti* larvae [16]. This difference occurred due to differences in varieties or species of bacilli isolated from different soils. Differences in varieties or species from local isolates of *B. thuringiensis* will affect their toxicity to test larvae because the types of toxins produced are different. As reported, the toxicity of *B. sphaericus* bacteria was influenced by several things, such as the type of toxin, the type of larvae, the age of the target larvae, and the type of growth media used [6].

*B. thuringiensis* strains produced a variety of parasporal inclusion during the sporulation process in its growth cycle. Maeda et al. [18], reported a relationship between toxin activity and crystal form, therefore ultrastructural observations could provide important clues [18]. *B. thuringiensis* subsp. *israelensis* had a variety of forms of parasporal inclusion Cry proteins that were expressed and contained specific insecticide protein coding genes for insects of the Diptera order, namely Cry4A, Cry4B, Cry11, P19, P20, Cyt1A, and Cyt2 [12].

*B. thuringiensis* subsp. *israelensis* strain H-14 ONR60A possessed a spherical parasporal body, containing components of the Cry4A, Cry4B, Cry11A, and Cyt1Aa genes [19]. The Cry4 gene has toxicity to the Diptera order [20]. Cyt toxin had cytolytic toxicity, capable of causing pore formation in the microvilli of midgut gastric epithelial cells of larvae, which leads to osmotic cell lysis and subsequent death of the host. Cyt toxin had toxicity to insects of the order Diptera [21]. *B. thuringiensis* subsp. *israelensis* has a very varied parasporal crystal morphology and protein content and is capable of being potential bacteria as a biolarvicide product against many insect orders [12]. Exosporium was a balloon-like layer which acts as a barrier to the outer permeability of spores and contributes to spore survival and virulence as well as interacts with host cells during infection [22]. *B. thuringiensis* subsp. *kurstaki* strain HD1 had bipyramidal and ovoidal parasporal inclusion forms. The parasporal body of *B. thuringiensis* subsp. *kurstaki* strain HD1 was one of the most complex *B. thuringiensis* isolates and has toxicity for insects of the order Lepidoptera [19]. *B. thuringiensis* subsp. *kurstaki* strain HD1 was the first to be commercialized and remains the most widely used product worldwide for the control of Lepidoptera pests from vegetable crops, agricultural crops, and forestry [19].

Recombinant *B. sphaericus* strain 2297 has two parasporal inclusions present in the exosporium in the form of cuboidal inclusions surrounded by endoplasm [23]. The production of Cyt1Ab1 protein in *B. sphaericus* strain 2297 was able to overcome resistance to binary *B. sphaericus* toxin in resistant mosquito populations. The Cyt1Ab1 protein is capable of inducing additional pores in the intestinal membrane [24], thus allowing binary toxins to pass through the membrane. Cry11A protein has the ability to kill mosquito species from the genera *Culex*, *Aedes*, and *Anopheles* [25]. Melo et al. [26] reported that the *B. thuringiensis* strains, in addition to producing parasporal toxins which are larvicidal, also produces chitinase enzymes which are important to be developed in the agricultural industry, as well as parasporin toxins which have a cytotoxic effect on cancer cells, therefore this bacteria has the potential to be developed in various fields in future [26].

## Conclusions

*B. thuringiensis* BK5.2 showed high toxicity against third instar larvae of *A. aegypti*. Morphologically, the dead larvae had shown damage to the abdomen and thorax. The results of detection through TEM and SEM had shown that the presence of flatcuboidal parasporal inclusion crystals was found. Researchers recommend that *B. thuringiensis* BK5.2 still needs further development research, if directed as biolarvicide against *A. aegypti* the DHF vector.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.

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Melanny Ika Sulistyowaty*, Retno Widyowati, Galih Satrio Putra, Tutuk Budiati and Katsuyoshi Matsunami

# Synthesis, ADMET predictions, molecular docking studies, and *in-vitro* anticancer activity of some benzoxazines against A549 human lung cancer cells

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**Abstract**

**Objectives:** This study aims to synthesize a series of benzoxazines (**1**–**5**) to be examined as an epidermal growth factor receptor (EGFR) inhibitor by *in-silico* study. The overexpression of EGFR causes the growth of normal lung cells to become uncontrollable, which may lead to cancer formation. We also conducted the absorption, distribution, metabolism, excretions and toxicity (ADMET) properties evaluation and also examined *in vitro* anticancer assay on human lung cancer cells line, which is A549.

**Methods:** Benzoxazines (**1**–**5**) were synthesized by reacting anthranilic acid and benzoyl chlorides. The structures of the compounds were determined with $^{1}$H, $^{13}$C-NMR, HRMS, UV and FT-IR spectrometric methods. Prediction of ADMET was using online pkCSM, and the molecular docking studies were using MVD with EGFR-TKIs as the target (PDB ID: 1M17). *In vitro* assay of anticancer activity was performed by MTT assay.

**Results:** Compounds **1**–**5** were successfully synthesized in good yields (71–84%). The ADMET prediction showed that benzoxazines are able to be absorbed through GIT, metabolized by CYP 450, and not hepatotoxic. The title compounds have a greater Moldock Score than Erlotinib, and **3** has the highest activity against A549 compared with other benzoxazines, $IC_{50}$=36.6 μg/mL.

**Conclusions:** Compound (**3**) more active as anticancer against Human cancer cells line compared with other benzoxazines.

**Keywords:** A549 cancer cell; ADMET prediction; benzoxazines; molecular docking; synthesis.

## Introduction

Lung cancer has the highest mortality case in the world among the other cancers. According to WHO data in 2018, a total of 26,095 people in Indonesia die from lung cancer each year, with 30,023 new cases, thus considered as a country with the highest cases in Southeast Asia [1]. Non-small cell lung cancer (NSCLC) is a type of lung cancer that often ensues nearly 75% all lung cancer cases. Many studies reported that NSCLC occurred due to the overexpression of epidermal growth factor receptor (EGFR) which caused the growth of normal lung cells to become uncontrollable [2]. In recent years, novel drugs known as EGFR-targeted therapies, or EGFR-tyrosine kinase inhibitors (TKIs), such as Gefitinib, Erlotinib, Afatinib and Osimertinib have succeeded in restraining the progression of lung cancer in some NSCLC patients [3].

Currently, several treatments for lung cancer are available, but a continuous treatment innovation is needed because some cases indicate the resistance to EGFR-tyrosine kinase inhibitors (TKIs) in some patients. One of the strategies undertaken by researchers to overcome cases of resistance to EGFR-TKIs, is first of all to combine the TKIs drug with several drugs that have other mechanisms such as Bortezomib, Everolimus, Bevacizumab, Tivantinib and Sorafenib. The second step is to create a new drug that has less side effects than the previous drug [4].

In the development of anticancer candidates, several studies found out that some compounds which possessed

***Corresponding author: Melanny Ika Sulistyowaty**, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, E-mail: melanny-i-s@ff.unair.ac.id
**Retno Widyowati,** Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. https://orcid.org/0000-0002-6166-1289
**Galih Satrio Putra,** Department of Pharmaceutical Chemistry, Stikes Rumah Sakit Anwar Medika, Sidoarjo, Indonesia
**Tutuk Budiati,** Faculty of Pharmacy, Widya Mandala Catholic University, Surabaya, Indonesia
**Katsuyoshi Matsunami,** Department of Pharmacognosy, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

benzoxazine ring are able to inhibit the growth of A549 cell line (Figure 1) [5–9]. Based on the molecular docking results of benzoxazine ring to the PDB 1M17 receptor, it is predicted that it has ability to inhibit EGFR-TKIs, such as Erlotinib [6]. Therefore, our research aims were to synthesize derivatives of benzoxazine and carried out the computational tests on ADMET predictions and molecular docking on the EGFR receptors. In addition, we also conducted bioassays against A549 human lung cancer cells line.

# Materials and methods

### Synthesis of benzoxazines derivatives

All chemicals used were analytical grade and obtained from Sigma-Aldrich. Anthranilic acid (10 mmol) was dissolved in pyridine and benzoyl chloride (1.5 eq) was added wisely at 0 °C, then being stirred for an hour at room temperature. The progress of the reaction was conducted by TLC method, with *n*-hexane and ethyl acetate (1:1). When the reaction was completed, solution of sodium bicarbonate (10%) was poured to the mixture. The product was recrystallized from ethanol 96% [10, 11].

Characterizations of the benzoxazines were performed using various spectroscopic methods. $^{1}$H-NMR and $^{13}$C-NMR spectra measurements were conducted using Bruker Ultrashield 600 spectrometer at 600 and 150 MHz, MS spectra were measured in QSTAR XL Nano-Spray™ with ESI mode. FT-IR spectra were recorded by Jasco FT-IR 5300. Ultraviolet spectra were analyzed using Shimadzu UV–Vis Spectrophotometer 1800. In addition, melting point of the compound was determined using Fisher-John Electrothermal Mel-Temp without correction.

### ADMET prediction study

Benzoxazines (**1**–**5**) and Erlotinid were drawn using Marvin sketch and saved as a smile data. The data were then inputted to the online pcKSM website (http://biosig.unimelb.edu.au/pkcsm/) in order to obtain ADMET prediction data.

### Molecular docking study

*In silico* study was performed by using MVD (Molegro® Virtual Docker version 5.5) and MMFF94 was used to optimize the 3D geometry of the compounds [12]. The benzoxazines and Erlotinid were docked into the active site of EGFR-TK domain (PDB ID: 1M17) [6, 13]. The validation of docking was performed by docking its native ligand (Erlotinid) into its active site EGFR-TK domain. The criteria of acceptance were the value of RMSD≤2.0 Å. After validation docking process, benzoxazines were docked into active site of this receptor. The evaluation was carried out using MolDock score. Then, it was shown that the smaller the score, the more stable binding between ligand and receptor was [14].

### Bioassay against human lung cancer cell

A549 cell line was cultivated in an enhanced medium, which is the combination of DMEM (Dulbecco's modified Eagle's Medium), 10% heat inactive FBS (fetal bovine serum), Amphotericin B and Kanamycin. Three days-old cells were employed as test substance. One microliter of samples (1% in DMSO) and 99 µL of A549 cells ($5 \times 10^3$ cells) were incubated at 37 °C for 72 h. After some steps of treatment, we measured the absorbance of the mixture by scanning it at $\lambda$: 540 nm with a 2,300 EnSpire Multimode plate reader, Perkin Elmer, Inc. The percentage (%) of the inhibition of cell growth was computed using Eq. (1).

$$\%\boldsymbol{inhibition} = \left[1 - \frac{(\boldsymbol{A\ sample} - \boldsymbol{A\ blank})}{(\boldsymbol{A\ control} - \boldsymbol{A\ blank})}\right] \times 100 \qquad (1)$$

The evaluation was performed in triplicate and reported as mean ± SE [9].

# Results

## Synthesis

### 2-(2-chlorophenyl)-4*H*-benzo-[1,3]-oxazin-4-one (2)

Obtained as white crystals, mp: 125–128 °C. FT-IR (KBr) $cm^{-1}$: 1765 (C=O lactone); 1,620 and 1,474 (C=C aromatic); 3,040 (=C–H aromatic); 1,614 (C=N); 1,315 (C–N). UV ($\lambda_{max}$): 216, 264, 306 nm. $^{1}$H-NMR (600 MHz, CDCl3, δ) 8.28 (d, *J*=7.9 Hz, 1H), 7.91 (d, *J*=7.6 Hz, 1H), 7.87 (dd, *J*=8.1, 7.4 Hz, 1H), 7.73 (d, *J*=7.9 Hz, 1H), 7.59 (t, *J*=7.6 Hz, 1H), 7.54 (d, *J*=8.0 Hz, 1H), 7.51 – 7.44 (m, 1H), 7.41 (t, *J*=7.6 Hz, 1H). $^{13}$C-NMR (151 MHz, $CDCl_3$, δ) 159.39, 156.76, 146.64, 136.80, 133.67, 132.48, 131.61, 131.28, 130.51, 129.13, 128.77, 127.61, 127.04, 117.19, 77.16. HRMS-ESI (*m/z*)=280.0137 $[M+Na]^+$ (calcd. for $C_{14}H_8O_2NClNa$: 280.0137).

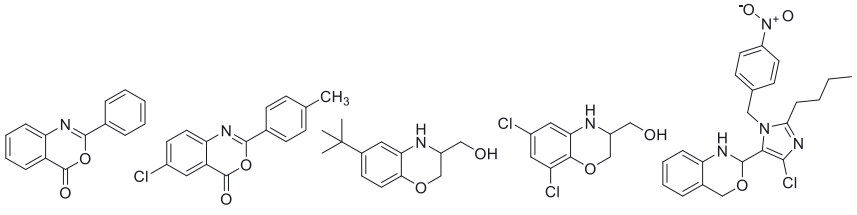


**Figure 1:** Some benzoxazines which have ability on inhibiting the growth of A549 human lung cancer cells line.

**2-(2,4-dichlorophenyl)-4*H*-benzo-[1,3]-oxazin-4-one (3)**

Yielded as white crystals, mp: 141–143 °C. FT-IR (KBr) $cm^{-1}$: 1767 (C=O lactone); 1,623 and 1,476 (C=C aromatic); 3,090 (=C–H aromatic); 1,620 (C=N); 1,315 (C–N); 1,029 (C–O–C) and 772 (C–Cl). UV ($\lambda_{max}$): 220, 280, 310 nm. $^1$H-NMR (600 MHz, $CDCl_3$, δ) 8.30 – 8.25 (m, 1H), 7.90 (d, *J*=8.4 Hz, 1H), 7.89 – 7.84 (m, 1H), 7.72 (d, *J*=8.1 Hz, 1H), 7.62 – 7.57 (m, 1H), 7.56 (d, *J*=2.0 Hz, 1H), 7.40 (dd, *J*=8.4, 2.0 Hz, 1H). $^{13}$C-NMR (151 MHz, $CDCl_3$, δ) 159.15, 146.50, 138.24, 136.88, 134.70, 132.55, 131.29, 129.29, 128.82, 127.64, 127.51, 117.15, 77.16. HRMS-ESI (*m/z*)=313.9748 $[M+Na]^+$ (calculated for $C_{14}H_7O_2NCl_2Na$: 313.9746).

**2-(3,4-dichlorophenyl)-4*H*-benzo-[1,3]-oxazin-4-one (4)**

Yielded as white powders, mp: 166–169 °C. FT-IR (KBr) $cm^{-1}$: 1760 (C=O lactone); 1,621 and 1,474 (C=C aromatic); 3,090 (=C–H aromatic); 1,620 (C=N); 1,324 (C-N); 1,076 (C–O–); C–Cl (770). UV ($\lambda_{max}$): 220, 244, 288, 302 nm. $^1$H-NMR (600 MHz, $CDCl_3$, δ) 8.42 (d, *J*=1.9 Hz, 1H), 8.26 (d, *J*=7.9 Hz, 1H), 8.14 (dd, *J*=8.5, 2.0 Hz, 1H), 7.86 (t, *J*=7.7 Hz, 1H), 7.70 (d, *J*=7.8 Hz, 1H), 7.60 (d, *J*=8.4 Hz, 1H), 7.56 (t, *J*=7.6 Hz, 1H). $^{13}$C-NMR (151 MHz, $CDCl_3$, δ) 159.12, 155.29, 146.69, 137.32, 136.95, 133.60, 131.02, 130.33, 130.23, 128.96, 128.92, 127.52, 127.39, 117.17. HRMS-ESI (*m/z*)=313.9748 $[M+Na]^+$ (calcd. for $C_{14}H_7O_2NCl_2Na$: 313.9746).

**2-(4-methoxyphenyl)-4*H*-benzo-[1,3]-oxazin-4-one (5)**

Yielded as white powders, mp: 150–152 °C. FT-IR (KBr) $cm^{-1}$: 1760 (C=O lactone); 1,621 and 1,474 (C=C aromatic); 3,090 (=C–H aromatic); 1,620 (C=N); 1,324 (C–N); 1,076 (C–O–); C–Cl (770). UV ($\lambda_{max}$): 220, 250, 294, 306 nm. $^1$H-NMR (600 MHz, $CDCl_3$, δ) 8.29 – 8.25 (m, 2H), 8.22 (dd, *J*=7.8, 1.1 Hz, 1H), 7.80 (td, *J*=8.1, 1.5 Hz, 1H), 7.67 – 7.63 (m, 1H), 7.48 (t, *J*=7.6 Hz, 1H), 7.01 (d, *J*=8.9 Hz, 2H), 3.90 (s, 3H). $^{13}$C-NMR (151 MHz, $CDCl_3$, δ) 163.31 (s), 159.80 (s), 157.17 (s), 136.49 (s), 130.30 (s), 128.57 (s), 127.71 (s), 126.94 (s), 122.60 (s), 116.76 (s), 114.17 (s), 55.52 (s). HRMS-ESI (*m/z*)=276.0632 $[M+Na]^+$ (calcd. for $C_{15}H_{11}O_3NNa$: 276.0631).

## ADMET prediction study

The absorption prediction of the title compounds by pkCSM application showed in Table 1 below:

The distribution prediction of benzoxazines using pkCSM application showed in Table 2 below:

The predictions of excretion of synthesized compounds using pkCSM application were shown in Table 3:

**Table 1:** Absorption prediction of compound **1**–**5** and Erlotinib.

| Compounds | Lipinski Rule of Five | Human intestinal absorption, % | Caco2 permeability (log Papp in $10^{-6}$ cm/s) |
|---|---|---|---|
| **1** | ✓ | 97.11 | 1.32 |
| **2** | ✓ | 95.36 | 1.33 |
| **3** | ✓ | 94.22 | 1.37 |
| **4** | ✓ | 95.15 | 1.38 |
| **5** | ✓ | 97.96 | 1.30 |
| Erlotinib | ✓ | 96.05 | 1.17 |

✓, Mr (Molecular weight)<500; HBA, Hydrogen bond acceptor≤10; HBD, Hydogen bond donor≤5; Log<5; MR, molar refractivity=120–140 Å

The prediction of toxicity of compounds **1**–**5** using the application of pkCSM as described below in Table 4:

## Molecular docking study

The Molecular docking of the title compounds toward EGFR-tyrosine as shown Table 5 below:

Figure 2 illustrated the hydrogen bond and steric interaction of tittle compounds into the active site of EGFR-tyrosine in 2D and 3D as shown below:

The $IC_{50}$ of benzoxazines **1**–**5** as shown in Table 6 below:

# Discussion

## Synthesis

Synthesis of the title compounds was started by dissolved anthranilic acid then added benzoyl chloride as described in Figure 3. Benzoxazines (**1–5**) were obtained in 60–84% yields. The synthesized compounds then being analysed their stuctures by using some method of spectrophotometry.

**Table 2:** Distribution prediction of benzoxazines and Erlotinib.

| Compounds | Volume distribution | BBB permeability Log BB | CNS permeability Log PS |
|---|---|---|---|
| **1** | −0.12 | 0.35 | −1.35 |
| **2** | −0.03 | 0.32 | −1.33 |
| **3** | 0.01 | 0.25 | −1.32 |
| **4** | 0.07 | 0.3 | −1.36 |
| **5** | −0.01 | 0.35 | −2 |
| Erlotinid | 0.07 | −0.51 | −3.40 |

**Table 3:** Metabolism and excretion prediction of the title compounds.

| Compounds | Substrates | | Inhibitors | | Excretion |
|---|---|---|---|---|---|
| | CYP 2D6 | CYP 3A4 | CYP 2D6 | CYP 3A4 | mL/min/kg |
| 1 | – | ✓ | – | – | 7.40 |
| 2 | – | ✓ | ✓ | – | 1.79 |
| 3 | – | ✓ | – | – | 1.50 |
| 4 | – | ✓ | – | – | 1.48 |
| 5 | – | ✓ | – | – | 7.13 |
| Erlotinib | – | ✓ | – | ✓ | 4.21 |

**Table 4:** Toxicity Prediction of benzoxazines **1**–**5** and Erlotinib.

| Compounds | AMES toxicity | Hepatoxicity | Maximal tolerated dose (human), mg/kg/day | Oral rat acute toxicity, mol/kg |
|---|---|---|---|---|
| **1** | – | – | 1.37 | 1.72 |
| **2** | – | – | 1.08 | 1.84 |
| **3** | – | – | 0.68 | 1.99 |
| **4** | – | – | 0.80 | 2.00 |
| **5** | – | – | 1.02 | 2.03 |
| Erlotinib | – | + | 4.25 | 2.68 |

## ADMET prediction study

Based on the prediction of absorption by pkCSM application (Table 1), the title compounds and Erlotinid can be absorbed in the digestive tract (>90%). It happens because they meet the requirements of the Lipinski Rule of Five (MW<500, hydrogen bond donor <5, hydrogen acceptors <10, log P<5, molar refractivity between 40 and 130) [15, 16]. Not only that, all tested compounds have Caco2 permeability value of >0.9 which means it has high permeability. Caco2 cell lines are human epithelial colorectal adenocarcinoma cells which are cell monolayers that are often used as a human intestinal mucosa model as *in vitro* assay to predict oral drug absorption [16].

From the result of the distribution prediction using pkCSM application (Table 2), the tested compounds have moderate volume of distribution. It means that the total concentration of drugs circulating in blood plasma and tissues are the same. It is categorized as a small volume distribution if the value is less than −0.15 log L/kg and as a large volume distribution if the value is >2.81>0.45 log L/kg [16]. The benzoxazines derivatives are predicted to be able to penetrate BBB and enter the bloodstream in the brain because they have log BB value more of than 0.3 and log PS of more than −2. On the other hand, Erlotinid is predicted not to be able to penetrate BBB and cannot enter the bloodstream in the brain because it has the log BB value of less than −0.1 and Log PS of less than −3. The molecular weight of Erlonitib is also twice bigger compared to the synthesized compounds [16].

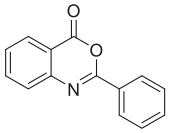
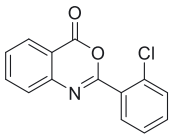
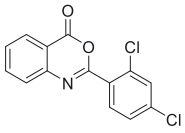
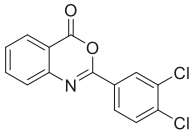
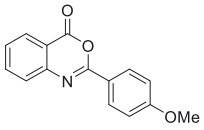
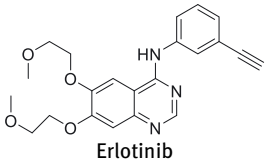
Benzoxazines are predicted to be able to penetrate BBB and enter the bloodstream in the brain because they are similar to narcotics class 1 [17, 18]. The starting material of benzoxazines was anthranilic acid, which is a precursor used in synthesizing narcotic-like compounds. There are several compounds classified as class 1 and often being misused as narcotic compounds which can be synthesized from anthranilic acid, namely Mecloqualone and Methaqualone. The structure of those compounds was similar to **1**–**5** [Figure 4].

Cytochrome 450 is responsible for metabolizing most drugs. Most drugs are metabolized by two isoforms of cytochrome 450, namely CYP 2D6 and CYP 3A4. The knowledge about whether a drug is a substrate of CYP 2D6/CYP 3A4 or else will relate to the presence of inducer and inhibitor of both isoforms. This will have an impact on the fluctuations of drug's bioavailability [19]. Based on the prediction of metabolism by pkCSM application, benzoxazines (**1**–**5**) and Erlotinib compounds are CYP 3A4 substrates and are not CYP 2D6 substrates. It means that the presence of CYP 3A4 inducers has the potential to reduce blood levels of benzoxazines **1**–**5** and Erlotinid. However, the presence of CYP 3A4 inhibitors will create the opposite effect.

Based on the prediction of metabolism and excretion using pkCSM application (Table 3), the rate of drug clearance (total clearance) in the body is a combination of hepatic clearance (liver metabolism and bilinary clearance) and renal clearance [16, 20]. The greater the total clearance, the faster the drug is excreted by the body. Compound **2**, **3** and **4** have low total clearance rate (<2 mL/min/kg) compared to **1** and **5**. This prediction is possible because benzoxazines **2**, **3**, and **4** contained halo-substitution (Cl atom) in benzene ring, so they are more difficult to be excreted by the body.

From data of the prediction of toxicity using the application of pkCSM (Table 4), benzoxazines **1**–**5** do not cause mutations. It is related to negative value of AMES toxicity predictions. The AMES test is widely used as a method for initial screening of mutagenic compound with the help of bacteria [16, 21]. The prediction results of pkCSM applications and mutagenic test of Erlotinib have been published as non-mutagenic compound, both by AMES test and by *in vitro* assay of mammalian mutation test [16, 22]. From the prediction of toxicity of pkCSM

**Table 5:** Molecular docking result of benzoxazines and their native ligand (Erlotinib).

| Compound | Moldock score, Kcal/mol | Docked Pose | Hydrogen bond | Amino acids residues | Steric interaction | Amino acids residues |
|---|---|---|---|---|---|---|
|  **1** | −65.89 ± 0.07 | √ | 1 | Met 769 | – | – |
|  **2** | −71.28 ± 0.03 | √ | 2 | Met 769<br>Gln 767 | – | – |
|  **3** | −73.29 ± 0.05 | √ | 1 | Met 769 | – | – |
|  **4** | −72.94 ± 0.02 | √ | 2 | Lys 721<br>Met 769 | – | – |
|  **5** | −68.86 ± 0.06 | √ | 1 | Thr 766 | 3 | Leu 694<br>Leu 768<br>Met 769 |
|  Erlotinib | −122.93 ± 0.06 | √ | 2 | Thr 766<br>Met 769 | 2 | Gly 695<br>Gln 767 |

application, the synthesized compounds are not hepatotoxic while Erlotinib causes hepatotoxicity. This result was in line with the fact that Erlonitib increases transaminases [16, 21].

MTD is an estimated safe dose limit for humans. These data are very helpful for the initial dose given in phase 1 clinical trials. Prediction of MTD of compounds **1**–**5** is less than 3 mg/kg/day which is categorized as low [13]. Meanwhile, the MTD prediction of Erlotinib is more than 3 mg/kg/day which is categorized as high. The dose of Erlotinib used for NSCLC cases is 150 mg, which means that it does not exceed the prediction of the maximum tolerated dose [16, 21, 23].

From the prediction evaluation of oral rat acute toxicity, the LD50 results of the synthesized compounds are lower than Erlotinid. This can happen because benzoxazines **1**–**5** have the same structure as Mecloqualone and Methaqualone with LD50 values of 250 mg/kg and 185 mg/kg [24]. Both compounds are narcotics drug class 1 which have a high potential for addiction with a potential effect of large lethality.

## Molecular docking study

Based on the results of the molecular docking study (Table 5), the most suitable predictor of binding with EGFR-TKs is Erlotinib as the native ligand. It has the lowest moldock score, which is −122.93 ± 0.06 Kcal/mol while benzoxazines **1**–**5** have moldock scores greater than Erlotinib. That means that the ability of benzoxazines in inhibiting the EGFR-TKI is not as great as Erlotinib.

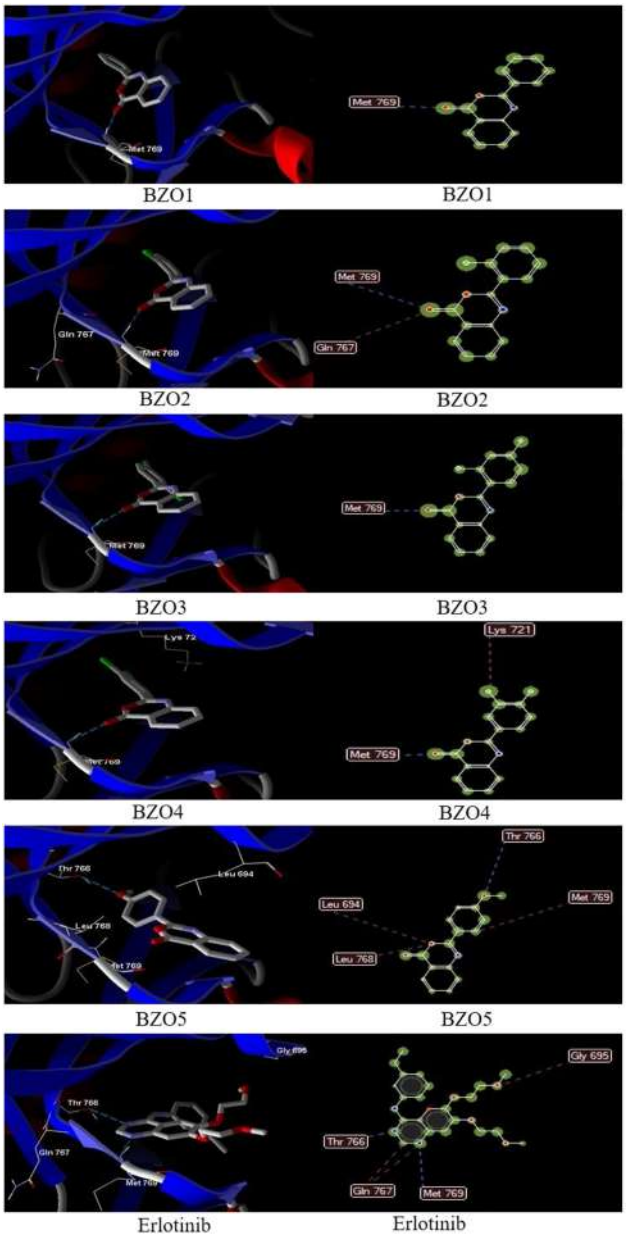



**Figure 2:** Hydrogen bond (blue dotted) and steric interaction (red dotted) of **1**–**5** and Erlotinib into the active site of EGFR-tyrosine. Left side (3D); Right side (2D). *In-vitro* anticancer activity.

From Figure 2, Erlotinib has a hydrogen bond with the amino acid Thr 766, Met 769 and steric interaction with amino acid Gly 695, Gln 767. This interaction is predicted to be the most important, so that Erlonitib has low binding energy. The interaction of Erlotinib with the presence of hydrogen bonds (2.89 Å) from the N atom as a hydrogen acceptor with Met 769 gives a low free energy contribution, followed by the N atom as a hydrogen acceptor with Thr 766 with a distance of 2.93 Å [6, 11]. Compound **1**, **2**, **3** and **4** also have a hydrogen bond (2.62–3.20 Å) with the amino acid Met 769 and an O atom as a hydrogen acceptor but don't have a hydrogen bond with the amino acid Thr 766.

**Table 6:** $IC_{50}$ of the title compounds.

| Compound | $IC_{50}$, *μg/mL* | $IC_{50}$, *μM* |
|---|---|---|
| **1** | 74.3 | >200 |
| **2** | 69.2 | >200 |
| **3** | 36.6 | 125 |
| **4** | >100 | >200 |
| **5** | >100 | >200 |
| Doxorubicin | 1.46 ± 2.3 | 2.68 |
| Erlotinib | – | – |

## *In vitro* anticancer activity

Based on the results of the *in vitro* evaluation on inhibiting the growth of A549 cell line using the MTT method (Table 6), the benzoxazines **1**–**5** possessed moderate activity as anticancer agent. Among the synthesized products, benzoxazine **3** had the greatest activity against A549 cell line, with the lowest $IC_{50}$ value of 36.6 μg/mL, which is categorized as the low activity category [25]. Doxorubicin as a positive control *in vitro* had an $IC_{50}$ value of 2.68 μM which is included in the strong activity category [25, 26]. The limitation of this *in vitro* examination was that we did not use erlotinib as a positive control, which is one of the drugs included in the NCCN Guideline for NSCLC cases [27]. In addition, the cytotoxic assay on the normal cell line to obtain the Selectivity Index (SI) value did not carried out because there was no tested compound with $IC_{50}$ values of <25 μM.

# Conclusions

The derivatives of benzoxazine were synthesized in good yields (60–84%). The ADMET prediction resulted that the compounds were able to be absorbed through GIT, metabolized by CYP 450, and not hepatotoxic. From the result of *in vitro* evaluation and also *in silico* study, from the

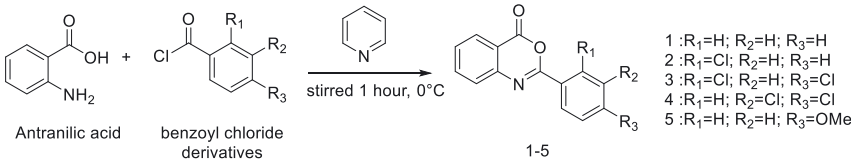



**Figure 3:** Synthesis of benzoxazines.

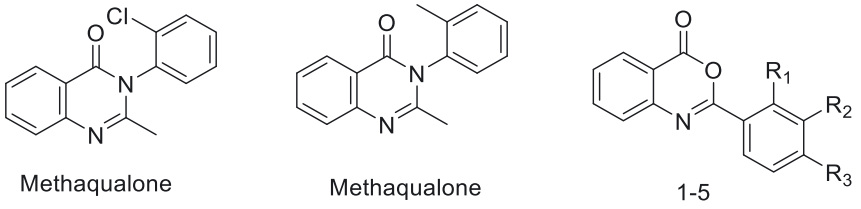



**Figure 4:** Chemical structures of Mecloqualone, Methaqualone and the synthesized compounds.

result of *in silico* and bioassay, compound **3** had strongly potential as anticancer activity compared other substituent, against human lung cancer cell line.

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**Competing interests:** Authors state no conflict of interest.
**Informed consent:** Not applicable.
**Ethical approval:** Not aplicable.

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Adinda Adelia Wulandari, Achmad Aziz Choiri, Fitria and Tri Widiandani*

# Thymoquinone and its derivatives against breast cancer with HER2 positive: *in silico* studies of ADMET, docking and QSPR

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**Abstract**

**Objectives:** The high prevalence of HER2-positive breast cancer has become a significant concern in the health sector. The problem is more complex with the side effects of breast cancer drugs currently used. Thymoquinone (TQ), the main bioactive compound in *Nigella sativa*, has been shown to have anticancer activity. However, it is necessary to modify the structure of the thymoquinone derivatives to improve drug bioavailability. This study uses an *in silico* approach to predict pharmacokinetic profile, docking, quantitative structure–properties relationship (QSPR) of new thymoquinone-derived compounds as candidates cytotoxic agent for breast cancer with HER-2 positive.

**Methods:** The prediction of ADMET was using pkCSM online. Molecular docking was used to determine thymoquinone derivatives activity using Molegro Virtual Docker version 5.5 by docking the thymoquinone derivatives to the HER2 receptor targets, PDB ID 3PP0 and QSPR analysis using the IBM SPSS 21 version.

**Results:** The 35 thymoquinone derivatives showed good physicochemical and absorption properties and not hepatotoxic, so they are suitable for oral drugs. The molecular docking of 35 thymoquinone derivatives against 3PP0 proteins showed better activity than thymoquinone. One of the thymoquinone derivatives, TQ 15, showed the largest negative RS value, meaning that is predicted to have the highest anticancer activity. Based on the QSPR analysis, the essential parameter in determining 35 thymoquinone derivatives activity was the lipophilic and steric parameter.

**Conclusions:** Based on *in silico* test, thymoquinone derivative, TQ 15, had the potential to be further developed as a HER2-positive breast cancer drug.

**Keywords:** breast cancer; HER2; Molegro Virtual Docker; pkCSM; QSPR; thymoquinone.

## Introduction

Breast cancer is the leading cause of cancer death in women worldwide, including in Indonesia, with an incidence of 42.1 per 100,000 population [1, 2]. Based on molecular analysis of breast cancer with gene expression profiling (GEP), breast cancer can be classified into several different subtypes such as HER2 overexpression or also known as HER2 positive.

Drugs that have been used in the treatment of breast cancer with HER2 positives such as trastuzumab, lapatinib, and neratinib have a variety of adverse events such as resistance and hepatotoxicity with trastuzumab [3], lapatinib [3, 4], and neratinib [5, 6]. The development of new drugs by utilizing chemical compounds from natural ingredients is an alternative to overcome problems in drugs that have been measured [7].

One of the plants known to have anticancer activity is black cumin (*Nigella sativa*) [8]. The anticancer activity in black cumin is related to the presence of thymoquinone (TQ), which is the main component of black cumin seed essential oil (28–45% of essential oil) [9]. However, thymoquinone has poor membrane penetration ability [10], slow absorption, and fast elimination in the body [11]. Modification of thymoquinone's chemical structure can be used as a solution to overcome these problems [12].

Abdellazeem et al. [13] have derivatized the thymoquinone compound into the *N*′-(4-hydroxy-2,5-dimethylcyclohexa-2,5-dien-1-yl) benzohydrazide structure. However, these compounds have lower anticancer activity even though they have better physicochemical properties than thymoquinone compounds. Therefore, this research was

***Corresponding author: Tri Widiandani**, Department of Pharmaceutical Chemistry, Faculty of Pharmacy Universitas Airlangga, Surabaya, Indonesia, Phone: +81803022660,
E-mail: tri-w@ff.unair.ac.id. https://orcid.org/0000-0002-0156-6095
**Adinda Adelia Wulandari, Achmad Aziz Choiri and Fitria,** Department of Pharmaceutical Chemistry, Faculty of Pharmacy Universitas Airlangga, Surabaya, Indonesia

carried out *in silico* with the further development of the structure of *N′*-(4-hydroxy-2,5-dimethylcyclohexa-2, 5-dien-1-yl) benzohydrazide by adding 35 different substituents by designs to obtain a derivative compound with better physicochemical and activity. The *in silico* test was performed by docking the thymoquinone derivatives to the HER2 receptor with PBD ID 3PP0. The 3PP0 structure is known to have low resolution and a low missing residue. In addition, HER2 receptors also have standard ligands, namely 2-(2-[4-({5-chloro6-[3(trifluoromethyl) phenoxy] pyridin-3-yl} amino)-5Hpyrrolo [3,2-d] pyrimidine-5yl] ethoxy}-ethanol (SYR127063) [14, 15] (Figure 1).

The *in silico* method in this research includes structural modification and analysis with the prediction of physicochemical properties, pharmacokinetic profile, activity against HER2 receptors and analysis of the quantitative relationship of the structure of the activity of thymoquinone derivatives.

# Materials and methods

### Computer tools and programs

This research uses Lenovo brand computers, Windows 7 operating system, 64 bit, Intel Core i3-3110M, CPU @ 2.40 GHz, 2.00 GB RAM. The programs used include Chem Draw 2-D 17.0, Chem Draw 3-D 17.0, Molegro Virtual Docker 5.5 (Molegro ApS), SMILES Translator, pkCSM, and IBM SPSS 21.

### Download the protein target

The molecular structure of the target protein is downloaded via the Protein Data Bank website (http://www.rcsb.org/pdb/home/home.do). This study used a protein target coded PDB 3PP0 because it is a tyrosine kinase receptor that can increase HER2 expression [16].

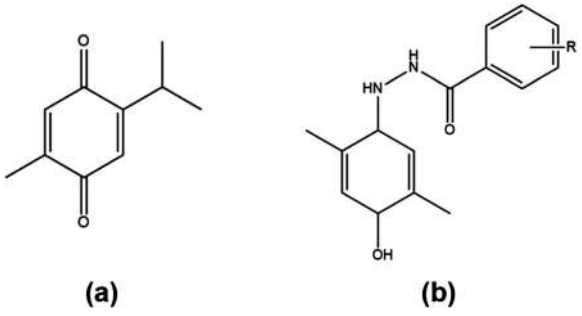


**Figure 1:** The structure of (a) thymoquinone compound and (b) the derivative of the thymoquinone compound modified by the addition of 35 substituents at the R position (Table 1).

### The physicochemical properties and pharmacokinetics of thymoquinone derivatives

The prediction of physicochemical properties was carried out using the Chem Draw 2-D 17.0 application and the pkCSM online tool. The results obtained in this study are BM (molecular weight), LogP, HBA (hydrogen bond acceptor), HBD (hydrogen bond donor), FRB (freely rotatable bond), and tPSA (topological polar surface area). Furthermore, the prediction of the pharmacokinetic profile of 35 thymoquinone derivative compounds along with comparators, i.e., thymoquinone using the pkCSM online tool application (http://biosig.unimelb.edu.au/pkCSM/prediction).

### Molecular docking of thymoquinone derivatives

Prediction of the activity of 35 thymoquinone derivative compounds along with comparators, i.e., thymoquinone was obtained by drawing these compounds using the Chem Draw 2-D 17.0 application, then copied to the Chem Draw 3-D 17.0 program. Next, each compound's minimum energy is measured and stored in the file mol2 {SYBYL2 (*. Mol2)}. Once stored, the docking process is carried out against the HER2 receptor target, coded as PDB 3PP0 using the version 5.5 Molegro Virtual Docker computer program. RMSD is used to control the docking of 3PP0 receptor. In Molegro Virtual Docker, RMSD can be run by native ligand redocked with 3PP0 receptor. The results obtained are a Rerank Score (RS), which describes the amount of energy needed to form bonds between ligands and receptors.

### The quantitative structure-properties relationship (QSPR) of thymoquinone derivatives

Prediction of HKSA thymoquinone derivatives is performed by IBM SPSS Statistic 21. There are three parameters in predicting HKSA, i.e., lipophilic (ClogP), steric (CMR), and electronic (Etot) parameters. Data on physicochemical properties (ClogP, CMR, and Etot) were included in the SPSS table and analyzed using linear and nonlinear regression with the dependent variable being the re-rank score and the independent variable being the physicochemical parameter.

# Results

## The physicochemical properties of thymoquinone derivatives

The prediction results of the physicochemical properties of the thymoquinone derivative compounds are shown in Table 2. Thirty-five thymoquinone derivative compounds are predicted to be suitable to be given orally based on the Lipinski Rule of 5. The requirements included in the Lipinski Rule of 5 law are molecular weight (BM) <500, LogP <5, hydrogen bond donors (HBD) <5, hydrogen bond acceptors (HBA) <10, freely rotatable bonds (FRB) <10, and polar surface area (tPSA) ≤140 Å.

**Table 1:** The chemical structure of thymoquinone derivatives, and thymoquinone as a reference compound.

| No. | R | Code | IUPAC name |
|---|---|---|---|
| 1 | H | TQ 1 | *N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 2 | Br (m), Cl (m) | TQ 2 | 3-Bromo-5-chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 3 | Br (p) | TQ 3 | 4-Bromo-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 4 | Br (p), I (o) | TQ 4 | 4-Bromo-*N*′-(4-hydroxy-2,5-dimethylphenyl)-2-iodobenzohydrazide |
| 5 | Br (o) | TQ 5 | 2-Bromo-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 6 | CF3 (p) | TQ 6 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-4-(trifluoromethyl)benzohydrazide |
| 7 | CF3 (m) | TQ 7 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-3-(trifluoromethyl)benzohydrazide |
| 8 | Cl (m) | TQ 8 | 3-Chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 9 | Cl (m), Cl (p) | TQ 9 | 3,4-Dichloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 10 | Cl (p) | TQ 10 | 4-Chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 11 | Cl (o), Cl (p) | TQ 11 | 2,4-Dichloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 12 | Cl (o) | TQ 12 | 2-Chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 13 | Cl (m), Cl (m) | TQ 13 | 3,5-Dichloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 14 | Cl (p), CF3 (m) | TQ 14 | 4-Chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)-3-(trifluoromethyl)benzohydrazide |
| 15 | Cl (m), CF3 (p) | TQ 15 | 3-Chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)-4-(trifluoromethyl)benzohydrazide |
| 16 | F (m), I (p) | TQ 16 | 3-Fluoro-*N*′-(4-hydroxy-2,5-dimethylphenyl)-4-iodobenzohydrazide |
| 17 | F (p) | TQ 17 | 4-Fluoro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 18 | F (o), I (p) | TQ 18 | 2-Fluoro-*N*′-(4-hydroxy-2,5-dimethylphenyl)-4-iodobenzohydrazide |
| 19 | F (m), I (m) | TQ 19 | 3-Fluoro-*N*′-(4-hydroxy-2,5-dimethylphenyl)-5-iodobenzohydrazide |
| 20 | I (p) | TQ 20 | 4-(Dimethylamino)-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 21 | CH3(p) | TQ 21 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-4-methylbenzohydrazide |
| 22 | OCH3 (o) | TQ 22 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-2-methoxybenzohydrazide |
| 23 | OCH3 (p) | TQ 23 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-4-methoxybenzohydrazide |
| 24 | Br (o), Cl (m) | TQ 24 | 2-Bromo-3-chloro-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 25 | T-butil (p) | TQ 25 | 4-(Tert-butyl)-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 26 | NH2 (p) | TQ 26 | 4-Amino-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 27 | OH (p) | TQ 27 | 4-Hydroxy-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 28 | CH3 (o) | TQ 28 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-2-methylbenzohydrazide |
| 29 | NO2 (p), CF3 (m) | TQ 29 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-4-nitro-3-(trifluoromethyl)benzohydrazide |
| 30 | NO2 (o), CF3 (m) | TQ 30 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-2-nitro-3-(trifluoromethyl)benzohydrazide |
| 31 | 3 N (CH3)2 | TQ 31 | 3-(Dimethylamino)-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 32 | C(Ch3)3) (p) | TQ 32 | 4-(Tert-butyl)-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| 33 | OCH(CH3)2 (p) | TQ 33 | *N*′-(4-hydroxy-2,5-dimethylphenyl)-4-isopropoxybenzohydrazide |
| 34 | CH3 (m), N-CH3 (p) | TQ 34 | 4-(dimethylamino)-*N*′-(4-hydroxy-2,5-dimethylphenyl)-3-methylbenzohydrazide |
| 35 | N (Ch3)2 (p) | TQ 35 | 4-(dimethylamino)-*N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide |
| **36** | **TQ** | **TQ** | ***N*′-(4-hydroxy-2,5-dimethylphenyl)benzohydrazide** |

## The pharmacokinetic properties of thymoquinone derivatives

The pharmacokinetic predictions of thymoquinone derivatives can be observed in Table 3. Based on Table 3, it can be seen that most of the TQ derivative compounds have good intestinal absorption and $CaCo_2$ permeability so that they can be used for oral use. Regarding toxicity, 35 TQ compound derivatives are not hepatotoxic. However, TQ 7, 25, 26, 29, 30 and 32 derivative compounds are mutagenic and carcinogenic because they give positive results on AMES toxicity.

## Molecular docking of thymoquinone derivatives

Activity prediction is performed with the Molegro virtual docker (MVD), which works by activating each ligand structure's poses using an algorithm. The software can identify the cavity or active site where the ligand binds to the receptor. In this study, the HER2 receptor was represented by a protein with the code 3PP0 found in cavities 1 [16]. Before we dock the TQ derivatives, we using RMSD to make sure that all the docking parameters are accepted to proceed. The RMSD value obtained is 0.44 Å so that we can continue to

**Table 2:** Physicochemical properties of thymoquinone derivatives.

| Derivative compounds | MW, g/mol | CLogP | HBD | HBA | FRB | Surface area | tPSA, Å | Lipinski rule of five requirement |
|---|---|---|---|---|---|---|---|---|
| TQ 1 | 256.305 | 2.76594 | 3 | 3 | 3 | 111.609 | 61.36 | Yes |
| TQ 2 | 369.646 | 4.18184 | 3 | 3 | 3 | 135.780 | 61.36 | Yes |
| TQ 3 | 335.20 | 3.52844 | 3 | 3 | 3 | 125.476 | 61.36 | Yes |
| TQ 4 | 461.097 | 4.13304 | 3 | 3 | 3 | 144.738 | 61.36 | Yes |
| TQ 5 | 335.201 | 3.52844 | 3 | 3 | 3 | 125.476 | 61.36 | Yes |
| TQ 6 | 324.302 | 3.78474 | 3 | 3 | 3 | 130.470 | 61.36 | Yes |
| TQ 7 | 324.30 | 3.78474 | 3 | 3 | 3 | 130.470 | 61.36 | Yes |
| TQ 8 | 290.75 | 3.41934 | 3 | 3 | 3 | 121.912 | 61.36 | Yes |
| TQ 9 | 325.19 | 4.07274 | 3 | 3 | 3 | 132.215 | 61.36 | Yes |
| TQ 10 | 290.75 | 3.41934 | 3 | 3 | 3 | 121.912 | 61.36 | Yes |
| TQ 11 | 325.19 | 4.07274 | 3 | 3 | 3 | 132.215 | 61.36 | Yes |
| TQ 12 | 290.75 | 3.41934 | 3 | 3 | 3 | 121.912 | 61.36 | Yes |
| TQ 13 | 325.19 | 4.07274 | 3 | 3 | 3 | 132.215 | 61.36 | Yes |
| TQ 14 | 358.75 | 4.43814 | 3 | 3 | 3 | 140.774 | 61.36 | Yes |
| TQ 15 | 358.75 | 4.43814 | 3 | 3 | 3 | 140.774 | 61.36 | Yes |
| TQ 16 | 400.191 | 3.50964 | 3 | 3 | 3 | 135.036 | 61.36 | Yes |
| TQ 17 | 274.30 | 2.90504 | 3 | 3 | 3 | 115.774 | 61.36 | Yes |
| TQ 18 | 400.19 | 3.50964 | 3 | 3 | 3 | 135.036 | 61.36 | Yes |
| TQ 19 | 400.19 | 3.50964 | 3 | 3 | 3 | 135.036 | 61.36 | Yes |
| TQ 20 | 382.201 | 3.37054 | 3 | 3 | 3 | 130.871 | 61.36 | Yes |
| TQ 21 | 270.332 | 3.07436 | 3 | 3 | 3 | 117.974 | 61.36 | Yes |
| TQ 22 | 286.331 | 2.77454 | 3 | 4 | 4 | 123.087 | 70.59 | Yes |
| TQ 23 | 286.331 | 2.77454 | 3 | 4 | 4 | 123.087 | 70.59 | Yes |
| TQ 24 | 369.646 | 4.18184 | 3 | 3 | 3 | 135.780 | 61.36 | Yes |
| TQ 25 | 312.413 | 4.06344 | 3 | 3 | 3 | 137.069 | 61.36 | Yes |
| TQ 26 | 271.32 | 2.34814 | 4 | 4 | 3 | 116.949 | 87.38 | Yes |
| TQ 27 | 272.304 | 2.47154 | 4 | 4 | 3 | 116.403 | 81.59 | Yes |
| TQ 28 | 270.332 | 3.07436 | 3 | 3 | 3 | 117.974 | 61.36 | Yes |
| TQ 29 | 369.299 | 3.69294 | 3 | 5 | 4 | 145.123 | 113.17 | Yes |
| TQ 30 | 369.299 | 3.69294 | 3 | 5 | 4 | 145.123 | 113.17 | Yes |
| TQ 31 | 299.374 | 2.83194 | 3 | 4 | 4 | 130.099 | 64.60 | Yes |
| TQ 32 | 312.413 | 4.06344 | 3 | 3 | 3 | 137.069 | 61.36 | Yes |
| TQ 33 | 314.385 | 3.55314 | 3 | 4 | 5 | 135.817 | 70.59 | Yes |
| TQ 34 | 313.401 | 3.14036 | 3 | 4 | 4 | 136.464 | 64.60 | Yes |
| TQ 35 | 299.374 | 2.83194 | 3 | 4 | 4 | 130.099 | 64.60 | Yes |
| TQ | 164.204 | 1.6669 | 0 | 2 | 1 | 71.962 | 34.14 | Yes |

docking the 35 TQ derivative with this receptor. The results of the prediction of the activity of 35 thymoquinone derivatives and molecular interaction of thymoquinone derivatives with amino acid residues at the active site of 3PPO are shown in Figure 5 and Table 4. Most of TQ derivatives showed better activity than thymoquinone compounds with compounds 1, 4, 5, 6, and 7, which provide the best physicochemical properties, ADMET and activity (Figures 2–4).

### The quantitative structure-properties relationship (QSPR) of thymoquinone derivatives

Prediction of HKSA for thymoquinone derivatives can be observed in Table 5. Prediction of HKSA aims to determine the parameters that have the most role in determining the compound's activity. In this study, the parameter that most determines thymoquinone derivatives activity as a breast anticancer with positive HER2 is the lipophilic (ClogP) and steric parameter (CMR).

## Discussion

In drug development, most drugs fail in clinical trials because of their ineffectiveness and their side effect. In this case, drug activity, pharmacokinetics and toxicity are critical to producing an effective, quality and safe drug. *In silico* methods aims to increase physicochemical properties, pharmacokinetic profile and activity of the drugs. *In silico* method can also significantly reduce the number of

**Table 3:** Pharmacokinetic profile of 35 thymoquinone derivatives compound.

| Derivative compounds | Parameter | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $CaCo_2$ permeability | Intestinal absorption | CNS permeability | CYP2D6 substrate | CYP3A4 substrate | Clearance | OCT2 substrate | AMES toxicity | LD50 | Hepatotoxicity |
| TQ 1 | 1.165 | 92.439 | −2.171 | No | Yes | 0.083 | No | No | 2.153 | No |
| TQ 2 | 1.262 | 89.051 | −1.919 | No | Yes | −0.360 | No | No | 2.226 | No |
| TQ 3 | 1.196 | 90.711 | −2.034 | No | Yes | −0.349 | No | No | 2.195 | No |
| TQ 4 | 1.237 | 90.078 | −1.933 | No | Yes | −0.591 | No | No | 2.288 | No |
| TQ 5 | 1.216 | 90.711 | −2.034 | No | Yes | −0.349 | No | No | 2.195 | No |
| TQ 6 | 1.257 | 89.166 | −2.014 | No | Yes | 0.271 | No | No | 2.208 | No |
| TQ 7 | 1.283 | 89.520 | −2.061 | No | No | −0.069 | No | Yes | 2.203 | No |
| TQ 8 | 1.211 | 90.778 | −2.057 | No | Yes | −0.313 | No | No | 2.189 | No |
| TQ 9 | 1.236 | 89.577 | 1.946 | No | Yes | 0.19 | No | Np | 2.289 | No |
| TQ 10 | 1.190 | 91.237 | 2.060 | No | No | 0.375 | No | No | 2.259 | No |
| TQ 11 | 1.262 | 89.931 | 1.995 | No | No | 0.178 | No | No | 2.254 | No |
| TQ 12 | 1.237 | 91.113 | 2.103 | No | No | 0.102 | No | No | 2.207 | No |
| TQ 13 | 1.257 | 89.118 | 1.942 | No | Yes | 0.337 | No | No | 2.220 | No |
| TQ 14 | 2.282 | 87.964 | 1.903 | No | Yes | 0.203 | No | No | 2.296 | No |
| TQ 15 | 1.303 | 87.505 | 1.899 | No | Yes | 0.206 | No | No | 2.231 | No |
| TQ 16 | 1.201 | 90.944 | −2.098 | No | Yes | −0.651 | No | No | 2.238 | No |
| TQ 17 | 1.161 | 92.091 | 2.212 | No | No | 0.355 | No | No | 2.146 | No |
| TQ 18 | 1.181 | 91.006 | 2.113 | No | No | 0.569 | No | No | 2.121 | No |
| TQ 19 | 1.227 | 90.485 | 2.095 | No | Yes | 0.665 | No | No | 2.133 | No |
| TQ 20 | 1.186 | 91.806 | −2.070 | No | Yes | −0.685 | No | No | 2.255 | No |
| TQ 21 | 1.213 | 91.358 | −2.080 | No | No | 0.063 | No | No | 1.690 | No |
| TQ 22 | 0.996 | 91.407 | −2.334 | No | No | 0.047 | No | No | 1.564 | No |
| TQ 23 | 1.042 | 91.832 | −2.350 | No | No | 0.443 | No | No | 1.570 | No |
| TQ 24 | 1.262 | 89.051 | −1.919 | No | Yes | −0.294 | No | No | 2.226 | No |
| TQ 25 | 1.233 | 90.817 | −1.809 | No | Yes | 0.018 | No | Yes | 2.199 | No |
| TQ 26 | 1.065 | 91.872 | −2.341 | No | No | 0.282 | No | Yes | 2.366 | No |
| TQ 27 | 1.096 | 91.595 | −2.354 | No | No | 0.398 | No | No | 2.200 | No |
| TQ 28 | 1.185 | 92.237 | −2.097 | No | Yes | 0.098 | No | No | 2.180 | No |
| TQ 29 | −0.250 | 86.114 | −2.210 | No | Yes | 0.276 | No | Yes | 3.504 | No |
| TQ 30 | −0.259 | 86.050 | −2.191 | No | Yes | −0.269 | No | Yes | 3.488 | No |
| TQ 31 | 1.059 | 86.114 | −0.227 | No | No | 0.267 | No | No | 1.734 | No |
| TQ 32 | 1.233 | 90.817 | −1.809 | No | Yes | 0.018 | No | Yes | 2.199 | No |
| TQ 33 | 1.010 | 91.313 | −2.254 | No | No | 0.485 | No | No | 1.598 | No |
| TQ 34 | 1.066 | 91.962 | −2.174 | No | No | 0.360 | No | No | 1.767 | No |
| TQ 35 | 1.062 | 92.164 | −2.248 | No | No | 0.351 | No | No | 1.733 | No |
| TQ | 1.533 | 97.797 | −2.199 | No | No | 0.225 | No | No | 1.625 | No |

failing compounds in clinical trials due to poor ADMET properties [17].

Doak et al. [18] stated that the good physicochemical properties that make a drug compound ideal for oral administration are based on the Lipinski rule of 5. Large BM and log P can cause lower water solubility, low passive cell permeability and cause problems related to drug metabolism. Furthermore, if the HBD value >5 will cause phospholipid membrane damage, and if the HBA value >10 will inhibit the amount of drug absorption in the body [19]. In this study, it is known that the physicochemical properties of the 35 thymoquinone derivatives appropriate with the Lipinski Rule of 5 so that they are suitable for oral administration.

In line with drug physicochemical properties, the pharmacokinetic profile (ADMET) of the TQ derivatives compound also shows a good result. Based on Table 3, most of TQ derivatives compound have a good intestinal absorption if the value >80% and $CaCo_2$ >0.9 (except TQ 29 and 30) [17].

The higher the intestinal absorption value, the higher the drug compound is absorbed into the intestinal tract. Meanwhile, the higher the $CaCo_2$ value, the higher the drug's permeability to penetrate the intestinal membrane.

**Table 4:** Interaction of thymoquinone derivatives with amino acid residues at active site of 3PP0.

| Code | Steric bonds | Electronic bonds |
|---|---|---|
| TQ 1 | Ser 728, Asn 850, Asp 845, Gly 727 | Leu 726 |
| TQ 2 | Asp 863, Glu 770, Ser 783, Met 774, Thr 798 | Glu 770, Asp 863 |
| TQ 3 | Asn 850, asp 808, Leu 726, Arg 849, Gly 727 | Leu 726, Arg 849 |
| TQ 4 | Arg 849, Serin 728, Leu 726, Cys 805 | Gly 729, Arg 849 |
| TQ 5 | Gly 727, Asp 808 | Lys 724 |
| TQ 6 | Thr 798. Lys 753, Asp 863, Glu 770 | Glu 770 |
| TQ 7 | Arg 728, Se 849 | Arg 728, Se 849, Asn 850, Asp 808 |
| TQ 8 | Met 774, Ser 783 | Asp 863, Glu 770 |
| TQ 9 | Ser 728, Gly 729, Arg 849 | Cys 805, Gly 729 |
| TQ 10 | Arg 849, Ser 728, Asp 808, Leu 726 | Ser 728, Asn 850 |
| TQ 11 | Ser 728, Arg 849 | Arg 849 |
| TQ 12 | Ala 730, Ser 728, Arg 849, Asn 850 | Asp 845, Asn 850 |
| TQ 13 | Ser 728, Cys 805, Leu 726 | Arg 849 |
| TQ 14 | Leu 726, Asp 808, Cys 805, Arg 849, Ser 728, Asn 850 | Cys 805, Arg 849 |
| TQ 15 | Pro 802, Ser 728, Leu 726 | Asp 808, Arg 849 |
| TQ 16 | Leu 726, Ser 728, Asn 850, Arg 849, Cys 805 | Cys 805, Arg 849 |
| TQ 17 | Ser 728, Asn 850, Asp 845, Ala 730. Gly 727 | Leu 726 |
| TQ 18 | Arg 849, Ser 728 | Arg 849, Cys 805 |
| TQ 19 | Leu 726, Ser 728 | Arg 849 |
| TQ 20 | Met 774, Ser 783 | Asp 863, Glu 770 |
| TQ 21 | Ser 783, Met 774 | Asp 863, Glu 770 |
| TQ 22 | Gly 727, Asp 808, Ser 728, Arg 849, Asn 850 | Cys 805 |
| TQ 23 | Ser 783, Met 774, Leu 796 | Asp 863, Glu 770 |
| TQ 24 | Lys 753, Thr 798, Ser 783, Met 774 | Glu 770, Asp 863, Lys 753 |
| TQ 25 | Thr 798, Asp 863, Glu 770, Lys 753, Thr 862 | – |
| TQ 26 | Ser 728, Asn 850, Ala 730, Gly 727, Asp 845 | Leu 726, Asp 845 |
| TQ 27 | Ser 728, Ala 730, Asp 845, Asn 850, Gly 727 | Leu 726, Asp 845, Asn 850 |
| TQ 28 | Ala 730, Asn 850, Ser 728, Arg 845 | Ser 728, Asn 850, Asp 845 |
| TQ 29 | Leu 726, Pro 802 | Cys 805 |
| TQ 30 | Leu 726, Asn 850, Gly 729, Arg 849, Ser 728 | Cys 805 |
| TQ 31 | Leu 726, Asp 808, Cys 805, Arg 849, Ser 728 | Arg 849, Cys 805 |
| TQ 32 | Ser 728, Arg 849, Ala 730, Asn 850, Cys 805, leu 726 | Ser 728, Asn 850 |
| TQ 33 | Ser 728, Asn 850, Arg 849, Cys 805, leu 726, Asp 808 | Arg 849 |
| TQ 34 | Cys 805, Leu 726, Asp 808, Arg 849, Ser 728 | Arg 849 |
| TQ 35 | Ser 728, Arg 849, Cys 805, Asp 808, leu 726 | Arg 849, Gly 729 |
| TQ | Val 734 | Thr 862, Asp 863, leu 796, Lys 753, Aa 751 |

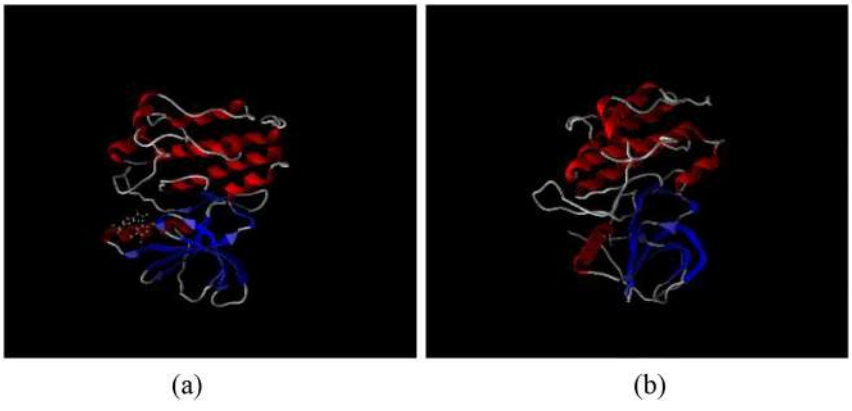


**Figure 2:** The 3D representation of the HER-2 receptor (PDB ID: 3PP0) in the backbone form with (a) derivatives compound of thymoquinone (TQ 4) and (b) thymoquinone as a reference compound.

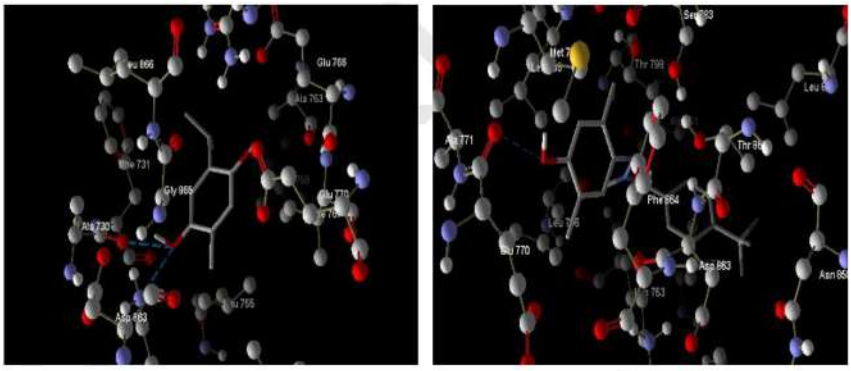


**Figure 3:** 2D molecular interaction of thymoquinone (a) and TQ 6 (b) with amino acid residues at active site of 3PP0.

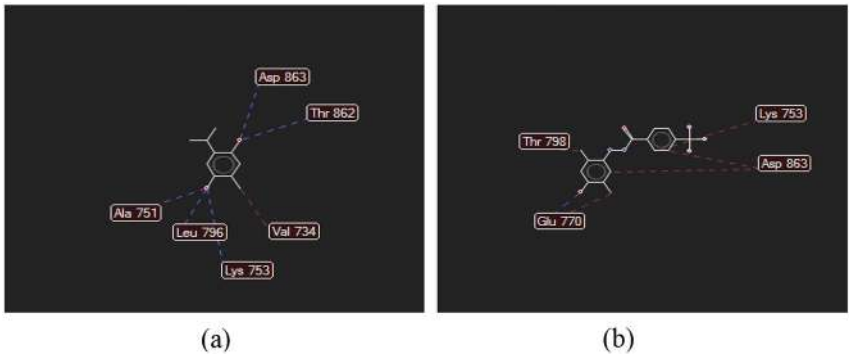


**Figure 4:** 2D molecular interaction of thymoquinone (a) and TQ 6 (b) with amino acid residues at active site of 3PP0.

Based on Table 3, most of the thymoquinone derivatives compounds have good absorption. Apart from these parameters, CNS permeability is an important factor for breast anticancer drugs with positive HER2. This is because breast cancer with positive HER2 can metastasize into the central nervous system, so the drug is needed to penetrate the central nervous system [9]. The central nervous system (CNS) permeability was used to measure blood–brain permeability and expressed as log PS. A log PS> –2 indicates drugs can be distributed into the central nervous system [17, 20]. Table 3 shows that 2, 9, 11, 13, 14, 15, 24, 25, 31, and 32 TQ derivatives are not distributed into the central nervous system. Regarding toxicity, it is known that TQ derivatives are not hepatotoxic, so this is an advantage when compared to drugs that are often used in breast cancer patients with positive HER 2, such as trastuzumab, lapatinib, and neratinib, which are hepatotoxicity.

**Table 5:** The HKSA equation for the thymoquinone derivative compound at the HER2 receptor with the protein code 3PP0.

| No. | Equation |
|---|---|
| 1. | RS=–5.548 ClogP – 77.109 n=35; *r*=0.105; *s*=36.9580; *F*=0.366; sig=0.550 |
| 2. | RS=–0.149 Etot – 80.890 n=35; *r*=0.071; *s*=37.0678; *F*=0.168; sig=0.684 |
| 3. | RS=–5.96 CMR – 41.682 n=35; *r*=0.086; *s*=37.0243; *F*=0.246; sig=0.623 |
| 4. | RS=–5.030 ClogP – 0.113 Etot – 69.952 n=35; *r*=0.117; *s*=37.4774; *F*=0.224; sig=0.801 |
| 5. | RS=–4.384 ClogP – 2.574 CMR – 58.465 n=35; *r*=0.109; *s*=37.5141; *F*=0.192; sig=0.826 |
| 6. | RS=–5.032 CMR – 0.111 Etot – 41.176 n=35; *r*=0.100; *s*=37.5489; *F*=0.162; sig=0.851 |
| 7. | RS=15.279 $ClogP^2$ – 90.025 ClogP + 31.809 n=35; *r*=0.302; *s*=35.9808; *F*=1.601; sig=0.217 |
| 8. | RS=15.335 $ClogP^2$ – 90.386 ClogP + 0.011 Etot + 31.526 n=35; *r*=0.302; *s*=36.5560; *F*=1.035; sig=0.391 |
| 9. | RS=15.672 $ClogP^2$ – 89.739 ClogP – 5.438 CMR + 74.003 n=35; *r*=0.308; *s*=36.4768; *F*=1.084; sig=0.370 |

The prediction of the activity of 35 thymoquinone derivatives against HER2 positive breast cancer receptors also has excellent results. The rerank score indicates the results of the activity prediction. The decreased rerank score, less energy of chemical structure needed to bind the receptor, and increased activity. Based on Figure 5, most of TQ derivatives compound have a lower rerank score than TQ, with the lowest rerank score is TQ 15. It is suspected that TQ 15 compound has a high similarity with the 3PP0 ligand standard. There are 3-CF3, 2-Cl groups, ether, amide, phenolic OH, and aromatic rings in the ligand standard's

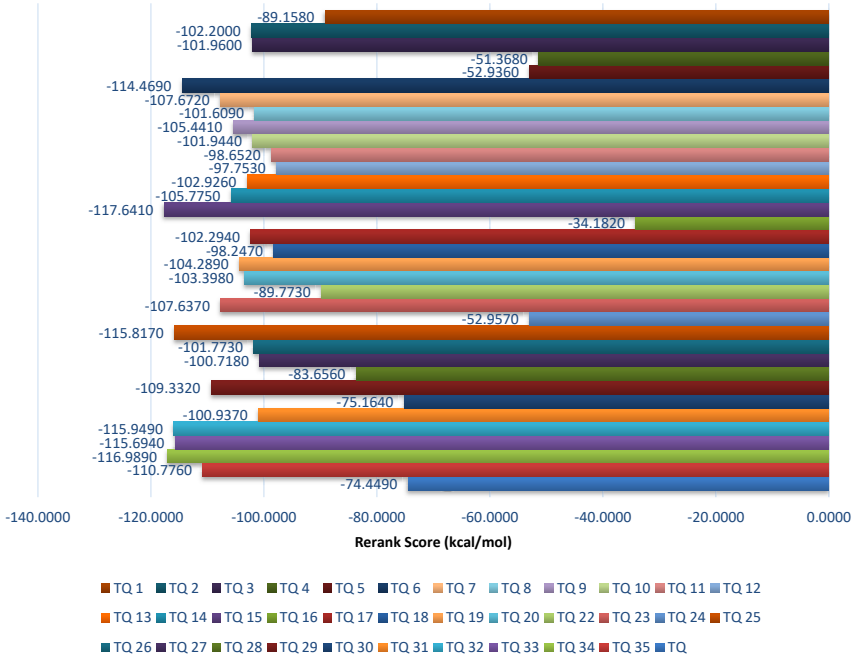


**Figure 5:** The graphs of *in silico* test results indicated by the rerank score parameter values.

chemical structure. In the TQ 15 structure, there are Cl groups, CF3 groups, amides, phenolic OH, and an aromatic ring [21]. Several amino acids residues interact with original ligands. Arg 849, Asn 850, Asp 845, Cys 805, Leu 726, and Ser 728 are amino acids that interact with original ligands.

Further research is QSPR (quantitative structure of properties relationship). Based on Table 5, the parameters that most influence the activity as breast cancer drugs with positive HER2 is electronic parameter shown from the regression equation with a correlation value (R) close to 0.2826, the highest degree of significance (*F*), and the lowest standard of deviation (*S*). In general, the higher the lipophilicity of a drug, tends to lead to higher the permeability of the drug to penetration into the membrane. Result of the research indicate that the derivatives of thymoquinone have higher lipophilicity than thymoquinone. Steric factors of a drug will influence the ability of the drug to approach and interact with the binding site. The bulky substituent can act as an interaction hinder between a drug and its binding site. The higher steric hindrance of the thymoquinone derivatives can decrease their ability to approach and interact with the binding site.

## Conclusions

Based on physicochemical properties, pharmacokinetic profile (ADMET), docking activity, and QSPR, it can be seen that most of TQ derivatives have excellent physicochemical properties, good ADME, nontoxic, and have better activity than TQ. TQ 15 and TQ 34 derivatives have the highest activity than the other derivatives, so that they have a great potential to be developed as positive HER2 breast cancer drugs.

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Jay P. Jazul*, Trisha Michaela G. Arciga, Mary Angelie C. Ante, Danavin Gwyneth B. Berlin, Loise Francoise L. Ravana, Samantha A. Reyes and Jashanjit Singh

# Assessment of patient understanding of their conventional cardiac medicines and herbal prepared/derived products: preliminary survey and interviews with selected community-dwelling elderly patients in the Philippines

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**Abstract**

**Objectives:** The aim of this study was to identify the patterns of medication load, its medication burden, coordination of healthcare and patient's understanding of their conventional cardiac medications and related herbal-derived preparations.

**Methods:** The study is a mixed-method both, quantitative and qualitative approach, which involved Filipino elderly patients (n=69) enrolled in the outpatient service of the National Center for Geriatric Health, Manila. Data were gathered through face-to-face surveys and interviews using a semi-structured questionnaire. Descriptive statistics were used during data analysis. Thematic analysis was also used to emphasize patterns in the responses of the participants.

**Results:** Respondents were knowledgeable on the name (86.9%), visual characteristics (78.3%), and indication and administration of their medicine (88.4%). The frequency of their doctor's information on the possible side effects of the medicines was noted. The almost negligible difference in the proportions of those who asserted during the information dissemination on the medication side effect by their doctors was observed (<10.5%). Association on the age and awareness of any interaction on the drugs they are taking (p=0.032) and an association between the gender and awareness of the doctor/pharmacists about other drugs the patient is taking (p=0.033) were observed. During thematic analysis, elderly respondents were keen on the physician's advice than that of the pharmacist. This is due to the limited knowledge of elderly patients on the role of pharmacists to conduct medication counseling.

**Conclusions:** The majority of the elderly patients recognized the purpose and extent of medication. It was noted that pharmacists play a limited role in understanding selected Filipino elderly patients on their medication. Lack of communication between the patient and the pharmacist was noted as preliminary findings in the study. Respondents were not yet informed of the responsibility of the pharmacist to provide information regarding their medication. Integration of pharmacists' care for geriatric health must be strengthened and highly recommended. Supervision by the healthcare professionals, particularly by the pharmacists, must be fully established.

**Keywords:** cardiovascular agents; elderly; geriatric assessment; health services for the aged; hospital; pharmaceutical service.

## Introduction

Patient understanding is the cornerstone of medication adherence. It is essential to address the lack of patient knowledge because it provides a practical approach. This is to improve patient compliance by directly guiding the patient's ability to do appropriate self-medication management. Taking medication requires it to achieve its optimal effect. It involves steps such as understanding the information of the drug before the drug intake. Health-seeking practices differ. Middle-income range patients consult their physician and purchase medications that the physician prescribes. On the other hand, people with less purchasing power tend to ask for health experience through their colleagues by sorting into herbal medicines.

***Corresponding author: Jay P. Jazul**, University of Santo Tomas, Faculty of Pharmacy, Manila, Philippines, E-mail: jpjazul@ust.edu.ph. https://orcid.org/0000-0001-8917-4397
**Trisha Michaela G. Arciga, Mary Angelie C. Ante, Danavin Gwyneth B. Berlin, Loise Francoise L. Ravana, Samantha A. Reyes and Jashanjit Singh,** University of Santo Tomas, Faculty of Pharmacy, Manila, Philippines

Depending on their social connections, some can speak to their problems by asking significant people for help [1]. The use of medicinal herbs is widely practiced in the Filipino culture. This was translated and developed into herbal derived-medicines that come as a capsule, tablet, syrup, essential oil, herbal tea, cream, and ointment for easier consumption as the common choice for self-medication therapy (Philippines Health Service Delivery Profile) [2]. The industry marketed this as a food and nutritional supplement. The rural community setting is still implemented by decoction, infusion, maceration and poultices. There is a limited review where the local pharmacists dispensed the herbal medicines through this traditional process.

One factor that affects the understanding of patients is the limited health literacy [3]. A study conducted by Davis et al. [4] stated that patient comprehension and misunderstanding are caused by medication errors, poor adherence and can lead to worse health outcomes. Patients with limited knowledge of drugs and taking more medications are more at risk of medication failure [4]. This factor affects the medication understanding and other health parameters such as status, health care use, outcomes and costs. Patients are expected to handle the information they are given and manage complex drug therapy at home. If they cannot make sense of the health information they are given, they cannot make proper health decisions [5]. The presence of a gap concerning health care professionals and their patients' literacy may be at risk for poor health outcomes [6]. In giving information, patients, especially the older ones, prefer a more explicit language explaining how to take medicines appropriately and correctly [6]. In giving information, patients, especially the older ones, prefer a more explicit language explaining how to take medicines appropriately and correctly [4]. For all patients, one should use layman phrases and terminology that the patient can comprehend. To do this effectively, appropriate language should be used. Patients will always receive and gather information. This may cause them to collect some of their information support from primary sources (such as the Internet), which is not related to the immediate doctor-patient consultation or pharmacist–patient consultation. This may not always be on a parallel; the patient can understand or be correct and cannot substitute through verbal communication. Variation in individual patient's understanding of different terminologies is also a factor [7]. All health care professionals shall adapt their language in a way that shall understandably convey the information and shall inform patients on their illness or condition, acknowledge informed decision-making, and not cause misunderstanding all at the same time [8]. Communication among prescribers and sufficient patient education are important in the reduction of ADR [9].

## Materials and methods

This is a quantitative and qualitative research study designed to measure the understanding of medication intake of the elderly patient enrolled in the outpatient service of the National Center for Geriatric Health, Manila. The researchers examined data gathered from surveys and interviews with the selected elderly patients residing in the various communities from August 2019 to March 2020. The study complied with the ethical standards of the University of Santo Tomas Faculty of Pharmacy Ethics Review Board. Permission to conduct an interview was also secured from the National Center for Geriatric Health. Participants aged 60 years old and above, absence or presence of hypertension, with or without related co-morbidity, currently taking one or more cardiac medications, with herbal prepared and derived products, without terminally ill condition, and can answer the questions and can speak and understand Filipino fluently were included in the study. Further assessment on their disease condition in relation to the use of cardiac medications will not covered in the study. Informed consent from the respondents was asked at the beginning of the study. Aside from written explanations, oral explanations regarding the study were also provided to guarantee a complete understanding of the details of the study. The researchers guaranteed that the information from the respondents would be treated confidential. Based on the standard normal variate of 1.96 at 5% Type 1 error and 10% absolute error through convenience sampling, 69 respondents were recruited [10]. The participants were screened through purposive sampling. Data gathering using face-to-face interview in a comfortable environment was conducted. The data collection tool composed of three portions: demographics, data questionnaire, and medication understanding questionnaire. The medication understanding questionnaire includes the following questions based on Hotham and Suppiah. To utilize it with local participants, it was translated into the Filipino language. Thematic analysis was used to determine, analyze and translate patterned meanings. Fisher's exact test was used to establish the association of different parameters and attributes using p-value $<0.05$ as a significant value.

## Results

A total of 69 elderly patients participated on this study. The socio-demographic characteristics of all participants are described in Table 1.

The mean age of these elderly patients understudy is 68.5 years old with a standard deviation of 5.7 years. The youngest among them is 60 years old while the oldest is 81 years old. Female participants outnumbered the male participants in the ratio of almost 7:3. The majority of these participants are hypertensive (72.5%) and they are taking an average of three [3] medicines per day. Also, the drugs they are taking are considered maintenance medicines, hence these are considered medication regularly. A bulk number of participants take their medications once daily (71.0%).

**Table 1:** Socio-demographic and medication profile of respondents (n=69).

| Characteristic | | n (%) | Mean (SD) |
|---|---|---|---|
| Age | | | 68.5 (5.7) |
| Gender | Female | 48 (69.6) | |
| | Male | 21 (30.4) | |
| Allergies | Seafood | 5 (7.2) | |
| | Other allergies | 2 (2.9) | |
| | Not known allergies | 46 (66.7) | |
| | No allergies | 16 (23.2) | |
| Medication | Number of drugs | | 2.8 (1.6) |
| Medication type | Short term | 3 (4.3) | |
| | Regular | 63 (91.4) | |
| | PRN | 3 (4.3) | |
| Medication frequency | Once a day | 49 (71.0) | |
| | Twice a day | 7 (10.1) | |
| | Thrice a day | 2 (2.9) | |
| | No information | 11 (15.9) | |

Based on Table 2, the majority of the elderly patients can identify the names of some or all of their medications and the dosage they need to take (86.9%). Likewise, they can visually recognize most or all of their medicines (78.3%), though some of them can simply distinguish one medicine from the other based on its size and color (14.5%). A positive affirmation was observed from the greater proportion of the participants (88.4%) on the question of whether they assure that they obtain the correct medicine every time they fill a prescription. Over half of the participants (56.5%) were aware of any interactions that might occur due to the medications they are taking. Participants noted during the interview on how frequently their physicians giving information on the possible side effects of the medicines they are taking. An almost negligible difference in the proportions of those who assert during the dissemination of medication side effect information by their doctors (<10.5%) were noted. However, a considerable no interaction response was noted among the pharmacists and elderly patients concerning possible adverse effects of the drugs they are taking (69%). Nevertheless, these elderly patients were still hesitant in revealing other drugs, such as herbal or supplements to the general practitioners (50%).

Participants monitored laboratory tests during doctor consultation based on the positive rate response. Based on Table 3, more than half (>50%) affirmed a change in their diet and the exercise level after the diagnosis. Moreover, a fourth of these elderly patients were considered adherent based on the fact that they never missed any single moment of their medication. However, for those who forget their medication for some reason, the majority applied the concept of procrastination, which is skipping the medicine for this day and taking it a day after (58.0%). About 4% of them simply take the medication once they remember.

**Table 2:** Participant responses to understanding of medication questions.

| Item number | Question | Response | n (%) |
|---|---|---|---|
| 1 | Do you know the names of all of your medicines? | Yes to all | 53 (76.8) |
| | | Some/most | 7 (10.1) |
| | | No | 9 (13.0) |
| 2 | Can you visually identify all of your medicines? | Yes to all | 52 (75.4) |
| | | Most of the time | 2 (2.9) |
| | | Recognition of size or color only | 10 (14.5) |
| | | No | 5 (7.2) |
| 3 | Do you always make sure that you get the right ones every time you fill a prescription? | Yes | 61 (88.4) |
| | | No | 8 (11.6) |
| 4 | Are you aware of any interactions between the drugs that you are on? | Aware | 39 (56.5) |
| | | Unaware | 30 (43.5) |
| 5 | How often does your doctor talk to you about side effects of your drug? | Always/Most of the time | 35 (50.7) |
| | | Does not/never | 34 (49.3) |
| 6 | How often does your doctor ask you about the side effects of your drugs? | Always/Most of the time | 38 (55.1) |
| | | Does not/never | 31 (44.9) |
| 7 | How often does your pharmacist talk to you about the side effects of your drugs? | Always/Most of the time | 21 (30.4) |
| | | Does not/never | 48 (69.6) |
| 8 | How often does your pharmacist ask you about the side effects of your drugs? | Always/Most of the time | 20 (29.0) |
| | | Does not/never | 49 (71.0) |
| 9 | Do you tell your doctor/pharmacist if you are on any herbal, supplements, or over-the counter drugs? | Always/Most of the time | 32 (46.4) |
| | | Does not/never | 37 (53.6) |

The association between the socio-demographic characteristics of the participants under study, such as age, gender, presence or absence of hypertension, and their responses to the understanding of medication questions and for cardiac medications are determined using Fisher's exact test and presented in Table 4.

## Association of patient understanding with the gender of the respondents

Association between the gender and awareness of the doctor/pharmacists about other drugs the patient is taking were noted (p=0.033) based on Table 5. Male participants

**Table 3:** Participants' responses on cardiac medications.

| Item number | Question | Response | n (%) |
|---|---|---|---|
| 1 | Do you monitor your health between doctor visits (blood pressure measurement, weight, sugar levels)? | Yes | 69 (100) |
| 2 | Since your diagnosis, have you changed your diet? | Yes | 36 (52.2) |
| | | No | 33 (47.8) |
| 3 | Since your diagnosis, have you changed your exercise level? | Yes | 41 (59.4) |
| | | No | 28 (40.6) |
| 4 | How do you make sure that you take your medications as per your prescription? What would you do if you have missed a dose of one of your medications? | Constant reminder | 6 (8.7) |
| | | Write schedule | 1 (1.4) |
| | | Be vigilant | 1 (1.4) |
| | | Take at once | 3 (4.3) |
| | | Skip then just take on another day | 40 (58.0) |
| | | Nothing, never missed | 18 (26.1) |

**Table 4:** Association of socio-demographic characteristics and responses to understanding of medication adherence and for cardiac medications.

| Question | | Fisher's exact test p-value | | |
|---|---|---|---|---|
| | | Age | Gender | Presence or absence of hypertension |
| Understanding of medication adherence | Know the names of all medicines | 0.820 | 0.128 | 0.383 |
| | Can visually identify all medicines | 0.870 | 0.074 | 0.354 |
| | Adequate prescription | 0.286 | 1.000 | 0.428 |
| | Awareness of any interactions in taking medication | 0.032* | 0.595 | 0.586 |
| | Frequency of doctor's advice on side effects of the drugs | 0.940 | 1.000 | 0.785 |
| | Frequency of doctor's inquiry about side effects of the drug | 0.940 | 1.000 | 0.785 |
| | Frequency of pharmacist's advice on side effects of the drugs | 0.653 | 0.187 | 0.585 |
| | Frequency of pharmacist's inquiry about side effects of the drug | 0.311 | 0.580 | 0.553 |
| | Awareness of the doctor/pharmacist about any herbal, supplement, or over-the counter drugs the patient are taking | 0.742 | 0.033* | 0.782 |
| Cardiac medication | Change of diet | 0.494 | 0.793 | 0.170 |
| | Change of exercise level | 0.429 | 0.788 | 0.167 |

*Significant at 0.05 level.

Based on Table 4, a significant association was observed between the age of the respondents and awareness of any interaction on the drugs they are taking (p-value = 0.032). Association between the gender and awareness of the doctor/pharmacists about other drugs the patient is taking was also noted (p-value = 0.033), as also shown in Table 5. This implies that elderly patients below 70 years old were keener on knowing the possible interactions of the drugs they are taking compared to those older than them, as stated in Table 6. Likewise, male participants tend to keep to themselves any herbal, supplement, or over-the-counter drugs they are taking unlike women who tend to inform their doctors or pharmacists of any information about these drugs. The questionnaire included presence and absence of hypertension. However, there was no association between the patient's diagnosis on hypertension to understand medication adherence and changes in their lifestyle during their intake of cardiac medication.

tend to keep to themselves any herbal, supplement, or over-the-counter drugs they are taking, unlike women who tend to inform their doctors or pharmacists of any information about these drugs they are taking. There were documented differences in the medication intake based on gender perspectives. The most important factors in health-related behaviors is attributed to gender. Women are more non-adherent than men according to the several studies. Others documented that women manages to stick to prescriptions better. Women were more expected to account on their filled prescription as compared to men [11] as stated in Table 5.

## Association of patient understanding with the age of the respondents

Based on Table 6, a significant association between the respondents' age and awareness of any interaction on the drugs they are taking was noted (p=0.032). It implies that elderly patients below 70 years old were keener on knowing their medicines' possible interactions compared to those older than them. In previous literature, older patients were more easily satisfied with care. Their expectation was based on their history of dealing with medication. It was proposed that the more aging population will more likely report their health shortcomings. On the other hand, the younger population preferred continuity of care given to them by their healthcare providers. Therefore, young adults preferred management and maintenance for the whole person other than disease and individual symptoms [12] as reflected in Table 6.

## Medication duration of the respondents

Around 63 respondents (91.4%) stated that they took their medicine regularly. Three respondents (4.3%) indicated that they were taking short-term treatment, while the other three respondents (4.3%) took their medication as needed.

## Knowledge on the names of their medicine

The majority (86.9%) can identify the names of some or all of their medications and the dosage they need. Two of the

**Table 5:** Association of gender and responses to medication understanding.

| Questions on medication understanding | Fisher's exact test (p-value) on gender association |
|---|---|
| Know the names of all medicines | 0.128 |
| Can visually identify all medicines | 0.074 |
| Awareness of any interactions in taking medication | 0.595 |
| Frequency of doctor's advice on side effects of the drugs | 1.000 |
| Frequency of doctor's inquiry about side effects of the drug | 1.000 |
| Frequency of pharmacist's advice on side effects of the drug | 0.187 |
| Frequency of pharmacist's inquiry about side effects of the drug | 0.580 |
| Awareness of the doctor/pharmacist about any herbal, supplement, or over-the-counter drugs the patient is taking. | 0.033* |

*Significant at 0.05 level.

**Table 6:** Association of age and responses to the medication understanding.

| Questions on medication understanding | Fisher's exact test (p-value) on age association |
|---|---|
| Know the names of all medicines | 0.820 |
| Can visually identify all medicines | 0.870 |
| Awareness of any interactions in taking medication | 0.032* |
| Frequency of doctor's advice on side effects of the drugs | 0.940 |
| Frequency of doctor's inquiry about side effects of the drug | 0.940 |
| Frequency of pharmacist's advice on side effects of the drug | 0.653 |
| Frequency of pharmacist's inquiry about side effects of the drug | 0.311 |
| Awareness of the doctor/pharmacist about any herbal, supplement, or over-the-counter drugs the patient is taking. | 0.742 |

*Significant at 0.05 level.

respondents claimed that he quickly recalled his drug and already managed the medicines due to its long-term use. The remaining respondents (13.0%) stated that they could not remember the names of the drug they are taking. Challenges in retaining the medication intake were documented.

## Visually identifying medicines

The elderly patients can visually recognize most or all of their medicines (78.3%). However, some can distinguish one pill from the other based on its characteristics, such as shape or color (14.5%). Most of the respondents described the features of the drug they are taking. However, two respondents stated a challenge in visualizing it due to poor eyesight and sheer numbers of different tablets, making it difficult for some to recognize the medicines.

## Frequency of medication administration

The majority of the respondents (71%) stated that their current medication was only taken once a day. 11 respondents (15.9%) had no information regarding the frequency of administering their medicine. A total of 7 respondents (10.1%) stated that they took their medication twice a day, while two (2.9%) of the respondents took their medication three times a day.

## Knowledge on the dosage form of their medicine

The majority knew the dosage form of their medication. Some even stated the exact dosage form as "capsule," "tablet," "syrup." There were 51 (73.1%) respondents who had previous information on their medicines' dosage form, while 18 (26.1%) cannot recall the dosage form of their drugs.

## Knowledge on the dosage strength of their medicine

More than half of the respondents (65.2%) knew the dosage strength of their medicine, while 24 (34.8%) respondents were unaware of their therapy's dosage strength. One-third of the respondents have no information or the unrecalled dose of their medications.

## Knowledge on the indication of their medicine

The majority were aware of their medicine (92.7%), while five respondents (7.2%) stated that they were not informed of the presentation of the medication they were taking.

## Knowledge of drug interactions

Most of the participants (56.5%) were aware of drug interactions. The remaining 30 (43.5%) respondents stated that they were unaware of what drug interactions are. The elderly patients have gained knowledge from patient information leaflets about the side effects of their drugs. Two of the respondents stated they should limit the medication intake because it may target their kidneys.

## Side effect information given by doctors

Some participants responded to how frequently their doctors informed them of the possible side effects of their medicines. The respondents stated that they didn't bother to ask questions or interact much with the doctor since they were checked during their clinic follow-up and assessment.

## Doctor–patient inquiries on side effects

There was an almost negligible difference in the proportions of those who assert when their doctors ask about their medications' side effects (<10%). About 54.4% of the participants reported that their doctor asks them about their drugs' side effects, and 45.6% stated that their doctors do not.

## Providing side effect information by pharmacists

The majority of the respondents (69.6%) stated that their pharmacist did not inform them regarding their drug's side effects. A respondent noted that the pharmacist does not

need to give drug information because it is the doctor's responsibility.

### Pharmacist–patient communication on asking side effects

More than half of the participants (69.6%) stated that their pharmacist did not inform them about the side effects of the drug, and 71% specified that the pharmacist does not ask them regarding the possible side effects of the medicines they take. Therefore, a negative response was observed when most of them considered no interaction between them and the pharmacists on the possible adverse effects of the drugs they are taking.

### Taking of herbal, supplements and OTC drugs

Almost half of the respondents (53.6%) stated that they did not inform their doctors when taking other medications, and 46.4% said that they tell their doctors when taking other drugs. It shows that some elderly patients are still hesitant in revealing other medications, such as herbal or supplements, they are taking.

### Thematic analysis

The respondents had the chance to elaborate on their perception on the use of medication. After completing the prepared interview questions, emergent themes were emphasized in thematic analysis as stated in Table 7. Participants have highlighted that doctors were more likely to give them dialog regarding medication side-effects than pharmacists. Participants were also keener to the doctor's advice than that of the pharmacist. It was also stated that a number of the participants were not aware of the responsibility of the pharmacist to give out information regarding their medication. However, in a similar study by Corre et al. [10], results of their interviews featured that pharmacists were more likely to start conversation on adverse medication effects than prescribers as reflected in Table 7.

## Discussion

The majority (86.9%) can identify some of their medications' names and the dosage, and the dosage form they need to take. The respondents can visually recognize most or all of their medicines (78.3%). However, some can distinguish one medicine from the other based on its characteristics like shape or color (14.5%). The majority of the participants (91.4%) stated that they took their medicine regularly. On the other hand, 4.3% indicated that they were taking short-term treatment while taking their medication as needed. Some even stated the exact dosage form "capsule," "tablet," "syrup." Seeing and visualizing their medication's physical attributes can decrease the medication burden, especially among elderly patients. Visual impairment can often lead to medication non-adherence, resulting in severe

**Table 7:** Thematic analysis based on the face-to-face interviews with the respondents.

| Theme | Patient comments/responses |
|---|---|
| **Doctor/patient communication breakdown:**<br>The respondents stated high attentiveness and trust in doctor's advice regarding their medication. They were complacent with their doctor's advice and did not usually ask further questions on their medication. Almost half of the respondents stated the presence of communication on its side effects with their doctor. | – The doctor always told me the use of my drug. I am always listening to them<br>– I am not here to ask question as long as I pay visit to the doctor's clinic.<br>– The doctor always explained to me different drugs for my illness every time I visited them. |
| **Pharmacist/patient communication breakdown:**<br>Almost all of the respondents stated that they did not have communication with their pharmacist on their medication. They expected pharmacist to only dispense their medicine and have not experienced being advised regarding their medication. | – We didn't mind talking on my medication. The doctor already knew it. There's no need for any interaction.<br>– There's no need to talk about my medication.<br>– Pharmacist didn't bother to ask about my medication.<br>– I didn't ask additional information. I just gave the prescription to the pharmacist. |
| **Informing doctor/pharmacist regarding herbal, supplements and over-the-counter drugs:**<br>Respondents did not usually inform their physician on other medications (herbal, supplement and over-the-counter drugs) | – The doctor didn't mind asking on any herbal supplements. If there's no question, I will give no answer.<br>– I didn't mention I am taking these herbal remedies. It seems it's okay with me so there's no need to inform them. |

consequences such as wrong medication and incorrect dosing (overdose and underdose). Factors like difficulty in identifying the tablet's color, shape, and size with its decreased acuity will affect the reduced medication. Anxiety-related medication intake was also documented among the visually impaired individuals [13].

There were 51 (73.1%) respondents who had information regarding their medicine dosage, while 18 (26.1%) cannot recall the dosage form of their medicine. Forty-five (65.2%) respondents knew the dosage strength of their medicine, while 24 (34.8%) respondents were unaware of the dosage strength. One-third of the respondents have no information or the unrecalled dose of their medications. The majority of the 69 respondents were aware of the indication of their medicine (92.7%). In comparison, five respondents (7.2%) stated that they were not informed on the indication of their medication. Most of the participants (56.5%) are aware of any interactions that might occur due to their medications. Almost an equal number of participants responded to how frequently their doctors informed them of the possible side effects of the medicines they are taking. An equal number of respondents (50.7%) stated that their doctor always asked them about their drugs' side effects. In comparison, the remaining 34 respondents (49.3%) stated that their doctor never asked them about their medication's side effects. Study shows that constant communication between the elderly patients and physician is encouraged to deal with the drug side-effects and to address such hurdle in medication intake [14]. A previous study suggested that geriatric patients can comprehend written instruction, but understanding the presentation and format may vary. Most of the elderly patients knew the prescribed medication and treatment [15]. They are poly medicated people where the chance of having medication error is high.

The almost negligible difference was noted in the proportions of those who assert when their doctors ask about their medications' side effects (<10%). Almost half of the participants (54.4%) reported that their doctor asked them about their drugs' side effects, and 45.6% stated that their doctors do not. The majority of the respondents (69.6%) stated that their pharmacist did not inform them regarding their drug's side effects. Furthermore, many of the respondents (71%) also stated that pharmacists did not ask them regarding their knowledge of their medication's side effects. The response was observed in these elderly patients when most of them considered no interaction between them, and the pharmacists regarding possible adverse effects of the drugs they are taking. Pharmacists must play an essential role in doing the medication counseling and education, especially among the elderly, based on the established literature where the pharmacists can provide geriatric care. The pharmacist can enhance the medication adherence and compliance further and reduce the medication-related issues such as wring intake of medicines and side effects [16]. It was also suggested that the immediate caregivers should consult the pharmacist. Based on the estimates, only 15% of the caregivers seek training on the pharmacist's appropriate medications [17]. In the Philippines, there are still currently a limited number of pharmacist-related clinic or service that will anchor medication management on its geriatric care level. The majority of the government hospitals and healthcare institutions do not cover most of the clinical pharmacy services due to the focus on the dispensing and drug distribution within its nursing unit [18]. Despite the differences in the government hospital structure, pharmaceutical care, based on patients' and pharmacists' relationship, must be coupled with the usual pharmacy administrative functions [19, 20]. Another factor is the noted shortage of pharmacists in the hospital setting due to the current and existing salary and migration due to the increased demand for pharmacy jobs abroad [21]. Acceptance of pharmacists in the healthcare industry to cater the elderly patients can be regarded as a challenge. Lack of communication between the pharmacists and the healthcare team is often attributed to the low acceptance rate, leading to the limited pharmaceutical care delivered to elderly patients [22]. It will be a high time and ample opportunity for the local pharmacists to put up a clinical pharmacy in promoting pharmacotherapy in a developing country to stimulate specific patient-care [23]. This includes communication skills, monitoring adverse drug-related events, and critical thinking as the Filipino clinical pharmacists' proposed competency values in hospital settings [24].

Nearly half of the respondents (46.4%) stated that they tell their doctors when taking other drugs (herbal, supplement or over-the-counter medications). In comparison, the remaining respondents (53.6%) prefer not to inform their doctor regarding these medications. There were noted barriers in giving information on the doctor's medication intake, such as emotional attributes, difficulty in logistics, and financial constraints [25]. In reality, communication between healthcare professionals and elderly patients is quite essential. In order to get information, healthcare professionals should initiate appropriate strategies to establish interrelationships with the patients. History of drug intake by patients is an essential activity by physicians and pharmacists. Patient's knowledge of medication varies [5]. Based on the qualitative assessment, healthcare professionals' behavior, patient hesitation, limited

education by allied health professionals, language obstacle, illiteracy of patients, and pharmacists' unavailability are the proposed factors affecting the patient's knowledge of medication [26].

Changes in eating habits and exercise levels suggest that these participants are concerned about their health. A related study noted that older patients are open to improving their lifestyle after the diagnosis of disease. It is recommended to do more follow up with the healthcare team [27]. More than half of the said participants said they changed their diet and exercise level after their diagnosis, 52.2%, and 59.4%, respectively. On medication adherence, six of the respondents said they always needed to be reminded to take their medicines. In cases where patients missed taking their prescribed medicines, it was noted that 58% of the respondents tend to skip their doses for the day and continue to take their medicines the following day or the day after. Skipping the dose is one of the most common problems encountered by the geriatric population due to emerging challenges such as the existing polypharmacy [15, 28].

During the thematic analysis, three emergent themes were highlighted. First was the patient–doctor communication breakdown based on the responses that there is an existing lapse of communication between patient and doctor on the drug's side effects. The second emergent theme was on patient–pharmacist communication and suggested that there is little to no communication between patients and pharmacists regarding the side effects of their medication. This is in connection to the previous study that there is still unfamiliarity with the activity by pharmacists [29]. Lastly, respondents do not usually inform their doctor or physician on their herbal preparations and over-the-counter drugs. Patronage on the use of herbal-derived products is established due to the limited time to consult the physician, thereby would lead to self-medication practice [30].

This is an initial study to assess the medication understanding and medication burden experienced by the local elderly patients enrolled in the National Center for Geriatric Health (NCGH). Patient recruitment from the said healthcare facility represents the common grounds among the elderly population within the Manila districts. NCGH is a government health institution created under the Philippine Law to cater to the outpatient elderly sectors previously admitted from the Department of Health (DOH) hospitals. The study does not cover the in-depth discussion on pharmacists' role like the usual suggestive dispensing of the alternative brand name of cardiac medicines or its substitution, which is the standard practice in the Philippines [31]. Such provision did not cover in the semistructured questionnaire. This usual activity (recognized by the elderly population due to the challenges in their financial capability) is a potential venue between the elderly patient and pharmacist in providing medication information. Assessment on the extent of drug information by the pharmacist did not cover in the questionnaire. The recent findings do not reflect the older patients' medication burden and the highlighted communication barrier with the physician and pharmacist as a whole. It is highly recommended to do the national mapping from other culturally centric locations such as Cebu City to represent the Visayas regions and Davao City to cover the Philippines' Mindanao regions. The scope of pharmaceutical and medical care differ from the suggested study sites. Distinctions between the government or public hospital or privately owned health care institutions must be recognized due to the differences in the command structure and healthcare objectives.

## Conclusions

Significant associations were noted between the respondents' age to awareness of any interaction on the drugs they are taking (p-value=0.032) and gender to the doctor/pharmacists' awareness about other drugs the patient is taking (p-value=0.033). Elderly participants can identify the physical nature of cardiac medicines, such as their dosage forms. The majority of the participants can still recall the use of their medicines. Comprehension of medication instruction was identified. There were existing delivery and hindrance of information to the physician or prescriber on the drug's side effects and extent of the use of herbal derived products and over-the-counter medicines. Older patients tend to follow lifestyle modification to improve their health outcomes. Initial results suggested that pharmacists still play a minimal role in understanding Filipino elderly patients on their medication due to a lack of communication between the patient and the pharmacist. Scope of pharmaceutical care was not observed in the study site, which results in the perceived limited communication between the older patients and pharmacists. Respondents are not informed of the responsibility of the pharmacist to provide information regarding their medication. Integration of pharmacists' care for geriatric health must be strengthened and highly recommended. Supervision by the healthcare professionals, particularly by the pharmacists, must be fully established. Conducting the study in other sites is recommended to further assess the differences or similarities from

one geographical location to another. A separate semi-structured questionnaire to assess pharmaceutical care's knowledge and extent toward geriatric patients is also suggested.

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Rodhiyatul Fithri, Umi Athiyah and Elida Zairina*

# The development and validation of the health belief model questionnaire for measuring factors affecting adherence in the elderly with hypertension

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**Abstract**

**Objectives:** This study aimed to validate the questionnaire on the health belief model questionnaire to assess health beliefs that could influence adherence to hypertension in the elderly.

**Methods:** The questionnaire was based on a study of the literature and discussion with experts. The questionnaire was then circulated via social media. Participants who met the following criteria were asked to participate in the study: (1) aged 60–79 years of age, (2) had antihypertensive medications in the last three months, and (3) had a mobile phone with an active number. The questionnaire consists of six domains: perceived susceptibility, perceived severity, perceived threat, perceived benefits, perceived barriers, and perceived self efficacy. The findings were grouped by domain and tested for reliability and validity using SPSS ver.24.

**Results:** Thirty participants completed the questionnaire. Each domain was tested for its reliability and validity at a value of 0.05. The result shows that each domain had a Cronbach's alpha value greater than 0.7, with a total score of 0.89 indicating that all domains in the questionnaire were reliable. Furthermore, of the 49 items in the questionnaire, only two items were invalid while the rest of the items demonstrated their validity based on the Pearson Correlation (>r table 0.361; $p<0.05$).

**Conclusions:** This self administered health belief model questionnaire was a valid and reliable instrument to assess health beliefs in elderly with hypertension.

**Keywords:** adherence; elderly; health belief model; hypertension.

## Introduction

The World Health Organization (WHO) has confirmed that hypertension is a severe medical condition that may increase the risk of heart, brain, and kidney diseases. Also, one of the main risk factors for hypertension is age >65 years. Hypertension is one of the leading causes of early death worldwide, with more than one million people suffering from hypertension in 2015, largely due to an increase in risk factors in these populations in recent decades [1]. Profoundly prevalence of hypertension is at aged 65 years or more. However, medication nonadherence increases at the age of 80 years or more [2, 3].

The Basic Health Research Indonesia (Riskesdas 2018) stated that the hypertension prevalence in Indonesia reached 34.1%, dominated by the elderly. About 427,218 people died due to hypertension. About 13.3% of people did not take medication, and 32.3% did not take medication regularly. Evidence showed that hypertension patients in Indonesia had a low level of medication adherence, caused illness belief, medication belief, and forgetfulness [4].

Several studies have reported that elderly hypertension has had low blood pressure control due to poor medication adherence, resulting in significant morbidity and use of health services [5–8]. The study revealed that more than half of the participants (55.9%) acknowledged some degree of medication non-compliance. Older age, living alone, and perceptions associated with treatment control were independently related with the need for

***Corresponding author: Elida Zairina**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; Innovative Pharmacy Practice and Integrated Outcomes Research (INACORE) Group, Universitas Airlangga, Surabaya, Indonesia; and Center for Patient Safety Research, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 031 5933150, E-mail: elida-z@ff.unair.ac.id. https://orcid.org/0000-0003-0845-4640

**Rodhiyatul Fithri,** Magister Program of Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

**Umi Athiyah,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Innovative Pharmacy Practice and Integrated Outcomes Research (INACORE) Group, Universitas Airlangga, Surabaya, Indonesia

adherence to treatment, with odds ratios ranging from 1.14 to 1.92 (p = 0.05) [2].

Adherence is affected by the patient's beliefs and wellbeing. The patient tends to follow the recommendation if they assume that hypertension is a controllable disease [9]. Greater awareness of the health beliefs of elderly people with hypertension can help pharmacist and researchers develop strategies to improve adherence to medications and manage blood pressure. This study aimed to validate the health belief model (HBM) questionnaire to measure health beliefs that could affect adherence in the elderly with hypertension.

## Materials and methods

The Committee of Ethical Approval in the Faculty of Nursing, Universitas Airlangga approved this study, with reference number 2090-KEPK. All participants gave their consent and were assured of confidentiality. It was a cross-sectional study conducted in September 2020. The questionnaire was built based on the literature review and discussion with experts. The questionnaire was then circulated via social media. Participants who met the following criteria were asked to participate in the study: (1) aged 60–79 years of age, (2) had antihypertensive medications in the last three months, and (3) had a cell phone with an active number.

The questionnaire consists of six domains: perceived susceptibility (eight items), perceived severity (five items), perceived threat (five items), perceived benefits (11 items), perceived barriers (12 items), and perceived self efficacy (eight items). The questionnaire consisted of 49 items and used A four-point Likert scale: 1 = strongly agreed, 2 = agreed, 3 = disagree, and 4 = strongly disagreed.

The findings were grouped by domain and tested for reliability and validity using SPSS ver.24. The validity of the item scale correlation was carried out to determine the degree to which the item was correlated with its hypothesized domain. The Pearson correlation coefficient values equal to or greater than the critical value table (r = 0.361; p = 0.05) were considered to be valid items [10]. The coefficient of reliability is an absolute number that can range from 0.00 to 1.00. The value of 1.00 indicates perfect consistency. A value of 0.00 indicates a complete lack of consistency [11]. A reliability coefficient of 0.70 or higher was accepted as proof of internal consistency for the instrument [12].

## Results

Thirty-two participants completed the questionnaire, and two of them were excluded due to inadequacy of age. The average age of participants was 66.47 ± 5.7 years. Most of the participants were female (63.3%), and the majority had tertiary education (36.7%). Most participants were retired (0.4%) and had hypertension of around 1–5 years (0.44%). Almost all participants had no comorbidity (0.44%). The characteristics of participants are shown in Table 1.

**Table 1:** Demographic characteristics of the participants (n = 30).

| Demographic data | Category | Number | Percentage, % |
|---|---|---|---|
| Age | Age – Mean (SD) | 66.47 (5.7) | – |
| Sex | **Women** | **19** | **63.3** |
| | Men | 11 | 36.7 |
| Education | Primary school | 4 | 0.13 |
| | Junior high school | 5 | 0.17 |
| | **Senior high school** | **11** | **36.7** |
| | Bachelor degree | 7 | 0.23 |
| | Master degree | 2 | 0.07 |
| | Doctoral | 1 | 0.03 |
| Occupation | Housewife | 9 | 0.3 |
| | Private employees | 2 | 0.07 |
| | Lecturer | 2 | 0.07 |
| | **Retired** | **12** | **0.4** |
| | Driver | 1 | 0.03 |
| | Farmer | 1 | 0.03 |
| | Teacher | 1 | 0.03 |
| | Odd jobs | 1 | 0.03 |
| | Unemployment | 1 | 0.03 |
| Duration of hypertension | One year | 3 | 0.1 |
| | **1–5 years** | **13** | **0.44** |
| | 5–10 years | 7 | 0.23 |
| | More than 10 years | 7 | 0.23 |
| Comorbidity | **None** | **13** | **0.44** |
| | Diabetes Mellitus type 1 or 2 | 5 | 0.17 |
| | Coronary heart disease | 1 | 0.03 |
| | Stroke | 1 | 0.03 |
| | Dyspepsia | 1 | 0.03 |
| | Maag | 1 | 0.03 |
| | Osteoarthritis | 3 | 0.1 |
| | Cataract | 1 | 0.03 |
| | Vertigo | 1 | 0.03 |
| | Gout | 1 | 0.03 |
| | Arrhythmia | 1 | 0.03 |
| | Hyperlipidaemia | 1 | 0.03 |

**Table 2:** The questionnaire reliability.

| Domain | Cronbach's alpha coefficient | Standard coefficient | Explanation |
|---|---|---|---|
| **Perceived susceptibility** | 0.897 | 0.70 | Reliable |
| **Perceived severity** | 0.743 | 0.70 | Reliable |
| **Perceived threat** | 0.761 | 0.70 | Reliable |
| **Perceived benefit** | 0.866 | 0.70 | Reliable |
| **Perceived barrier** | 0.849 | 0.70 | Reliable |
| **Perceived self efficacy** | 0.710 | 0.70 | Reliable |
| **All domain** | 0.898 | 0.70 | Reliable |

**Table 3:** The questionnaire validity.

| Item | Domain | Statements | Pearson correlation | r table | Explanation |
|---|---|---|---|---|---|
| 1 | Perceived susceptibility | Susceptibility of uncontrollable blood pressure caused by taking medication improperly and irregularly. | 0.727 | 0.361 | VALID |
| 2 | | Susceptibility of heart disease caused by taking medication improperly and irregularly. | 0.825 | 0.361 | VALID |
| 3 | | Susceptibility of stroke caused by taking medication improperly and irregularly. | 0.760 | 0.361 | VALID |
| 4 | | Susceptibility of a peripheral blood vessel caused by taking medication improperly and irregularly. | 0.743 | 0.361 | VALID |
| 5 | | Susceptibility of nerve disorder caused by taking medication improperly and irregularly. | 0.780 | 0.361 | VALID |
| 6 | | Susceptibility of renal disorder caused by taking medication improperly and irregularly. | 0.616 | 0.361 | VALID |
| 7 | | Susceptibility of retina disorder caused by taking medication improperly and irregularly. | 0.758 | 0.361 | VALID |
| 8 | | Susceptibility of brain disorder caused by taking medication improperly and irregularly. | 0.891 | 0.361 | VALID |
| 9 | Perceived severity | Anxiousness of blood pressure caused by taking medication incorrectly and irregularly. | 0.787 | 0.361 | VALID |
| 10 | | Concern of blood pressure condition. | 0.577 | 0.361 | VALID |
| 11 | | Fine with not taking any medications. | 0.629 | 0.361 | VALID |
| 12 | | Deterioration of health caused by improper use of medication. | 0.766 | 0.361 | VALID |
| 13 | | Deterioration of health caused by not taking the medication regularly. | 0.791 | 0.361 | VALID |
| 14 | Perceived threat | High-risk of a complication caused by not taking medication. | 0.673 | 0.361 | VALID |
| 15 | | High-risk of a complication caused by not taking medication correctly. | 0.851 | 0.361 | VALID |
| 16 | | Have no risk of a complication caused by taking medication irregularly. | 0.561 | 0.361 | VALID |
| 17 | | Have a health risk caused by increasing medication without a healthcare professional's approval. | 0.723 | 0.361 | VALID |
| 18 | | Have a health risk caused by reducing medication without a healthcare professional's approval. | 0.770 | 0.361 | VALID |
| 19 | Perceived benefit | Improved health caused by taking medication correctly. | 0.717 | 0.361 | VALID |
| 20 | | Improved health caused by taking the medication regularly. | 0.739 | 0.361 | VALID |
| 21 | | Healthier by taking medication and exercising regularly. | 0.793 | 0.361 | VALID |
| 22 | | Healthier by taking medicines, fruits, and vegetables. | 0.818 | 0.361 | VALID |
| 23 | | Healthier by taking medication and white meat. | 0.547 | 0.361 | VALID |
| 24 | | Healthier by taking medication and either boiled or grilled foods. | 0.667 | 0.361 | VALID |
| 25 | | Red meat is harmless to my blood pressure. | 0.406 | 0.361 | VALID |
| 26 | | Fatty or high cholesterol foods are safe for blood pressure. | 0.626 | 0.361 | VALID |
| 27 | | Healthier by taking medication and reducing salt intake (less than 1 tsp/day). | 0.439 | 0.361 | VALID |
| 28 | | Healthier by taking medication and not smoking. | 0.766 | 0.361 | VALID |
| 29 | | Healthier by taking medication and not drinking alcohol. | 0.851 | 0.361 | VALID |
| 30 | Perceived barrier | Uncomfortable due to cough while taking medication. | 0.518 | 0.361 | VALID |
| 31 | | Uncomfortable caused by polyuria while taking medication. | 0.564 | 0.361 | VALID |
| 32 | | Uncomfortable due to insomnia while taking medication. | 0.654 | 0.361 | VALID |
| 33 | | Uncomfortable due to dizziness or vertigo while taking medication. | 0.701 | 0.361 | VALID |
| 34 | | Uncomfortable caused by stomach ache while taking medication. | 0.772 | 0.361 | VALID |
| 35 | | Uncomfortable due to nausea or vomiting while taking medication. | 0.782 | 0.361 | VALID |
| 36 | | Uncomfortable due to constipation while taking medication. | 0.831 | 0.361 | VALID |
| 37 | | Uncomfortable caused by diarrhea while taking medication. | 0.765 | 0.361 | VALID |
| 38 | | Difficult to remember when taking medication as scheduled. | 0.457 | 0.361 | VALID |
| 39 | | Difficult to remember if the medication has been taken. | 0.601 | 0.361 | VALID |
| *40 | | Not knowing about how to use the medication. | 0.305 | 0.361 | INVALID |
| *41 | | Not knowing about the time to use the medication. | 0.348 | 0.361 | INVALID |
| 42 | Perceived self efficacy | Easy to controlled blood pressure due to regular checks. | 0.607 | 0.361 | VALID |

**Table 3:** (continued)

| Item | Domain | Statements | Pearson correlation | r table | Explanation |
|---|---|---|---|---|---|
| **43** | | Able to neither smoke nor drink alcohol. | 0.440 | 0.361 | VALID |
| **44** | | Able to exercise regularly. | 0.678 | 0.361 | VALID |
| **45** | | Able to consume fruits and vegetables regularly. | 0.479 | 0.361 | VALID |
| **46** | | Able to avoid either fatty or cholesterol food. | 0.706 | 0.361 | VALID |
| **47** | | Able to avoid red meat. | 0.695 | 0.361 | VALID |
| **48** | | Able to reduce salt intake (less than 1 tsp/day). | 0.454 | 0.361 | VALID |
| **49** | | Able to take medication as prescribed. | 0.516 | 0.361 | VALID |

*Item number 40 and 41 dropped because invalid.

All questionnaire scale data were entered into SPSS ver.24, and then analyzed for its reliability and validity at a significance level of 0.05. The result shows that each domain had a Cronbach's alpha value greater than 0.7, with the overall score was 0.89 indicating that all domains in the questionnaire were reliable [11, 12]. Furthermore, from 49 items in the questionnaire, two items were invalid and dropped from the questionnaire. All of the 47 items in the questionnaire were valid based on the Pearson Correlation ($r>0.361$; $p<0.05$) [10]. Items reliability and validity are shown in Tables 2 and 3.

## Discussion

This study evaluated a newly developed instrument to determine the illness beliefs and treatment beliefs of elderly with hypertension. This instrument was developed with experts and tested for internal consistency reliability and validity using SPSS ver.24 software. However, this instrument suggested testing in a larger sample to represent all elderly with hypertension in Indonesia.

Limitations of Cronbach's alpha as a sole index of reliability, showing how it is not invariant with variations of the scale length, interitem correlation, and the characteristics of the sample [13]. This study proposed that it be presented alongside other complementary statistical measures (such as the outcomes of factor analyses) where appropriate [14]. This study cannot perform the exploratory factor analysis (EFA) as it did not meet the minimum sample size requirements.

The study found that the ratio of a sample size to the number of variables exhibited an inverse relationship. Using the coefficient of congruence criteria mentioned above, and a fixed number of factors, a small number of variables needs a greater minimum sample size than a large number of variables [15]. Both Cattell (1978) and Nunnally (1967), suggested various ratios of participants to variables ranging from 3 to 1, 10 to 1, and even higher for such an index [16, 17]. However, a study summarized that the sample size of 30 could assess reliability by using Cronbach's alpha, given that the scale items have strong correlations [18].

The value of "r table" $p = 0.05$ with 30 respondents is 0.361, with Pearson correlation coefficient values equal to or greater than the critical value table ($r = 0.361$; $p = 0.05$) considered to be a valid item [10]. In Table 3, item 40 (Pearson correlation 0.305) and number 41 (Pearson correlation 0.348) the Pearson correlation is less than 0.361 (r table), so item 40 and 41 are considered invalid and dropped from the questionnaire.

## Conclusions

Finally, it was concluded that this study was sufficient to demonstrate that this questionnaire was accurate and valid so that calculating factors influencing adherence based on the health belief models in elderly with hypertension could be used. Also, more supportive data analysis is warranted to improve this questionnaire's application to the elderly with hypertension in Indonesia.

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**Ethical approval:** The Committee of Ethical Approval in the Faculty of Nursing Universitas Airlangga approved this study, with reference number 2090-KEPK.

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Denny Ardhianto, Suharjono*, Soedarsono and Umi Fatmawati

# Analysis of the side effect of QTc interval prolongation in the bedaquiline regimen in drug resistant tuberculosis patients

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**Abstract**

**Objectives:** Indonesia is one of the top 20 countries with the highest prevalence of drug resistant tuberculosis (DR-TB) worldwide with a percentage of new cases of 2.4% and retreatment of 13%. Bedaquiline (BDQ) is one of the drugs that used in the individual long regimen treating DR-TB. BDQ is also combined with levofloxacin (LFX) and/or clofazimine (CFZ) that can cause QTc interval prolongation. The aim was to study the differences in the use of BDQ regimens to the lengthening of the QTc interval and to study risk factors (diabetes, hypokalemia, sex, BMI, and age) in BDQ regimen.

**Methods:** This study was an observational retrospective study with a total sampling method, which was conducted at Dr. Soetomo General Hospital Surabaya. Samples from this study were patients diagnosed with DR-TB at Dr. Soetomo General Hospital Surabaya in the period of January 2015–December 2019 who used BDQ regimen and met the inclusion criteria. The ECG data were analyzed from the mean of each group (BDQ regimen and risk factors), also analyzed using statistical analysis.

**Results:** Data obtained from total sample in this study were 73 patients. The most widely used different regimens in this study were the combination of BDQ + LFX by 36 patients (49.3%), BDQ + LFX + CFZ by 16 patients (21.9%), BDQ by 11 patients (15.1%) and BDQ + CFZ 10 patients (13.7%). Out of 73 patients, 52 patients (71.2%) experienced lengthening of the QT interval and grade 1 of QTc interval prolongation occurred in most patients and also the onset was mostly one month after using BDQ regimen. The side effects of QTc interval prolongation from groups of combination and risk factors were no difference in each month ($p>0.05$).

**Conclusions:** This study can be concluded that there were no differences in the QTc prolongation between the groups of BDQ regimen (BDQ, BDQ + LFX, BDQ + CFZ and BDQ + LFX + CFZ) and the groups of risk factors.

**Keywords:** bedaquiline; dr-tb; qt interval.

## Introduction

Along with the increasing rate of TB case detection in Indonesia, it is necessary to pay attention to the emergence of DR-TB cases among TB cases due to noncompliance to medication and transmission from DR-TB patients. DR-TB treatment aims to cure patients and reduce the transmission of *M. tuberculosis* [1]. However, there are still obstacles to achieve this goal. This can be due to patient noncompliance in treatment, one of which is due to side effects of drugs experienced by the patient. Every year there was an increase in the number of patients who dropped out of treatment at Dr. Soetomo General Hospital Surabaya with a total number of 35 patients from January to September 2018.

The treatment management of DR-TB is divided into two types, namely short-term therapy (9–11 months) and long-term therapy using an individual regimen (20–24 months). Patients who are intolerant and do not meet the criteria for short-term therapy and are diagnosed with pre-extensively drug resistant tuberculosis (pre XDR-TB) or extensively drug resistant tuberculosis (XDR-TB) should apply long-term therapy. Pre XDR-TB refers to TB that resistant to rifampicin and isoniazid with one of the fluoroquinolones or one of the second-line injectable drugs. XDR-TB involves resistance to rifampicin and isoniazid with one of the fluoroquinolones and one of the second-line injection drugs. The drugs used in individual therapy regimens are at least five effective drugs. As a new drug, BDQ should be included in such regimens plus four other drugs based on the sensitivity of

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 812 1733 877, E-mail: suharjono@ff.unair.ac.id
**Denny Ardhianto,** Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Soedarsono,** Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Umi Fatmawati,** Department of Pharmacy, Dr. Soetomo General Hospital, Surabaya, Indonesia

each patient. The BDQ regimen used is generally combined with the drug class of fluoroquinolone and CFZ.

One risk of Bedaquiline relates to the heart. It may cause an increase in incidence of prolonged QTc interval on the electrocardiogram [2, 3]. The drug in the class of fluoroquinolone that is often used as a combination is LFX. CFZ and LFX used in combination also have a side effect of prolonged QTc interval. In TB treatment there are risk factors that can increase the potential for prolonged QTc interval such as age, sex, BMI, diabetes mellitus, electrolyte imbalance, especially low potassium levels which can also be caused by side effects of the OAT used [4].

An electrocardiogram (ECG) describes the phases of the atrial and ventricular action potentials. The QTc interval can be measured as the interval from the start of the QRS complex to the end of the T wave when it returns to baseline. The QTc interval represents the electrical ventricular system and is a combined measure of cardiac depolarization and repolarization. QTc values are considered normal for men when they are in the range between 350 and 450 ms and for women when they are between 360 and 470 ms [3]. An increase in the QTc interval above 500 ms will lead to two to three times of the possibility of the incidence of torsade de pointes, a polymorphic type of heart rhythm disorder that is fatal to the patient, where the heart may stop suddenly. Therefore, WHO recommends caution when administering these drugs and recommends to put strict monitoring procedures in place [5]. Dr. Soetomo General Hospital is one of the referral hospitals located in East Java Province that has MDR-TB Polyclinic. Drugs for DR-TB therapy include BDQ, LFX and CFZ. This study aims to analyze the effect of differences in the use of BDQ regimens (BDQ, BDQ + LFX, BDQ + CFZ, and BDQ + LFX + CFZ) and risk factors (DM, hypokalemia, sex, BMI, age) on prolonged QTc interval.

## Materials and methods

This research has been accepted through an ethical process and has been approved by the Ethics Committee of Dr. Soetomo General Hospital, Surabaya as stated in a letter number 1803/KEPK/I/2020. This was an observational study with a cross sectional study design. Retrospective data collection was carried out by observing the data on the medical records of DR-TB patients at MDR-TB Polyclinic, Dr. Soetomo General Hospital, Surabaya. The samples of the study were patients with a diagnosis of DR-TB according to the inclusion criteria and underwent treatment at MDR-TB Polyclinic of Dr. Soetomo General Hospital, Surabaya from January 2015 to December 2019.

The inclusion criteria for this study were DR-TB patients who received the BDQ regimen from January 2015 to December 2019 at the MDR TB Polyclinic of Dr. Soetomo General Hospital, Surabaya, and had a complete medical records which included patient profiles, drugs used, laboratory examinations, and ECG examinations. The exclusion criteria for this study were patients with a history of prolonged QTc interval, patients who ever received moxifloxacin, delamanid, terfenadine, digoxin, amiodarone, or azole antifungals, new patients who used BDQ for less than 6 months, the patients died or dropped out during BDQ use and patients with a history of DCFC, atrial fibrillation, acute coronary syndrome diagnosis.

Descriptive analyzes for the prevalence of severity, incidence of prolonged QTc interval, mean prolonged QTc interval, time of occurrence of prolonged QTc interval and risk factors (age, sex, BMI, DM and hypokalemia) were presented in table. Analysis of differences in the use of BDQ, BDQ + LFX combination, BDQ + CFZ combination and BDQ + LFX + CFZ combination and BMI risk factor for the side effects of prolonged QTc interval will use the one-way ANOVA statistical test if the data are normally distributed. If the data are not normally distributed, the difference analysis will use the Kruskal-Wallis statistical test. Then the risk factors for hypokalemia, sex, DM and age were analyzed using the independent *T* sample test.

## Results

There were 155 patients who received the BDQ regimen while 73 patients met the inclusion criteria. A total of 73 DR-TB patients who were treated as outpatients at the MDR-TB Polyclinic of Dr. Soetomo General Hospital, Surabaya and received the BDQ regimen were assigned into several diagnoses including MDR-TB 48 (65.7%), pre-XDR TB 24 (32.8%), and XDR-TB 1 (1.3%). The patients' demographic data consisting of sex, age, and patient risk factors were analyzed, and the results are listed in Table 1.

Based on the severity of the incidence of prolonged QTc interval mostly occurred in grade 1 in each month as presented in Table 2. Severity divided into three levels, grade 1 for normal QTc value – 480 ms, grade 2 for 481–500 ms, grade 3 for above 500 ms. Furthermore, the onset of prolonged QTc interval was mostly found in the first

**Table 1:** Patient demographic.

| Characteristics | | | Total | Percentage |
|---|---|---|---|---|
| DR-TB type | | MDR TB | 48 | 65.7 |
| | | Pre-XDR TB | 24 | 32.8 |
| | | XDR TB | 1 | 1.3 |
| Risk factor | Sex | Female | 32 | 43.8 |
| | | Male | 41 | 56.1 |
| | Age | Elderly (>60) | 11 | 15 |
| | | Adult (20–59) | 62 | 84.9 |
| | BMI | Severe underweight (<16) | 7 | 9.5 |
| | | Underweight (16–18.49) | 17 | 23.2 |
| | | Normal (18.5–24.9) | 39 | 53.4 |
| | | Overweight (25–29.9) | 10 | 13.9 |
| | DM | | 42 | 57.5 |
| | Hypokalemia | | 18 | 24.6 |

month. Thus it can be said that prolonged QTc interval was mostly occurred in the first month after the patient received the BDQ regimen. In the first month, prolonged QTc interval was mostly found at grade 1 so that according to the management of side effects by USAID and KNCV, monitoring for electrolyte levels and electrolyte correction was necessary so that QTc may not get worse [6].

Based on the results of this study, the onset of QTc interval side effects showed the highest percentage in the first month. Then based on the data on the level of severity, grade 1 data was mostly found. Thus, it was indicated that the percentage of the incidence of QTc interval side effects was mostly found under 480 ms.

One of the regimens used for DR-TB patients is the BDQ regimen. The BDQ regimen administered in this study was BDQ plus other anti-TB drugs, a combination of BDQ LFX plus other anti-TB drugs, a combination of BDQ CFZ plus other anti-TB drugs and a combination of BDQ LFX CFZ plus other anti-TB drugs.

The use of BDQ dosage in the first two weeks was 400 mg every day, and the following week until the 25th week used 200 mg, three times a week. The dose of LFX administered is 750 mg daily. The dosage of CFZ administered was 100 –300 mg every day depending on body weight for two months, after which the dosage was reduced to 100 mg per day. All drugs used were oral tablet of 100 mg of BDQ tablets, 250 mg of LFX tablets and 100 mg of CFZ tablets. The BDQ regimen that was most widely used in this study was the BDQ + LFX combination for 36 patients (49.3%).

The patient's ECG data that had been taken were then statistically tested using one-way ANOVA for 6 months in each month (M1, M3, M4, and M5). The M2 and M6 data were not normally distributed, so the Kruskal-Wallis test was performed. Based on the statistical results presented in Table 3, it can be concluded that there was no difference in the QTc interval for each regimen ($p>0.05$).

Statistical tests on risk factors for diabetes, hypokalemia, sex, and age did not show any differences ($p>0.05$). Meanwhile, the BMI showed a difference in the third month ($p<0.05$), but there was no difference in the other months. The results of the analysis of differences for BDQ regimens and risk factors are shown in Table 3.

**Table 2:** QTc interval prolongation side effect.

| QTc interval prolongation side effect | Total, n | Percentage, % |
|---|---|---|
| QTc interval prolongation occurred | 52 | 71.2 |
| **Severity** | | |
| *M1 (Month 1)* | | |
| Grade 1 | 11 | 57.9 |
| Grade 2 | 5 | 26.3 |
| Grade 3 | 3 | 15.8 |
| *M2 (Month 2)* | | |
| Grade 1 | 10 | 40 |
| Grade 2 | 7 | 28 |
| Grade 3 | 8 | 32 |
| *M3 (Month 3)* | | |
| Grade 1 | 12 | 63.2 |
| Grade 2 | 1 | 5.3 |
| Grade 3 | 6 | 31.6 |
| *M4 (Month 4)* | | |
| Grade 1 | 10 | 52.6 |
| Grade 2 | 6 | 31.6 |
| Grade 3 | 3 | 15.8 |
| *M5 (Month 5)* | | |
| Grade 1 | 6 | 40 |
| Grade 2 | 3 | 20 |
| Grade 3 | 6 | 40 |
| *M6 (Month 6)* | | |
| Grade 1 | 1 | 11.1 |
| Grade 2 | 5 | 55.5 |
| Grade 3 | 3 | 33.3 |
| **Onset** | | |
| M1 | 24 | 46.2 |
| M2 | 14 | 26.9 |
| M3 | 8 | 15.4 |
| M4 | 3 | 5.8 |
| M5 | 2 | 3.8 |
| M6 | 2 | 3.8 |

## Discussion

Sampling was conducted in March 2020 at Dr. Soetomo General Hospital, Surabaya, and obtained 155 DR-TB patients who used the BDQ regimen, but only 73 people met the inclusion criteria of this study and made as samples. The BDQ regimen was only administered when patients previously received short-term therapy were not tolerant of more severe side effects such as prolonged QTc interval. QT interval can be affected by heart rate, so it is necessary to use a formula to synchronize the QT interval with heart rate [7]. In this study, the data taken were QTc interval corrected using Bazett's formula if the heart rate was normal and Framingham if the heart rate was abnormal. Routine ECG examination was carried out every month at MDR-TB Polyclinic of Dr. Soetomo General Hospital.

The mechanism of prolonged QTc interval triggered by administration of drugs occurs due to blockade of voltage-gated potassium channels, especially the fast component of the delayed rectifier potassium current (IKr) released by hERG (the human ether-a-go-go-related gene) which results in the accumulation of potassium in myocytes which may lead to delayed cardiac repolarization [8, 9].

**Table 3:** Statistical analysis to BDQ regimen and risk factors.

| Group | Prolongation mean, ms | QTc mean, ms | Month | p-Value |
|---|---|---|---|---|
| **Regimen** | | | | |
| | | | M1 | 0.547 |
| BDQ | 26.5 ±21.6 | 439.4 ±18.4 | M2 | 0.279 |
| BDQ + LFX | 12.9 ±10.9 | 436.7 ±16.2 | M3 | 0.693 |
| BDQ + CFZ | 22.2 ±18.9 | 453.3 ±26.2 | M4 | 0.786 |
| BDQ + LFX + CFZ | 25.1 ±13.9 | 446.2 ±24.4 | M5 | 0.613 |
| | | | M6 | 0.417 |
| **DM** | | | | |
| | | | M1 | 0.059 |
| | | | M2 | 0.737 |
| DM | 19.7 ± 17.8 | 440.5 ± 21.4 | M3 | 0.643 |
| Non DM | 18.6 ± 13.1 | 442.8 ± 19.4 | M4 | 0.323 |
| | | | M5 | 0.517 |
| | | | M6 | 0.329 |
| **Hypokalemia** | | | | |
| | | | M1 | 0.050 |
| | | | M2 | 0.159 |
| Hypokalemia | 19.8 ± 15.6 | 447.1 ± 13.4 | M3 | 0.961 |
| Non hypokalemia | 18.9 ± 15.8 | 439.8 ± 22.0 | M4 | 0.552 |
| | | | M5 | 0.241 |
| | | | M6 | 0.741 |
| **Sex** | | | | |
| | | | M1 | 0.397 |
| | | | M2 | 0.386 |
| Male | 19.3 ± 17.8 | 438.1 ± 20.2 | M3 | 0.841 |
| Female | 19.0 ± 12.5 | 445.8 ± 20.2 | M4 | 0.885 |
| | | | M5 | 0.589 |
| | | | M6 | 0.432 |
| **BMI** | | | | |
| | | | M1 | 0.101 |
| Severe underweight | 16.9 ± 8.6 | 432.1 ± 21.8 | M2 | 0.279 |
| Underweight | 23.7 ± 15.4 | 446.2 ± 24.2 | M3 | 0.027 |
| Normal | 15.5 ± 13.9 | 439.7 ± 16.4 | M4 | 0.936 |
| Overweight | 27.6 ± 22.8 | 447.6 ± 25.9 | M5 | 0.089 |
| | | | M6 | 0.417 |
| **Age** | | | | |
| | | | M1 | 0.897 |
| | | | M2 | 0.073 |
| Elderly | 21.4 ± 12.5 | 447.4 ± 23.3 | M3 | 0.683 |
| Adult | 19.4 ± 16.3 | 441.2 ± 20.3 | M4 | 0.100 |
| | | | M5 | 0.943 |
| | | | M6 | 0.415 |

The mean QTc interval in the group without diabetes mellitus was slightly longer than the group with diabetes mellitus. This could be possible because there were more female patients in the non-DM group by 17 (54.8%), whereas there were 15 female patients (35.7%) in the DM group. In addition, in the non-DM group, the number of patients who were administered a combination of three drugs, namely BDQ + LFX + CFZ, was more than the DM group. This could increase the risk of prolonged QTc interval due to synergistic drug interactions [10]. Hyperglycemic condition will lead to overproduction of reactive oxygen species resulting in reduced activity in the IKr ion channels [11, 12].

Hypokalemia is a risk factor for prolonged QTc interval through a mechanism to reduce IKr activity or so called a hERG channel with excessive inactivation and block of sodium channels [13–15]. The highest mean prolonged QTc interval in the hypokalemia group was 19.8 ± 15.6 ms compared to the non hypokalemia group. The highest mean QTc was found in the hypokalemia group, namely 447.1 ± 13.4 ms compared to the non hypokalemia group. The number who experienced prolongation was greater in the hypokalemia group as many as 16 patients (88.9%) compared to the non hypokalemia group of 37 patients (66.1%). This suggested that the risk factor of hypokalemia could increase the QTc interval.

The presence of comorbidities such as diabetes mellitus was more prevalent among male respondents of 27 patients (65.9%) compared to female respondents of 15 patients (46.9%) wherein sex was a risk factor for prolonged QTc interval. Another risk factor was the presence of hypokalemia. It was found more in the male group of 10 patients (24.4%) compared to the female group of seven patients (21.9%). However, based on the mean QTc interval, the highest value was found among women of 445.9 ± 20.3 ms while for men it was 438.1 ± 20.2 ms. This is due estrogen hormone which can prolong the QTc interval through a suppression mechanism on the rapid (IKr), slow (IKs), and inward rectifier (IK1) ion channels so as to extend the action potential and consequently prolong the QTc interval [16]. In addition to this, male patients have a greater tendency to have smoke habits than female patients. One study stated that smoking increased the risk of prolonged QTc interval and there was a significant difference in QTc interval between before smoking and after smoking [17]. One of the components of cigarettes is nicotine, which is known as an inhibitor of non specific potassium ion channels, so that the potassium ion rate is inhibited and causes prolongation of the QTc interval [18, 19]. In the male group, the mean age was 50.5 years, while in the female group the mean age was 46.3 years. Older patients have the potential to experience a decrease in several body functions such as excretion, so that the drugs or metabolites that should be excreted are still remain in the body and cause more potential side effects [20].

Increasing BMI will increase the risk of prolonged QTc interval. This could be due to reduced ventricular repolarization [21]. The incidence of prolonged QTc interval among

DR-TB patients at Dr. Soetomo General Hospital was mostly found in the overweight group, namely 27.6 ± 22.9 ms. The highest mean QTc interval was also found in the overweight group, namely 447.6 ± 25.9 ms. In the overweight BMI group, all patients had risk factors for diabetes mellitus (100%), while risk factors for hypokalemia found in two patients (20%). BDQ and CFZ are lipophilic drugs which are stored in fatty tissue [22]. In general, fat-soluble drugs can pass through cell membranes faster than water-soluble drugs [23]. The drug, which accumulates in the fatty tissue, passes out of the tissue slowly so that it circulates in the bloodstream for several days even after a person stops taking the drug [24]. Therefore, in the overweight group who had a higher lipid concentration and took the drug, the maximum QTc would take longer to appear. The buildup of concentration when the drug from the fat is released and the new drug is introduced will further increase the risk of prolonged QTc interval. This was presented in the results of this study where the highest number of QTc above 500 ms was found among three patients (30%). After statistical test using one-way ANOVA was conducted, it was found that there was a difference at the third month. After that, further test was carried out using post hoc and it was found that there were differences in the data of the severe underweight, underweight, and overweight groups ($p<0.05$). Based on the mean QTc interval in the third month, there was an increase in the QTc interval with the highest increased in the overweight group. Thus, it can be said that a concomitant increase in BMI had the potential to prolong the patient's QTc interval. There was no difference in normal group since the mean in this group was less than the underweight group. This can be due to the smaller number of female respondents (35.9%) compared to other groups. Besides, it could also be caused by less number of patients who experienced hypokalemia (17.9%).

The elderly are more at risk of experiencing prolonged QTc interval with a mean prolongation of 21.39 ± 12.49 ms, while in the adult age group it was 19.5 ± 16.3 ms. There was a higher mean QTc interval in the elderly, namely 447.4 ± 23.3 ms, while in adults it was 441.4 ± 20.3 ms. According to a previous study, the older you are, the higher the risk of having a prolonged QTc interval [25]. Age affects the function of the autonomic nerves, namely increasing sympathetic nerve activity and decreasing parasympathetic activity which can change the pattern of repolarization in the heart [26]. The highest number of prolonged QTc interval was found in the elderly group, namely 10 patients (90.9%), followed by adults of 42 patients (67.7%). Likewise, QTc above 500 ms was mostly found in the elderly group of three patients (27.3%), and among adults of 14 patients (23.3%).

In this study, the highest mean prolonged QTc interval in the BDQ group was 26.5 ± 21.6 ms, comorbids such as diabetes mellitus could increase the effect of prolonged QTc interval. There were seven patients with diabetes mellitus in the BDQ group (63.6% of patients in the BDQ group). In addition to the comorbid factor of diabetes mellitus, four patients (36.3%) in the BDQ group had a higher risk of hypokalemia than the other groups. Furthermore, the mean prolonged QTc interval in the BDQ + LFX + CFZ group was the highest, followed by the BDQ + CFZ and BDQ + LFX group. A study stated that the addition of OAT CFZ significantly increased the incidence of a side effect of prolonged QTc interval [27]. The highest mean QTc interval for the BDQ + LFX + CFZ combination group was 446.2 ± 24.4. This is consistent with previous study which stated that the addition of a drug that had a side effect of prolonged QTc interval would provide a greater prolongation effect [28].

Based on the data of this study, there was a fluctuation in the average QTc interval on ECG examination every month that can be seen in Figure 1. There were patients who experienced an increase in the QTc interval in M2 then did not experience a prolongation in the following month, after that there was an increase in the QTc interval in the following month. This can be caused by the factor of potassium levels in these patients. When the QTc interval was prolonged, the patient's potassium level was lower (2.8 mmol/l) than before (3.8 mmol/l). Besides, the patient also had hyperglycemia or an increase in blood sugar levels during the month when the QTc interval was prolonged (blood sugar of 228 mg/dl). The incidence of prolonged QTc interval decreased as the blood sugar levels decreased from a previously high levels (blood sugar of 138 mg/dl).

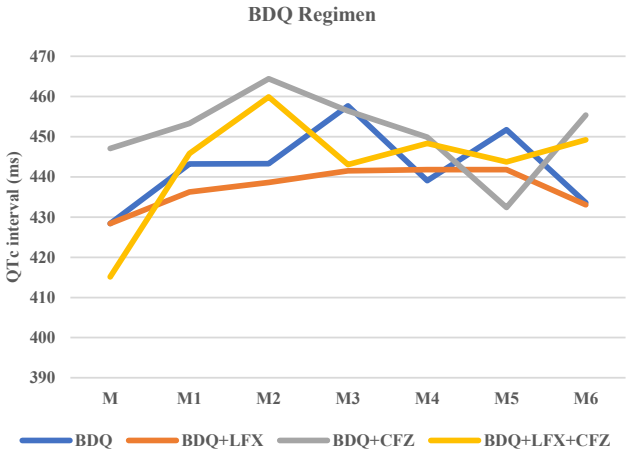


**Figure 1:** QTc interval mean of BDQ regimen every month, M, baseline; M1, first month; M2, second month; M3, third month; M4, fourth month; M5: fifth month; M6: sixth month.

This study had several limitations, namely the small number of samples taken which were not in balance with other groups and could not describe the incidence of side effects throughout Indonesian society. Then, the data taken for this study were only during the use of BDQ for six months due to the use of BDQ in individual DR-TB therapy for six months, while the half-life of BDQ and its metabolites from peripheral tissue by 5.5 months was not measured, so it is possible that BDQ still caused side effects including prolonged QTc interval. This study was conducted using retrospective data, the data taken were only limited based on existing medical records.

## Conclusions

It can be concluded from this study that there was no difference in the incidence of side effect of prolonged QTc interval in use of BDQ regimens (BDQ, BDQ + LFX, BDQ + CFZ and BDQ + LFX + CFZ). In addition, the presence of risk factors of diabetes mellitus, hypokalemia, sex, BMI, and age also did not lead to a difference in the incidence of side effect of prolonged QTc interval.

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Juni Ekowati*, Kholidah Febriani, Itsna N. A. Yaqin, Adinda A. Wulandari, Indra H. Mulya, Kholis A. Nofianti and Achmad Syahrani

# Shallot skin profilling, computational evaluation of physicochemical properties, ADMET, and molecular docking of its components against P2Y12 receptor

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**Abstract**

**Objectives:** Medicinal plants are a source of many compounds that are useful in the pharmaceutical field for novel drug development. Polyphenols and the flavonoid group in plants are known to have several activities, such as relieving cardio vascular disease (CVD). The outer skin of the shallot which is disposed of as waste is known to have an antiplatelet activity which was tested *in vitro* assay. To date, there is no study reported on the ADMET profile and physicochemical properties of the active component of the shallot skins.

**Methods:** The extraction of shallot skins was conducted by ultrasonic irradiation using ethanol. The phytochemical screenings were carried out by TLC and color reaction. The profiling of its active ingredient was presented by GC-MS, HPLC and spectrophotometry UV–vis. Whereas their physicochemical properties were analyzed by ChemDraw 17.00 program and the ADMET predictions were studied using pkCSM online tool. The MVD program was operated in the docking study on protein P2Y12 (PDB ID 4PXZ).

**Results:** The extract showed the presence of polyphenol, flavonoids, quercetin, natalensine-3,5-dinitrobenzoate; bis [2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine, benzo[a] heptalene, *N*-(trifluoroacetyl) methyl-*N*-deacethyl-colchicine. The ADMET prediction data displayed that the compounds in the extract have good absorption so that they can be used in the oral and transdermal routes. Some components in the extract have lower MDS than clopidogrel.

**Conclusions:** The ultrasonicated shallot skin extract can be used as additional resources of the active pharmaceutical ingredients and to have the potency to be developed as an oral or transdermal preparation.

**Keywords:** ADMET; cardiovascular disease; P2Y12 receptor; quercetin; shallot skin profilling; ultrasonic extraction.

## Introduction

*Cardiac Vascular Disease* (CVD), especially coronary heart disease, greatly contribute to the mortality rate across the globe, and patient medical costs continue to increase due to an increase in the number of sufferers [1, 2]. This disease occurs due to impaired blood flow to the myocardium due to platelet aggregation, thrombus, and the accumulation of oxidative damage to Low Density Lipid (LDL) by Reactive Oxygen Species (ROS) [1, 3]. Oxidant stress causes endothelial dysfunction and thrombus formation [4].

Drugs used to treat coronary heart disease are thrombolytic, antiplatelets and several antioxidants [5, 6]. Although they can treat coronary heart conditions due to thromboembolism, these drugs also have undesirable side effects such as intracranial bleeding, nausea, dyspnea, and it was reported that the patient had resistance to aspirin as an antiplatelet [7, 8]. Therefore, alternative therapies are needed to overcome the above problems with mild side effects.

Medicinal plants are a source of many chemical compounds that are useful in the pharmaceutical field for novel drug development, including polyphenols, the flavonoid class. The flavonoid group are known to have several activities, such as antibacterial and antioxidant [9, 10]. One of the natural ingredients that is widely used in daily food is shallots. Shallots have the active compound i.e. polyphenol quercetin as an antibacterial [11]. Not only the tuber part of the shallot, the outer skin of the shallot which is disposed of as waste is also known to have anti-inflammatory [12]

***Corresponding author: Juni Ekowati**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 81332041503, E-mail: juni-e@ff.unair.ac.id
**Kholidah Febriani, Itsna N. A. Yaqin, Adinda A. Wulandari, Indra H. Mulya, Kholis A. Nofianti and Achmad Syahrani,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia

and antimicrobial activity [13, 14]. It was also reported that there is antioxidant activity of the ethanolic extract from shallot skins using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method [15]. Apart from being antibacterial and antioxidant, the activity of shallot extract as an antiplatelet which was tested *in vitro* has also been revealed by Ro et al. [16]. These things show that the shallot skins has the potency as an active pharmaceutical ingredient (API).

Beside the activity, prospective drug compounds also need to be investigated regarding their physicochemical properties and pharmacokinetic profile, including absorption, distribution, metabolism, and excretion as well as its toxicity (hereinafter referred to as ADMET) to humans [17]. The pharmacokinetic profile of a drug could been influenced by the physicochemical properties [18]. Lipnski et al. has formulated several criteria regarding the physical and chemical properties of compounds that can demonstrate its oral bioavalability, consisting of: the ability to accept and donate hydrogen, molecular weight, and log p [19].

However, until now, there no research on the physicochemical and pharmacokinetics (ADMET) of the active ingredients of shallot skins. The effects of administering the extract on the gastrointestinal tract also need to be studied to ensure its safety in oral use. Therefore, this study aims to find out the component of shallot skin, its physicochemical properties prediction, and its pharmacokinetics (ADMET) prediction.

Pharmacokinetic profile analysis (ADMET) *in silico* is able to be conducted with the help of the online pkCSM program [20]. Prediction with the online pkCSM program has advantages over other software such as SwissADME, since there are more pharmacokinetic parameters that can be predicted with the online pkCSM program [21, 22] The greater number of parameters will have an impact on the broader information obtained to support the next drug development process.

Based on the research of Ro et al. [16] which states that shallot skins extract has antiplatelet activity *in vitro*, this study also evaluated the inhibition mechanism of the P2Y12 receptor by *in silico* test (PDB ID 4pxz). P2Y12 is a main receptor and the distinctive P2 goal for clinically allowed antiplatelet drugs (herein named as P2Y12 inhibitors) [17, 23].

# Materials and methods

The waste from shallot skins obtained from traditional markets is collected, washed, then dried at room temperature, and powdered using a blender. Previously, the species of shallot skin were examined at the Materia Medica Batu institute, and it was found that the shallot species was *Allium cepa* L. Ethanol p.a. (Merck, Germany) was used as solvent of extraction.

### Extraction

The powder then extracted in ethanol using the ultrasonic method. First, 80 g of shallot skin powder soaked in 500 mL Erlenmeyer with 350 mL 96% ethanol, then performed ultrasonic at high power and temperature at 40 °C for 30 min. The extraction product is then filtered using a Buchner funnel under vacuum; the filtrate is accumulated in a different Erlenmeyer. Second, the extracted pulp was put back into the Erlenmeyer 500 mL and added with 300 mL of 96% ethanol. The same process then carried out like the previous process. The extracted filtrate collected and carried out at a rotary evaporator. This ultrasonic extraction was repeated 14 times (until the filtrate did not react with $FeCl_3$, this is indicated by the solution remains clear).

### Phytochemical screening

Screening of flavonoid content was carried out by Thin layer Chromatography (TLC) method, using stationary phase silica gel GF254, the mobile phase butanol-acetic acid glacial-water (4:1:5) and ammonia vapor was used as color reagent. While the polyphenol group was detected by solution $FeCl_3$ 2%.

### Chromatographic profile

Examination of chemical compounds carried out by Gas Chromatography – Mass Spectrometry (GC–MS). The sample was weighed 100 mg, dissolved 2 mL of p.a. ethanol, then vortexed for 2 min, centrifuged at 3,000 rpm for 5 min. The filtrate was injected into 0.1 µL GC–MS, under optimum conditions. The instrument used in this study was Agilent 6980N Network GC system with auto sampler with detector Agilent 5973 inert MSD Inlet split 1/100. Run at a temperature of 250 °C, 50 °C programmed oven for 5 min, an increase of 10 °C every minute to 280 °C for 15 min, the rate in the column is 1 mL/min constant, Aux is 250 °C, MS Quad 150 °C, MS Source 230 °C, solvent delay 0 min, Wiley library version 7.0, and sample injection volume is 0.1 µL.

### Polyphenol assay

Polyphenol content test was carried out by spectrophotometric method. A standard solution of Gallic acid was made with a level of 5–25 ppm. Each with a pipette of 1.0 mL put into the vial, added 0.5 mL of Folin–Ciocalteu, left for 5 min, and then added 2 mL of 10% sodium carbonate solution. After that the absorbance was measured at $\lambda$ = 770 nm. Sample preparation was carried out by weighing 50 mg of the sample, dissolved in 50 mL of ethanol, then pipetting 1 and 10 mL, the dilution of the sample was piped 1.0 mL and then put into the vial. Furthermore, 0.5 mL of Folin–Ciocalteu was added, the mixture was 5 min, then added 2 mL of 10% sodium carbonate solution, the mixture was added 10 min before measuring the absorbance (at $\lambda$ = 765 nm).

### Quercetin content assay

Quercetin content test was carried out by High Pressure Liquid Chromatography (HPLC). Qualitative analysis was performed by comparing the identical retention time of the sample solution chromatogram with the quercetin standard solution chromatogram at the

same HPLC conditions. Quercetin standards were made of a standard solution of 50 ppm, pipette 0.6, 0.8, 1, and 1.2 mL, each put into a 5 mL volumetric flask, then diluted with solvent to the mark line, so that the concentrations solutions are 6, 8, 10, and 12 ppm. The ethanol extract was filtered by a 0.45 µm filter membrane and sonicator for 20 min. After that, each solution was injected into the HPLC system at a certain mobile phase and flow rate. The chromatogram is recorded and a calibration curve is made between the area of the peak and the concentration. From the measurement results, the area obtained is recorded, then the levels are calculated using a calibration curve (linear regression equation): $y = a + bx$.

### Physicochemical and ADMET prediction

Physicochemical prediction was carried out by ChemDraw version 17.00, while the ADMET prediction was carried out by the *online* program, pkCSM that can be accessed from http://biosig.unimelb.edu.au/pkcsm/prediction. These test was ran in ASUS A407UA BV032T Intel core i-3 7th-7020U 2.30 GHz, Windows 10 64 bit.

### Docking study

The docking study was carried out using Molegro Virtual Docker program version 5.5. (Molegro ApS). Some of the steps involved in Molecular Docking program were: obtaining the receptor, ligand preparation, method validation, and docking studies. The receptor used in this study was the $P_2Y_{12}$ receptor, which can been downloaded from the Protein Data Bank (http://www.rcsb.org). This $P_2Y_{12}$ receptor has the code for PDB 4PXZ with 6AD_1201[A] as native ligand. The ligands that used in this study were the compounds obtained from shallot skins that was known from GC–MS and quercetin test. The ligands structure drew in ChemDraw 2D version 17.00 and copying into ChemDraw3D version 17.00 to get the 3D structure. The best conformation was determined from MMFF94, and then saved in sybil.mol2 extension. The docking process, native ligands, namely 6AD_1201[A] for $P_2Y_{12}$ was redocked to the suitable binding site [22]. The results of the docking studies could be detected visually by comparing the structure of the ligands and receptor $P_2Y_{12}$ (6AD_1202[A]) in the binding site. This resulted in the interaction energy between ligand and receptor was then called as MolDock scores (MDS). The minimum energy denotes the best binding pose between the functional moiety of the ligand and the amino acid residue of the receptor [17].

# Results

## Extraction

The extraction of shallot skin in 96% ethanol by ultrasonic method produces as much as 13.149 g of thick extract. The screening phytochemical extract showed that the extract contained flavonoid and polyphenol compounds. The plate TLC showed the black spot, which is product reaction of phenolic moiety with $FeCl_3$. Whereas that plate showed yellowish spot which showed flavonoid content.

## Chromatographic profile

The results of examination of chemical compounds by GC–MS show in Table 1, which show that Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine has the highest percentage. The measurements were also carried out to determine the presence and levels of quercetin and polyphenol (which using Gallic acid as the standard) in the ethanol extract of shallot skins as shown in Table 2.

## Physicochemical and ADMET prediction

The *in silico* test was carried out to calculate the physicochemical and pharmacokinetic properties of the compounds contained in the shallot skins as shown in Table 3. The molecular weight ranges from 204.272 to 495.479. Log p value, which is a lipophilicity parameter, ranges from 1.988 to 8.417. The bond rotation, HBA, and HBD respectively ranges from 0 (Benzo[a]heptalene) until 7 (*N*-(trifluoroacetyl)methyl-*N*-deacethyl-Colchicine and Querc-etin), from 0 (Benzo[a]heptalene) until 10 (Natalensine, 3,5-dinitrobenzoate), and from 0 (Benzo[a]heptalene) until 5 (Quercetin).

## Docking study

Figure 1 shows P2Y12 (PDB ID: 4PXZ) with the ligand reference: 6AD-1201. The docking study was carried out in cavity 2 Vol 74.752. While Figure 2 shows the interaction between ligands and amino acids at $P_2Y_{12}$ receptors.

**Table 1:** Examination of chemical compounds by GC–MS.

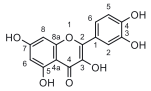
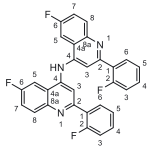
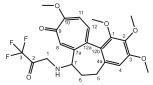
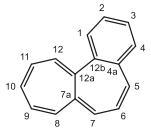
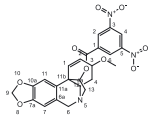
| RT | Compound name | %Normality | Qual |
|---|---|---|---|
| 28.34 | Natalensine, 3,5-dinitrobenzoate | 13.43% | 30 |
| 28.85 and 33.05 | Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine | 36.90% | 35 |
| 29.13 | Benzo[a]heptalene | 17.43% | 95 |
| 29.30 | *N*-(trifluoroacetyl)methyl-*N*-deacethyl-colchicine | 32.23% | 35 |

**Table 2:** Quercetin and polyphenol content in extract.

| Content | Quantity in extract Mean %(b/b) ± RPD |
|---|---|
| Quercetin | 4.61 ± 2.43 |
| Polyphenol | 11.14 ± 5.12 |

RPD: relative percent difference.

**Table 3:** Physicochemical and pharmacokinetic prediction.

| Compound | Structure | MW | Boiling point, K | Melting point, K | Log p | Bond rotation | HBA | HBD | PSA | Absorption | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Water solubility | Intestinal absorption |
| Quercetin |  | 302.238 | 1135.37 | 970.62 | 1.988 | 1 | 7 | 5 | 122.108 | −2.925 | 77.207 |
| Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl] amine |  | 495.479 | 1225.17 | 896.7 | 8.417 | 4 | 3 | 1 | 208.617 | −3.577 | 93.87 |
| *N*-(trifluoroacetyl) methyl-*N*-deacethyl-colchicine |  | 467.44 | 1076.21 | 754.77 | 3.4565 | 7 | 7 | 1 | 187.966 | −3.781 | 93.15 |
| Benzo[a]heptalene |  | 204.272 | 643 | 367.64 | 2.24 | 0 | 0 | 0 | 94.932 | −3.691 | 99.286 |
| Natalensine, 3,5-dinitrobenzoate |  | 495.444 | – | – | 2.8678 | 5 | 10 | 0 | 203.836 | −4.896 | 94.254 |

| Compound | Absorption | | Distribution | | | Metabolism | | Excretion | Toxicity | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Skin permeability | Caco-2 permeability | VDss (human) | BBB permeability | CNS permeability | CYP2D6 substrate | CYP3A4 substrate | Total clearance | AMES toxicity | Hepato toxicity | LD50 |
| Quercetin | −2.735 | −0.229 | 1.559 | −1.098 | −3.065 | No | No | 0.407 | No | No | 2.471 |
| Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl] amine | −2.735 | 1.165 | −0.826 | 0.343 | −0.819 | No | Yes | −0.275 | No | Yes | 3.335 |
| *N*-(trifluoroacetyl) methyl-*N*-deacethyl-colchicine | −2.716 | 1.139 | 0.833 | −1.231 | −3.21 | No | Yes | 0.479 | No | Yes | 2.992 |
| Benzo[a]heptalene | −1.645 | 1.539 | 0.207 | 0.619 | −1.986 | No | Yes | 0.205 | No | No | 1.573 |
| Natalensine, 3,5-dinitrobenzoate | −2.737 | −0.006 | 0.235 | −0.891 | −2.55 | No | Yes | 0.489 | Yes | Yes | 2.252 |

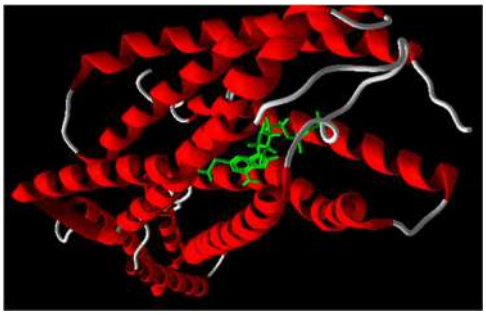


**Figure 1:** $P_2Y_{12}$ receptor with PDB: ID 4PXZ by which the binding site of the reference ligand and protein will be occupied by the test compounds.

Table 4 revealed the docking results of all tested compounds, ((2R, 3S, 4R, 5R)-5-(6-amino-2-(methylthio)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl) methyltrihy-drogen diphosphate against $P_2Y_{12}$ receptor. Clopidogrel, Quercetin and Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine. Quercetin and Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine has the similarity amino acid with ((2R, 3S, 4R, 5R)-5-(6-amino-2-(methylthio)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl trihydrogen diphosphate or Clopidogrel.

## Discussion

The outer skins of shallot have known to have anti-inflammatory [12], antimicrobial activity [13, 14] and antioxidant activity [15]. Apart from being antibacterial and antioxidant, the activity of shallot extract as an antiplatelet test also tested *in vitro* by Ro et al. [16]. This study

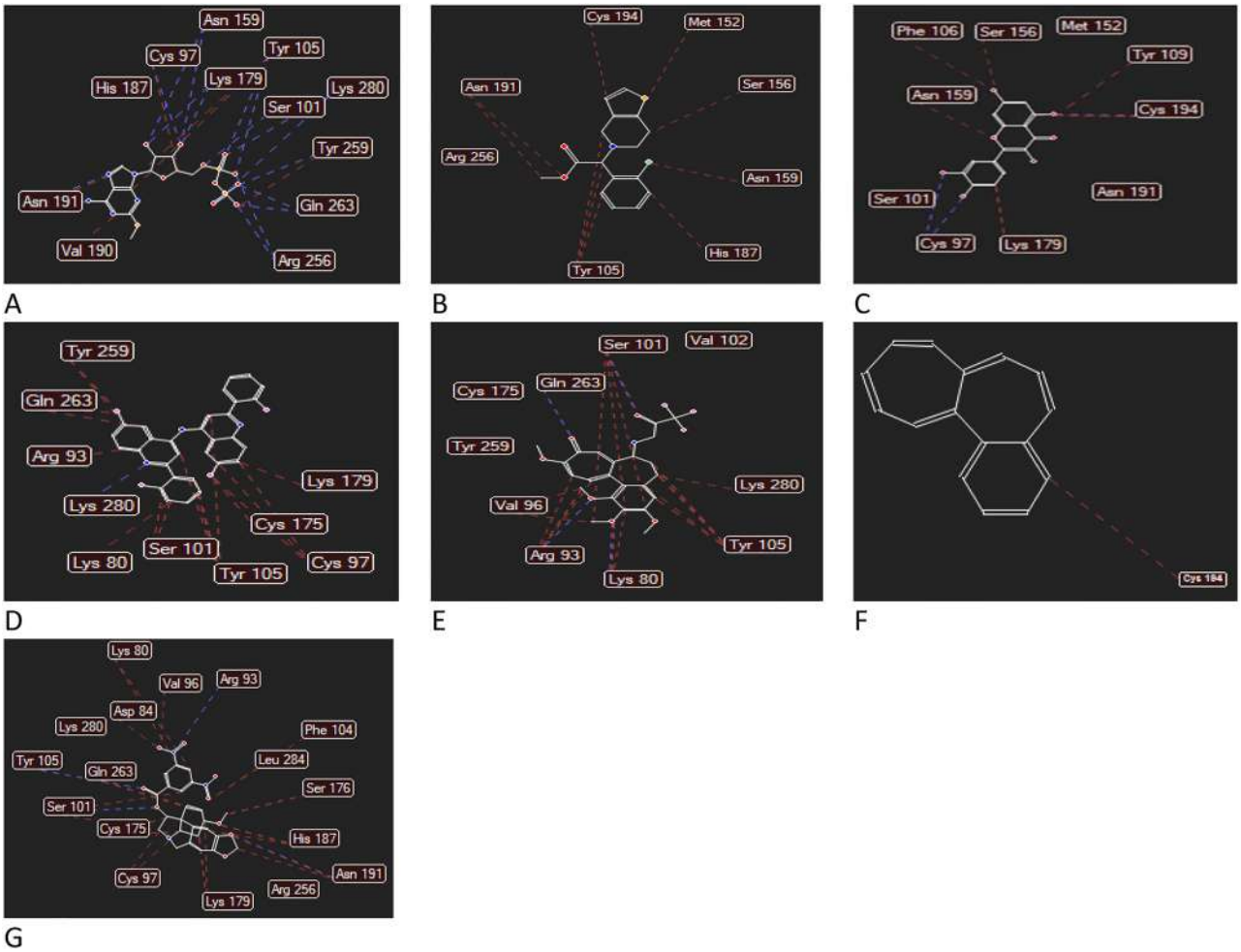



**Figure 2:** Map of the interaction between shallot skins compound and $P_2Y_{12}$ receptor: (A) ((2R, 3S, 4R, 5R)-5-(6-amino-2-(methylthio)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl trihydrogen diphosphate, (B) Clopidogrel, (C) Quercetin, (D) Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine, (E) *N*-(trifluoroacetyl)methyl-*N*-deacethyl-Colchicine, (F) Benzo[a]heptalene, and (G) Natalensine, 3,5-dinitrobenzoate.

**Table 4:** Results of the docking of the test ligand at the binding site of P2Y12 receptor.

| Compound name | MDS | Amino acid |
|---|---|---|
| ((2R, 3S, 4R, 5R)-5-(6-amino-2-(methylthio)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl trihydrogen diphosphate | −157.334 | Arg 256, Asn 159, Asn 191, Cys 97, Gln 263, His 187, Lys 179, Lys 280, Ser 101, Tyr 105, Tyr 259, Val 190 |
| Clopidogrel | −128.010 | Arg 256, Asn 159, Asn 191, Cys 194, His 187 Met 152, Ser 156, Tyr 105 |
| Quercetin | −116.863 | Asn 159, Asn 191, Cys 97, Cys 194, Lys 179, Met 152, Phe 106, Ser 101, Ser 156, Tyr 109 |
| Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine | −157.041 | Arg 93, Cys 97, Cys 175, Gln 263, Lys 80, Lys 179, Lys 280, Ser 101, Tyr 105, Tyr 259 |
| *N*-(trifluoroacetyl)methyl-*N*-deacethyl-colchicine | −122.252 | Arg 93, Cys 175, Gln 263, Lys 80, Lys 280, Ser 101, Tyr 105, Tyr 259, Val 96, Val 102 |
| Benzo[a]heptalene | −101.726 | Cys 194 |
| Natalensine, 3,5-dinitrobenzoate | −150.457 | Arg 93, Arg 256, Asn 191, Asp 84, Cys 97, Cys 175, Gln 263, His 187, Leu 284, Lys 80, Lys 179, Lys 280, Phe 104,Ser 105, Tyr 105, Val 96 |

was conducted to determine the benefits of domestic waste shallot skins in the provision of raw materials for Active Pharmaceutical Ingredient.

In this study, the extraction of shallot skins carried out by ultrasonic methods, which the ultrasonic waves were emitted by passing through the medium conducted the waves by inducing vibrational motion of the molecules. The distance between molecules can vary to be closer or farther as the result of the oscillatory motion of the molecules. If the ultrasonic waves in the medium become more intense, a point will be reached where the intramolecular force of the fluid cannot maintain its molecular structure intact. As a result, the molecular structure of the liquid will break down and a cavity is formed [24].

Cavitation is a mechanical activation process that removes the attraction between molecules in the liquid. Once formed, the tiny air bubbles in the cavity will absorb energy from the ultrasonic waves and make the cavity bigger. As the cavity got bigger, the air bubbles inside could no longer absorb ultrasonic energy. Finally, the fluid around the cavity will enter and break the air cavity. The physical characteristic of the irradiated mixture are vital for the cavitation efficiency, and also for the appropriate transfer of acoustic energy to reactants [25–27].

The ultrasonic profiling using the GC-MS method on shallot skin extract (Table 1) showed that the content of Natalensine-3,5-dinitrobenzoate was 13.43%; Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine as much as 36.90%, Benzo[a]heptalene as much as 17.43% and *N*-(trifluoroacetyl)methyl-*N*-deacethyl-Colchicine as much as 32.23%.

Jose et al. [28] stated that the phenyl ring and three methoxyl groups of the colchicine derivative contribute additives to the binding strength of colchicine and its analogues with tubulin, which will affect cancer. Kaivan et al. [29] stated, the use of colchicine for short-term myocardial infarction would reduce the size of the infarction compared to placebo. From this, it could been seen that shallot skin has great potential as API in the treatment of cancer.

Healthy food contain high levels of natural phenols in fruits, vegetables, cereals, tea, and coffee. Fruits such as grapes, apples, pears, cherries, and berries contain up to 200–300 mg of polyphenols per 100 g of fresh weight (0.2–0.3% w/w) [30]. Literatures reported the biological activities of polyphenols such as antioxidants, antibacterial, antineoplastic, antithrombotic, and vasodilating activities [31].

One example of phenolic compound is ferulic acid, which used as antithrombolysis. ADP-induced platelet aggregation test shows that the stronger antithrombolysis activity is attributable to its moiety [32, 33]. Therefore, further research on polyphenols as antithrombolysis is necessary. In this research, extraction of shallot skin, which carried out ultrasonic, had the amount of polyphenols of 11.14% ± 5.12% w/w in the extract.

In relation to the activity of flavonoids as antiplatelet, structure-activity relationship analysis showed that antiaggregation activity of flavonoids are highly rely on the C-ring structure that represent the compounds class. If double bond is present between C2 and C3, it increases antiaggregation activity of flavonoids in case of non-methylated flavonoids. Most active flavonoids possess hydroxyl group at the position 6. Methylation of rings A and B decreases antiplatelet activity [34]. Flavonoid have several mechanisms of action such as change of bilayer function, change in ROS concentrations and oxidative stress, change of intracellular $Ca^{2+}$ concentration, inhibition of enzymes

(phospholipase C, cAMP phosphodiesterase, cyclo-oxygenase, thromboxane A2 synthase) [35].

Quercetin which is usually found in the food consumed, scientifically reported to have anticancer, antiviral, and antimicrobial activity. The use of quercetin is able to decrease CVD risk, LDL (plasma low-density lipoprotein), hypertension, and risk of ischemic heart disease. Its antiplatelet activity also indicated from the ability to inhibit platelet aggregation upon ex vivo post-supplementation and *in vitro* addition [36].

The absorption of active ingredients in the gastrointestinal tract is affected by the physicochemical characteristic of the drug, the dosage form used, and the anatomy and physiology of the absorption site [37]. Passive diffusion is influenced by the size and shape of the molecule, the rate of ionization, and the solubility of a drug in fat. Meanwhile, active ingredients that are weakly alkaline will be absorbed at a more alkaline pH, namely in the small intestine [38].

Predicting the solubility of active ingredients in water significantly contribute to the drug absorption after oral administration and is a consideration in parenteral drug administration. This is useful in the manipulating and testing process in the drug design and development process and is crucial for the bioavailability of drugs in the blood [39]. The ADMET profile of a drug is also related to its physicochemical properties [40, 41]. In Table 3, there are various parameters of physicochemical properties, it is known that the water solubility of Quercetin is $2.925 \times 10^{-4}$ mol/L; Bis [2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine is $3.577 \times 10^{-4}$ mol/L; *N*-(trifluoroacetyl)methyl-*N*-deacethyl-colchicine is $3.781 \times 10^{-4}$ mol/L; Benzo[a] heptalene is $3.691 \times 10^{-4}$ mol/L, and Natalensine, 3,5-dinitrobenzoate is $4{,}896 \times 10^{-4}$ mol/L.

The greater the solubility of the drug in fat (log p), the higher the absorption of the drug into the body's membrane. However, the drug must still be slightly hydrophilic in order for extracellular fluids to be transported and to be distributed throughout the body [42]. Based on Lipinski's law, log p of the active ingredients in the extract, apart from Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine, all of which meet these requirements. Related to Rule of Five [19], the compound Bis [2-(2-fluorophenyl)-6-fluoroquinolin-4-yl] amine is a compound that meets these criteria because the number of hydrogen bond donors (HBD) of each compound <5 and number of hydrogen bond acceptors (HBA) of each compound <10.

tPSA is a molecular descriptor as a parameter for intestinal absorption and drug penetration into the blood brain barrier [43]. From Table 3 It is known that two compounds from shallot skin extract, namely Quercetin and Benzo[a]heptalene, have tPSA values <140 Å. So, that compounds meet Veber's law requirements. Caco-2 permeability is an absorption model that uses monolayer Caco-2 cells as an *in vitro* model predicting the absorption of an orally administered drug. [20]. The compounds in the shallot skins have good permeability apart from 3,5-dinitrobenzoate-Natalensine, this indicates that the compounds in the shallot skins have the potency to be used orally and also have the potential if used through the transdermal route.

The volume of distribution (VDss) is the theoretical the volume by which the drug is dissolved in the body. The high VDss indicates that the majority of the drug is in the tissue [20]. The compounds in the shallot skins are predicted to have different VDss values so that some of the shallot skins compounds will survive in the blood vessels and most of them in the tissues, a good antiplatelet compound is expected more distributed in blood vessels than in tissues.

The drug ability to permeate the Central Nervous System (CNS) was calculated as blood-brain permeability (logPS), which compounds with log pS>−2 are considered to have access on CNS, while compounds with logPS<−3 are unable to penetrate [20]. Of the five test compounds, Quercetin and *N*-(trifluoroacetyl) methyl-*N*-deacethyl-colchicine had a logPS value <−3 meaning the compound was predicted not to permeate the central nervous system. Meanwhile, the other three compounds had a logPS value >−2, which means that the test compounds were predicted to penetrate the central nervous system.

CYP450 substrates, namely CYP2D6 and CYP3A4. are important to identify because CYP450 inhibitors can dramatically alter the pharmacokinetics of drugs metabolized by CYP450 [20]. It was found that apart from Quercetin, the test compound became a CYP3A4 substrate, whereas for CYP2D6, the five compounds did not become a substrate for CYP2D6.

Total clearance is related to bioavailability, and it is important to determine the dosage level to reach a steady-state concentration. Total clearances are expressed in logs (mL/min/kg) [20]. The test results showed that the five test compounds had a total clearance value stated in logs (mL/min/kg) of −0.275 to 0.489.

Toxicity is a pharmacokinetic parameter that is important to determine before designing a drug in order to create a drug that is not only effective and of good quality, but also safe to use. Many compounds can cause hepatotoxicity such as certain drugs, laboratory chemicals and some of herbal medicines [44]. In the shallot skins extract, it is known that Quercetin and Benzo[a]heptalene compounds are not hepatotoxic. Rat Oral Acute Toxicity (LD50) is the amount of compound given at once that can cause

the death of 50% of a group of test animals (mol/kg) [20]. The five test compounds have an LD50 value between 1.573 and 3.335.

Prediction of antiplatelet activity was carried out at the P1Y12 receptor, a G1-protein on platelet membrane surface receptors. It stimulated adenylyl cyclase inhibition and intracellular calcium mobilization [45, 46]. The first generation of P2Y12 receptor inhibitors is the thienopyridine ticlopidine class, which has the side effect of neutropenia. The second generation is the clopidrogel, which is highly metabolized by the CYP450 enzyme [47].

Based on the *in silico* test against the $P_2Y_{12}$ receptor, it is known that, quercetin and Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine has amino acids similar to ((2R, 3S, 4R, 5R)-5-(6-amino-2-(methylthio)-9H-purine-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyltri-hydrogen dip hosphate or clopidogrel, which used as standard. Bis[2-(2-fluorophenyl)-6-fluoroquinolin-4-yl]amine has an MDS value that is close to the standard, whereas quercetin although it has a greater MDS value than the standard, so that its binding ability is smaller, it still does not eliminate the possibility that quercetin can be used against the $P_2Y_{12}$ receptor as antiplatelet. After going through the *in silico* test phase, the shallot skins extract content should be tested *in vivo*. It was concluded that the ultrasonic shallot skin extract can be used as new source of the active pharmaceutical ingredient and are predicted to have the potency as antiplatelet in an oral or transdermal preparation.

## Conclusions

The ultrasonic shallot skin extract can be used as new source of the active ingredient for drug development and are predicted to have the potency to be developed as an oral or transdermal preparation.

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**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical approval:** Not applicable.

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Widya Handayani, Suharjono* and Mohammad Yogiarto

# Analysis of HMGB-1 level before and after providing atorvastatin standard therapy in coronary artery disease patients with type-2 diabetes mellitus compared to without type-2 diabetes mellitus

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**Abstract**

**Objectives:** Coronary artery disease (CAD) is one of the main causes of death from cardiovascular disease, because heart attacks result in atherosclerosis which causes narrowing of the arteries. Atorvastatin has a pleiotropic effect as anti-inflammatory through one of the target levels of High Mobility Group Box-1 (HMGB-1). This prospective observational study aimed to analyze the effect of atorvastatin on serum HMGB-1 levels in CAD.

**Methods:** Samples were collected from prospective observation pre–post study in May–July 2018 with consecutive sampling method. Serum HMGB-1 levels were measured in patients with CAD who were given atorvastatin for CAD with type-2 diabetes mellitus compared without type-2 diabetes mellitus in a patient ward. Blood was collected on admission day and before the patient left the hospital. After centrifugation, serum samples were stored at –80 °C before measurement. We used an ELISA kit (IBL International) to determine HMGB-1 concentrations. This research protocol has been approved by the Ethical Committee of Dr. Soetomo General Hospital, Surabaya.

**Results:** We enrolled 38 patients and divided them into two groups which 19 patients on CAD with type-2 diabetes mellitus and 19 patients without diabetes mellitus. Serum HMGB-1 levels in CAD with type-2 diabetes mellitus were increased significantly ($p = 0.049$) and not significantly decreased in CAD without type-2 diabetes mellitus ($p = 0.480$). The HMGB-1 level was not significantly different between the two groups ($p = 0.210$).

**Conclusions:** HMGB-1 levels after providing atorvastatin in CAD with type-2 diabetes mellitus increased significantly, meanwhile, in CAD without type-2 diabetes mellitus did not decrease significantly. The HMGB-1 level was not significantly different between the two groups. Longer time and more point for the collected sample needed for further research.

**Keywords:** atorvastatin; coronary artery disease; diabetes mellitus; HMGB1; inflammatory marker.

## Introduction

The 2016 Heart Disease Statistics and Stroke, American Heart Association (AHA), reports that 15.5 million people over the age of 20 years in the United States have coronary heart disease (CHD). It is a common thing that there is an increase with age, for men and women estimated at approximately every 42 s, Americans will suffer from myocardial infarction (MI) [1].

The increase in the incidence of CHD can be caused by the increasing prevalence of many risk factors, such as diabetes, which is one of the most critical risk factors [2]. The prevalence of diabetes in patients with CHD reaches 50% in many countries [3]. Diabetic patients show an increased risk of atherosclerosis in CHD for various reasons, including metabolic factors, such as hyperglycemia, dyslipidemia, and insulin resistance, which cause endothelial cell dysfunction and vascular smooth muscle [4].

Statins are well known for reducing cardiovascular events and death in patients with CAD or who are at high risk of cardiovascular disease. In addition to reducing low-density lipoprotein (LDL) cholesterol, statins have pleiotropic effects such as reduced thrombus formation,

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia, E-mail: suharjono@ff.unair.ac.id
**Widya Handayani,** Universitas Airlangga, Surabaya, East Java, Indonesia
**Mohammad Yogiarto,** Cardiovascular, Department of Cardiology, Faculty of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

improved endothelial function, and reduced inflammation [5]. Statins use the anti atherosclerotic effect independently of the hypolipidemic mechanism because mevalonate metabolism produces a series of isoprenoids that are vital for distinguishing the cellular function from cholesterol synthesis to control cell growth and differentiation; the barriers to HMG–CoA reductase have beneficial pleiotropic effects. Thus, statins significantly reduce coronary events, both in primary and secondary prevention, to be the most efficient hypolipidemic compounds that can reduce mortality in coronary patients [6].

Atorvastatin is the safest statin-associated with kidney function because it results in the least number of patients for two years of treatment with new onset of microalbuminuria (10.9%), then followed by rosuvastatin (14.3%) and pravastatin (26.6%) [7]. Atorvastatin is more effective in patients with CHD with LDL cholesterol, non HDL cholesterol, and triglycerides than simvastatin [8]. Some studies say atorvastatin has a protective effect on renal function [9]. In the GREek study of Atorvastatin and Coronary heart disease Evaluation (GREACE), administration of atorvastatin aggressively induces an increase in creatinine clearance (CrCL) after six months, and that effect continues for 48 months [10]. In the "*New Target*" study showed atorvastatin administration increased eGFR depending on the dose of administration and increased significantly in patients who received a dose (80 mg/day) compared with those who received a low dose (10 mg/day) [11]. In patients with type-2 diabetes mellitus, high-dose atorvastatin induces a decrease in strong CRP levels. CRP decrease is mainly independent of the effects on lipid reduction and changes in IL-6 levels. The pleiotropic effect of high doses of atorvastatin in inflammation can increase cardio-protective effects in high-risk patients [12].

HMGB-1 is a molecule associated with damage, which shows the potential role of this protein in the pathophysiology of acute coronary syndromes [13]. In the experimental model, the incidence of acute coronary and cerebrovascular syndromes shows that HMGB-1 is not only involved in strengthening the inflammatory response during acute ischemic injury, but also in the process of remodeling a recovery after ischemia. HMGB-1 has been independently proven to be associated with mortality cardiac in elevation myocardial infarction ST-segment. A number of clinical studies have shown that HMGB-1 concentrations are increased in patients with acute coronary syndromes compared to stable coronary artery disease (CAD) and healthy volunteers. HMGB-1 is independently associated with a large number of noncalcified plaques in patients with a stable CAD for predictions of coronary plaque complexity [14]. The study found an association between increased HMGB-1 and severe angiographic stenosis and complex lesions. Increased HMGB-1 is associated with future cardiovascular mortality in patients with (unstable angina) UA/(non ST-segment elevation myocardial infarction) NSTEMI. This finding suggests that HMGB-1 can be involved in the development of acute coronary syndromes [13].

Two clinical studies show that serum HMGB-1 levels are significantly higher in patients with CHD with diabetes mellitus than without diabetes [15]. In the study of post infarct patients, HMGB-1 levels of diabetic patients were significantly higher than those of nondiabetics [16]. Levels of HMGB-1 in plasma were higher in the type-2 diabetes mellitus group than in the normal glucose tolerance (NGT) group [17]. Periodic measurements of HMGB-1 can be useful for evaluating changes in inflammatory status, estimating risks during follow-up, and directing hospitalization and outpatient care [13]. Studies show that circulating HMGB-1 at the time of admission can be a strong predictor of independent and strong cardiovascular mortality in patients hospitalized for UA/NSTEMI within 24 h after onset of chest pain and measurements of HMGB-1 upon admission can improve early risk stratification in patients with UA/NSTEMI [13].

## Materials and methods

The study was to analyze the pleiotropic anti inflammatory effect of atorvastatin on serum HMGB-1 levels in coronary artery patients with type-2 diabetes mellitus and without type-2 diabetes mellitus. The study was conducted at the Functional Medical Staff of Cardiovascular and Vascular Dr. Soetomo Hospital, Surabaya, from May to July 2018. This research has received ethical conduct from the Health Research Ethics Committee of Dr. Soetomo Hospital, Surabaya, based on Ethical Clearance No. 0216/KEPK/IV/2018.

Sampling was performed by consecutive sampling, and a total of 38 patients were then randomized into two groups of 19 patients in the group of CAD patients with type-2 diabetes mellitus and coronary artery patients without type-2 diabetes mellitus. The blood sample was taken from both groups when the patients are at the admission and before discharge from hospital, then the serum HMGB-1 level is measured by the ELISA method, and the measurement results are in the form of HMGB-1 levels in units of ng/mL.

Inclusion criteria in this study: male or women patient >18 years old, patient who diagnosed CAD in CAD without type-2 diabetes mellitus group and also diagnosed type-2 diabetes mellitus in CAD with type-2 group, patient or patient family sign informed consent exclusion criteria in this study: Patient with malignancy, diagnosed autoimmune, receiving glucocorticoid, streptokinase, and NSAID except acetosal.

Data processing results of this study were conducted based on characteristic data and serum levels of HMGB-1 in each group of patients. Data are presented in the form of tables and drawings, and

statistical analyzes are performed using a paired *t*-test to determine the difference between pre- and post-atorvastatin levels. Also, statistical analysis with independent *T*-test was used to determine the comparison of serum levels of HMGB-1 in both groups, namely the group of CAD patients with type-2 diabetes mellitus and CAD without type-2 diabetes mellitus.

## Results

The average age of patients in this study was 60.95 ± 9.85 years in the group of CAD patients with type-2 diabetes mellitus and 56.74 ± 10.50 years in the coronary artery group without diabetes mellitus type-2 who did not have a significant difference between the two groups (p = 0.210), while the average age of patients is 58.85 ± 10.17 years. The number of male patients in the group of CAD patients with type-2 diabetes mellitus (68.4%) and the group CAD without type-2 diabetes mellitus (84.2%) was more dominant than the number of female patients in both groups CAD patient with type-2 diabetes mellitus and CAD patient group without type-2 diabetes mellitus, but the sexes in the two groups were spread evenly (p = 0.45), where the total number of male patients was 76.3%, and women were 23.7%. Patients with a history of active smokers in the group of patients with CAD with diabetes mellitus type-2 were 47.4 and 52.6% in the group of patients with CAD without type-2 diabetes mellitus with a total number of patients who smoked 50.0% (p = 1.00). History of hypertension is a risk factor for CAD, has a prevalence of 20 patients (52.6%), in the group of patients with CAD with type-2 diabetes mellitus as many as 12 patients (63.2%), and in the group of patients with CAD without type-2 diabetes mellitus with a total of eight patients (42.1%). History of dyslipidemia is a risk factor for CAD, has a prevalence of 11 patients (28.9%), in the group of patients with CAD with type-2 diabetes mellitus as many as three patients (15.8%) and in the group of CAD patients without type-2 diabetes mellitus with a total of eight patients (42.1%) (Tables 1 and 2).

In the group of patients with CAD patients with type-2 diabetes mellitus, after receiving atorvastatin during hospital treatment the mean serum HMGB-1 level increased from 14.85 ± 5.78 to 17.40 ± 4.81 ng/mL, statistically this shows a significant difference after the Wilcoxon signed-ranks test was conducted (p = 0.049). In the group of patients with type-2 diabetes mellitus, after receiving

**Table 1:** Demographics patient.

| Demographics | CAD + DM type 2 (n=19) | CAD non DM type 2 (n = 19) | Total (n = 38) | p Score |
|---|---|---|---|---|
| Age, years | 60.95 ± 9.85 | 56.74 ± 10.50 | 58.85 ± 10.17 | 0.21 |
| **Gender, %** | | | | 0.45 |
| Man | 13 (68.4%) | 16 (84.2%) | 29 (76.3%) | |
| Woman | 6 (31.6%) | 3 (15.8%) | 9 (23.7%) | |
| Smoking | 9 (47.4) | 10 (52.6%) | 19 (50%) | 1.00 |
| **Disease history – total, %** | | | | |
| Hypertension | 12 (63.2%) | 8 (42.1%) | 20 (52.6%) | 0.33 |
| Dyslipidemia | 3 (15.8%) | 8(42.1%) | 11 (28.9%) | 0.15 |

**Table 2:** Treatment patient.

| Treatment – total, % | CAD + DM type 2 (n = 19) | CAD non DM type 2 (n = 19) | Total (n = 38) | p Score |
|---|---|---|---|---|
| Clopidogrel | 11 (57.9%) | 11 (57.9%) | 22 (57.9%) | 1.00 |
| Ticagrelor | 10 (52.6%) | 8 (42.1%) | 18 (47.4%) | 0.75 |
| Rivaroxaban | 0 (0.0%) | 1 (5.3%) | 1 (2.6%) | 1.00 |
| ACE-I | 16 (84.2%) | 14 (73.7%) | 30 (78.9%) | 0.69 |
| ARB | 3 (15.8%) | 5 (26.3%) | 8 (21.1%) | 0.69 |
| CCB | 2 (10.5%) | 2 (10.5%) | 4 (10.5%) | 1.00 |
| β-Blocker | 12 (63.2%) | 16 (84.2%) | 28 (73.7%) | 0.27 |
| Fondaparinux | 1 (5.3%) | 0 (0.0%) | 1 (2.6%) | 1.00 |
| Enoxaparin | 6 (31.6%) | 6 (31.6%) | 12 (31.6%) | 1.00 |

atorvastatin during hospitalization, the mean serum HMGB-1 level decreased which was not statistically significant from 16.31 ± 4.93 ng/mL to be 15.26 ± 4.15 ng/mL, conducted paired T-test (p = 0.480) (Table 3).

The levels of HMGB-1 in the group of CAD patients with type-2 diabetes mellitus compared to the group of CAD patients with type-2 diabetes mellitus before administration of atorvastatin did not show a significant difference (p = 0.175). The mean serum HMGB-1 level after administration of atorvastatin in the group of CAD patients with type-2 diabetes mellitus was higher than the CAD without type-2 diabetes mellitus group, which was 17.40 ± 4.81 vs. 15.26 ± 4.15 ng/mL. Mann–Whitney test has carried out that the difference was not statistically significant with a value of p>0.05 (p = 0.209). The mean difference in levels of the group of CAD patients with type-2 diabetes mellitus compared to the CAD without type-2 diabetes mellitus patients did not show a significant difference through the T-test (p = 0.210) (Table 4).

## Discussion

This study was conducted to analyze the effect of atorvastatin on serum levels of HMGB-1 as one of the biomarkers in the inflammatory process that occurs in CAD with type-2 diabetes mellitus compared to type-2 diabetes mellitus.

Based on measurements of serum levels of HMGB-1 in all patients, mean serum levels of HMGB-1 in the group of CAD patients with type-2 diabetes mellitus showed a significant increase (p = 0.049). HMGB-1 levels before atorvastatin administration were 14.85 ± 5.78 ng/ml and after atorvastatin administration which was 17.40 ± 4.81 ng/mL. It was found that 14 patients (73.68%) of the CAD group with diabetes mellitus had elevated serum levels of HMGB-1. There were five patients (26.32%) who experienced a decrease in serum levels of HMGB-1 during hospitalization (Figure 1).

Whereas in the CAD without type-2 diabetes mellitus patients group, the average HMGB-1 level showed a non significant decrease (p = 0.480), before atorvastatin is 16.31 ± 4.93 ng/mL and after atorvastatin administration is 15.26 ± 4.15 ng/mL. Also, 11 patients (57.90%) from the CAD without type-2 diabetes mellitus group experienced elevated serum HMGB-1 levels after administration of atorvastatin during hospitalization, and nine patients reduced serum HMGB-1 levels after administration of atorvastatin during hospitalization (Figure 2).

The mean difference in the level of groups of patients with CAD with type-2 diabetes mellitus compared to type-2

**Table 3:** Result of HMGB-1 serum level in coronary artery disease with type-2 diabetes.

| Initial code | Pre | Post | Difference | % Difference |
|---|---|---|---|---|
| 1A | 9.1 | 16.7 | 7.6 | 83.52 |
| 2A | 12.6 | 11 | −1.6 | −12.70 |
| 3A | 10.6 | 24.7 | 14.1 | 133.02 |
| 4A | 19 | 13.5 | −5.5 | −28.95 |
| 5A | 18 | 16.8 | −1.2 | −6.67 |
| 6A | 15 | 17.9 | 2.9 | 19.33 |
| 7A | 10.9 | 13.6 | 2.7 | 24.77 |
| 8A | 11 | 12.7 | 1.7 | 15.45 |
| 9A | 33.6 | 18.4 | −15.2 | −45.24 |
| 10A | 17.1 | 18.7 | 1.6 | 9.36 |
| 11A | 19.8 | 16.3 | −3.5 | −17.68 |
| 12A | 9.4 | 14.7 | 5.3 | 56.38 |
| 13A | 11.5 | 16 | 4.5 | 39.13 |
| 14A | 11 | 14 | 3 | 27.27 |
| 15A | 11.3 | 15.6 | 4.3 | 38.05 |
| 16A | 15.4 | 20.9 | 5.5 | 35.71 |
| 17A | 11.4 | 19.9 | 8.5 | 74.56 |
| 18A | 15.5 | 16.9 | 1.4 | 9.03 |
| 19A | 19.9 | 32.3 | 12.4 | 62.31 |
| Average ± SD | 14.85 ± 5.78 | 17.40 ± 4.81 | 2.55 ± 6.52 | 17.20% |

**Table 4:** Result of HMGB-1 serum level in coronary artery disease without type-2 diabetes mellitus.

| Initial code | Pre | Post | Difference | % Difference |
|---|---|---|---|---|
| 1B | 13.6 | 21 | 7.4 | 54.41 |
| 2B | 20.1 | 11.6 | −8.5 | −42.29 |
| 3B | 18.4 | 10.9 | −7.5 | −40.76 |
| 4B | 18.9 | 11.4 | −7.5 | −39.68 |
| 5B | 14.5 | 7.5 | −7 | −48.28 |
| 6B | 13.4 | 13.7 | 0.3 | 2.24 |
| 7B | 21.5 | 11.7 | −9.8 | −45.58 |
| 8B | 24.9 | 10.5 | −14.4 | −57.83 |
| 9B | 14.7 | 16.5 | 1.8 | 12.24 |
| 10B | 12.7 | 15 | 2.3 | 18.11 |
| 11B | 11.7 | 16.5 | 4.8 | 41.03 |
| 12B | 7.6 | 13.3 | 5.7 | 75.00 |
| 13B | 13.2 | 17.2 | 4 | 30.30 |
| 14B | 10.8 | 17.5 | 6.7 | 62.04 |
| 15B | 13.2 | 15.8 | 2.6 | 19.70 |
| 16B | 23.9 | 21.4 | −2.5 | −10.46 |
| 17B | 18.2 | 20.4 | 2.2 | 12.09 |
| 18B | 14.2 | 15.6 | 1.4 | 9.86 |
| 19B | 24.3 | 22.4 | −1.9 | −7.82 |
| Average ± SD | 16.31 ± 4.93 | 15.26 ± 4.15 | −1.05 ± 6.33 | 6.42% |

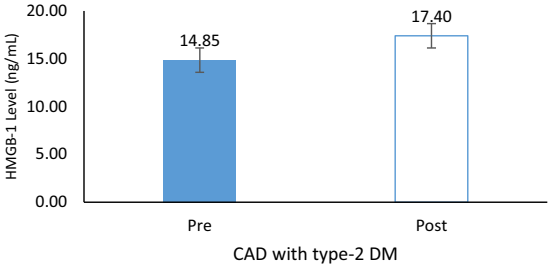


**Figure 1:** HMGB-1 level before and after providing atorvastatin in coronary artery disease with type-2 diabetes mellitus.

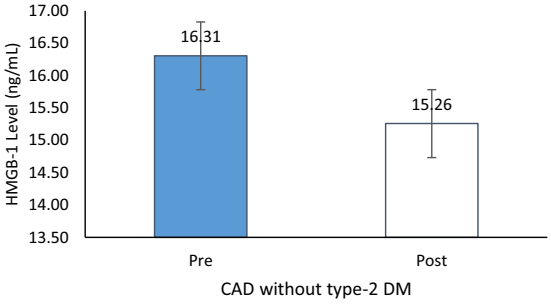


**Figure 2:** HMGB-1 level before and after providing atorvastatin in coronary artery disease without type-2 diabetes mellitus.

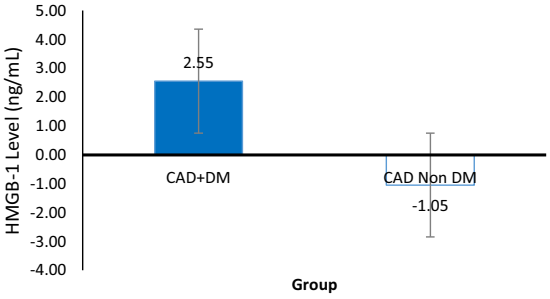


**Figure 3:** HMGB-1 level difference in coronary artery disease with type-2 diabetes mellitus and coronary artery disease without type-2 diabetes mellitus.

diabetes mellitus patients did not show a significant difference ($p = 0.210$). HMGB-1 levels with the administration of atorvastatin did not show a significant difference in the two groups (Figure 3).

Increased levels of HMGB-1 in patients with CAD with type-2 diabetes mellitus (14 patients) were more than the CAD without type-2 diabetes mellitus group (11 patients). The importance of innate immunity in inflammation, the mediator of innate immunity, plays an essential role in the development of type-2 diabetes mellitus. HMGB-1, a

nonhistone nucleic protein that plays a role in damage associated with molecular patterns, is related to the pathogenesis of type-2 diabetes mellitus. HMGB-1 can signal through receptors for products end of advanced glycation (RAGE) and toll-like receptors (TLRs) to activate the factor-κB signalling pathway (NF-κB) thus contributing to the inflammatory response in type-2 diabetes mellitus [17]. Plasma HMGB-1 levels were higher in the type-2 diabetes mellitus group than in the nonglucose tolerance group. Serum HMGB-1 concentration was also higher in obese subjects than in normal-weight subjects in the nonglucose tolerance group and type-2 diabetes mellitus [17]. Increased HMGB-1 in diabetic patients causes oxidative stress, disorders of endothelial repair, and can also cause smooth muscle vascular dysfunction and thrombosis [15]. Good control of blood glucose and lipids can reduce HMGB-1 levels and delay the development of atherosclerosis [18].

Patients who have several risk factors for CHD have elevated levels of HMGB-1. Hyperglycemia has been shown to increase HMGB-1 expression while HbA1c levels can represent an average blood glucose level of 90 days, HbA1c can be used to predict the severity of CHD and unacceptable cardiovascular risk, especially in CHD patients with type-2 diabetes mellitus [18].

Based on the patient's risk factors, hypertension had the highest prevalence in both groups (22%) followed by dyslipidemia (11%). Risk factors for hypertension or in complications can cause endothelial damage that triggers endothelial dysfunction, which can also trigger the development of hypertension. Vascular endothelial cells are activated by HMGB-1 lead to the expression and secretion of intercellular adhesion molecule 1 (ICAM-1), granulocyte colony stimulating factor (G-CSF), receptor for advanced glycation end products (AGER), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein (MCP-1), interleukin-8 (IL-8), tissue plasminogen activator (tPA), and plasminogen activator inhibitor 1 (PAI-1) [19].

Extracellular HMGB-1 does not only act as a cytokine, but HMGB-1 also interacts with toll-like Receptors (TLRs) and AGER. Studies show that immunity and inflammatory responses caused by AGE-ligation and AGER interactions can increase cellular reactive oxygen species (ROS) production and persistent activation of transcription factors, nuclear factor kappa-B (NF-kB) [19]. Studies say advanced glycation end products (AGEs) cause oxidative damage to the signaling pathways that lead to hypertension can change with age. Some CRP gene loci have a significant relationship with genetic susceptibility to hypertension. A study supports that AGEs induce oxidative inflammatory reactions contributing to genetic susceptibility to hypertension [18].

Based on the patient's risk factors, hypertension had the highest prevalence in both groups (22%) followed by dyslipidemia (11%). Risk factors for hypertension or in complications can cause endothelial damage that triggers endothelial dysfunction, which can also trigger the development of hypertension. Vascular endothelial cells are activated by HMGB-1 lead to the expression and secretion of ICAM-1, VCAM-1, G-CSF, E-selectin, MCP-1, AGER, TNF-α, IL-8, PAI-1, and tPA. Extracellular HMGB-1 does not only act as a cytokine, but HMGB-1 also interacts with TLRs and AGER. Studies show that immunity and inflammatory responses caused by AGE-ligation and AGER interactions can increase ROS production and persistent activation of transcription factors, nuclear factor kappa-B (NF-κB) [19]. Studies say AGEs cause oxidative damage to the signaling pathways that lead to hypertension can change with age. Some CRP gene loci have a significant relationship with genetic susceptibility to hypertension. A study 1 supports that AGEs induce oxidative inflammatory reactions contributing to genetic susceptibility to hypertension [19]. Some patients who have a history of dyslipidemia suffer from an increase in HMGB-1 levels. Study on male Syrian golden hamsters, high levels of HMGB-1 detected is positively correlated with upregulation of RAGE in lung tissue from hyperlipidemic animals [20]. Hyperlipidemia can stimulate the extracellular release of HMGB-1 if a decrease in hyperlipidemia can reduce the expression of HMGB-1 [18]. Targeted LDL cholesterol should be below 70 mg/dl for patients with CHD [21].

Some patients with a history of active smokers also have elevated levels of HMGB-1. Exposure to cigarette smoke can trigger the recruitment of inflammatory cells, and the production of proinflammatory cytokines increases significantly in bronchoalveolar lavage and lung tissue, and this effect depends on the TLR-4 signaling pathway [22]. Thus HMGB-1 activates the NF-κB and JNK/p38 pathways through TLR-4/MyD88 dependent signaling and induces an inflammatory response in the lungs revealed in cigarette smoke [22]. HMGB-1 is increased in skeletal muscles with exposure to chronic cigarette smoke. HMGB-1 increases muscle cell ceramide levels and impaired mitochondrial function and insulin, effects that are alleviated by ceramide inhibition [23]. Also, inhibition of ceramide partially prevents reduced insulin and glucose tolerance in mice and protects mitochondrial function in skeletal muscles. Overall, these data indicate that HMGB-1 can be a relevant signal mediator in the metabolic response to cigarette smoke exposure [23].

Based on the results of research that has been done after obtaining atorvastatin during hospitalization, the mean serum levels of HMGB-1 groups of patients with type-2 diabetes mellitus have increased from 14.85 ± 5.78 to 1.40 ± 4.81 ng/mL, which statistically showed a significant difference (p = 0.049). And the group of patients CAD without type-2 diabetes mellitus decreased from 16.31 ± 4.93 to 15.26 ± 4.15 ng/mL which did not statistically show a significant difference (p = 0.480).

## Conclusion

After receiving atorvastatin during hospitalization, the mean serum levels of HMGB-1 in the group of patients with CAD with type-2 diabetes mellitus experienced a statistically significant increase (p = 0.049) and the group of patients with CAD without type-2 diabetes mellitus has decreased which statistically does not show a significant difference (p = 0.480). HMGB-1 levels with the administration of atorvastatin did not show a significant difference in the two groups.

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Ira Purbosari, Bambang Zubakti Zulkarnain*, Muh Aminuddin and Umi Fatmawati

# Analysis of matrix metalloproteinase-9 levels among acute heart failure patients with ACE inhibitor therapy (Dr. Soetomo Regional General Hospital, Surabaya)

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**Abstract**

**Objectives:** Heart disease is a clinical condition characterized by specific signs such as joint inflammation, weakness, and shortness of breath. Left ventricular remodeling can be experienced by patients with heart failure wherein a change in myocyte and nonmyocyte components occurs. One of the biomarkers in heart disease with myocardial fibrosis is matrix metalloproteinase-9 (MMP-9). Common therapy that is often given to patients with heart failure is ACE inhibitors. This main objective of this research is to investigate the effect of ACE inhibitor therapy on the degrees of MMP-9 as a biomarker among patients with heart disease.

**Methods:** This research applied one group pretest–posttest design to analyze the variation in the levels of MMP-9 as a biomarker for heart function. Twenty-three subjects with acute heart disease met that inclusion also exclusion criteria, who were selected using nonrandom sampling. Statistical analysis was conducted to specify the levels of MMP-9 before, after the administration of therapy.

**Results:** The most widely used ACE inhibitor drug was ramipril for 15 patients (65%), and the least used ACE Inhibitor drug was captopril for two patients (9%). Meanwhile, the mean MMP-9 levels before therapy was (1,915.26 pg/mL ± 260.84), and the mean MMP-9 levels after therapy was (1,916.93 pg/mL ± 383.12). The statistical analysis result revealed no significant difference in the degrees of Matrix Metalloproteinase-9 accumulation (p=0.378).

**Conclusions:** There was no significant reduction in the levels of Matrix Metalloproteinase-9 after pretest and posttest.

**Keywords:** ACE inhibitor; heart failure; matrix metalloproteinase-9.

## Introduction

Heart disease is a complex clinical condition due to functional or structural damage of the heart regarding blood flow from and to the ventricles. Such disease is characterized by specific symptoms such as shortness of breath, ankle swelling, and weakness [1, 2]. Those symptoms are due to functional or structural heart defects that experience increased pressure or decreased cardiac output. The prevalence of heart failure continues to grow which affects up to 6–10% of patients aged >65 years. Based on Basic Health Research data in 2018, the prevalence of heart disease based on a doctor's diagnosis within the population of all ages in East Java Province was the second largest after West Java Province by 151,878 [3, 4].

Left ventricular remodeling can be experienced by patients with heart failure where in a change in myocyte and nonmyocyte components occurs. Nonmyocyte remodeling occurs due to changes in the extracellular matrix. These changes are characterized by the formation of fibrotic tissue, which may increase the ventricular wall's stiffness, decrease the rate of relaxation and ventricular filling, and affect contractility. An imbalance between the matrix metalloproteinase enzyme, which is required in the degradation process of an enzyme, and its inhibitor in the tissue also has a significant role in fibrosis, which is stimulated by RAAS activation, especially aldosterone. Matrix metalloproteinase (MMP) is more dominant among patients with dilative cardiomyopathy and

***Corresponding author: Bambang Zubakti Zulkarnain**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 8113419355,
E-mail: bambang-s-z@ff.unair.ac.id
**Ira Purbosari,** Department of Pharmacy, Universitas PGRI Adi Buana, Surabaya, Indonesia
**Muh Aminuddin,** Department of Cardiology and Vascular, Faculty of Medicine, Airlangga University, Surabaya, Indonesia
**Umi Fatmawati,** Department of Pharmacy, Universitas PGRI Madiun, Madiun, Indonesia

volume overload. In the long term, systolic–diastolic dysfunction is accompanied by neurohormonal activation and left ventricular remodeling which may lead to worsening heart failure. Drugs that inhibit RAAS activation in the class of ACE inhibitors can be administered to inhibit the progressivity [5–7].

Based on ACC/AHA 2017, the predictive value of myocardial fibrosis biomarkers such as MMP-9 not only predicts hospitalization and mortality regarding heart failure but also additives for the levels of natriuretic peptides. Compared with another biomarker of fibrosis, namely soluble sST2 among patients with chronic heart disease, the predictive contribution of MMP-9 in addition to existing clinical risk factors is lower. Zinc suspended endopeptidases involve Angiotensin Converting Enzyme inhibitor and MMP-9. Both of them stimulate left ventricular remodeling and also the process to convert angiotensin I to form angiotensin II. There is a different affinity binding between the two inhibitors. ACE inhibitors and MMP-9 can be applied to evaluate left ventricular remodeling [1, 8].

Currently, there are no studies that try to analyze changes in MMP-9 levels before and after the administration of ACE inhibitor therapy with patients with acute failure in Indonesia. Based on the background described above, this research aims to investigate the changes in MMP-9 levels before and after the administration of ACE inhibitor therapy among heart disease patients.

MMP-9 is one of the biomarkers for heart failure with myocardial fibrosis. This function is important in the degradation of components of heart disease, atherosclerosis, and myocardial infarction, which is associated with inflammation, diabetic micro vascular complications, and cardiac dysfunction. Therefore, evaluation toward the administration of ACE Inhibitors with acute heart disease can be performed through the analysis of changes in MMP-9 levels [9, 10].

Based on the background, the involvement of clinical pharmacy staff in the heart failure team has a significant effect on therapy reconciliation, education, ensuring management consistency in order to improve patient comfort and therapy adherence, and decreasing medication errors [11]. Therefore, MMP-9 as a biomarker can be clinically useful to determine therapeutics effects and prognosis of heart failure. At inpatient department of Vascular and Cardiology Medicine of Dr. Soetomo Regional General Hospital Surabaya, ACE inhibitor therapy is usually administered to patients with heart failure. However, there has been no evaluation of treatment by using MMP-9 as a marker. Base on this background, there is a need to conduct a study to analyze MMP-9 levels after the administration of ACE inhibitors in hospitalized patients with heart disease.

## Materials and methods

This research must be ensured that it can be carried out based on an ethical clearance provided and has approved by the Research Ethics Committee of Dr. Soetomo Regional General Hospital, Surabaya, based on a decree letter number 1227/KEPK/I/2019. Several kinds of ACE inhibitors were involved in the intervention within the current study, namely captopril, lisinopril, ramipril. MyBioSource Human Matrix Metalloproteinase-9 Elisa kit was used to test the levels of MMP-9. The analytical test was conducted using SPPS 21.0. ACE inhibitor class drugs were administered to the patients while undergoing treatment at inpatient department of Vascular and Cardiology Medicine of Dr. Soetomo Regional General Hospital, Surabaya.

This was a prospective observational study conducted by observing and recording the laboratory data of the patients. The samples were patients with acute heart failure undergoing treatment at inpatient department of Vascular and Cardiology Medicine of Dr. Soetomo Regional General Hospital, Surabaya in May–July 2019 who met the inclusion criteria. Twenty-three subjects were involved in the current study. This research aims to analyze the levels of MMP-9 between before and after the administration of ACE Inhibitors therapy among the study subjects. The inclusion criteria were male or female patients, ≥18 years of age, received ACE inhibitors, subjects with acute heart failure, subjects with NYHA class II–IV heart disease, signed the informed consent form. The exclusion criteria were patients with malignancy and renal failure which is predicted to increase the expression of Matrix Metalloproteinase-9. Drop out criteria were patients who underwent replacement therapy with Ace inhibitors during the follow-up period and patients who died during an observation.

Study subjects were selected through nonrandom sampling method. The probability of the unit to be taken as a sample was not defined. Consecutive sampling was applied for each patient who met the criteria regarding the levels of matrix Metalloproteinase-9 using the Human Matrix Metalloproteinase-9 ELISA kit with the Sandwich-ELISA principle. The study data were processed in the SPSS 21.0 program. Furthermore, statistical analysis was conducted to define the variation of the degrees of MMP-9 between before and after the administration of ACE inhibitor therapy. The data normality test was carried out using the Shapiro–Wilk test.

## Results

This study has been confirmed to be able to conduct based on an ethical clearance provided by the Ethics Committee of Dr. Soetomo Regional General Hospital, Surabaya and further were conducted in May–July 2019. This study engaged 23 patients as the study samples, who were consisted of 8 (39%) female patients and 15 (61%) male patients. Heart disease was the most common etiology of heart failure in this study, which occurred in patients (36%). Patient demographic data to analyzed consisted of gender, age, and risk factors. The results are presented in Tables 1 and 2.

In this study, the ACE inhibitor class therapy drug obtained by patients was divided into three types of drugs,

**Table 1:** Distribution of the gender of patients with heart failure who received ACE inhibitors.

| Gender | Total | Percentage, % |
|---|---|---|
| Male | 15 | 61.0 |
| Female | 8 | 9.0 |

**Table 2:** Distribution of the age of heart failure patients with ACE Inhibitors.

| Age range, years | Total | Percentage, % |
|---|---|---|
| 18–24 | 1 | 4.0 |
| 25–49 | 3 | 13.0 |
| 50–59 | 9 | 39.0 |
| 60–69 | 7 | 31.0 |
| 70–79 | 2 | 9.0 |
| >80 | 1 | 4.0 |
| Total | 23 | 100.0 |

namely ramipril, lisinopril, and captopril. The most widely used ACE inhibitor drug was ramipril for 15 patients (65%), and the least used ACE Inhibitor drug was captopril for two patients (9%). The distribution of the use of ACE inhibitor is presented in Figure 1.

Further finding showed that the mean MMP-9 levels before therapy was (1,915.26 pg/mL ± 260.84) and the mean MMP-9 levels after therapy was (1,916.93 pg/mL ± 383.12). Based on Wilcoxon's statistical analysis, it was found no significant change in the levels of MMP-9 between before and after the administration of ACE inhibitors (p=0.378) with a *Z* count of −0.882. Such finding can be seen in Figure 2. Since there was no significant change in means that, thus there was no worsening or progression of fibrosis in the myocardial infarction.

## Discussion

Based on ACC/AHA 2017, MMP-9 is a kind of myocardial fibrosis biomarkers which can predict hospitalization and

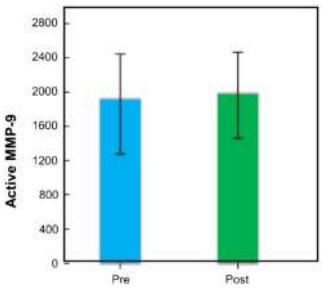


**Figure 2:** Comparison of the levels of matrix metalloproteinase-9 (MMP-9) between.

mortality among patients who experience heart disease. In relation to this, the higher the levels of MMP-9 levels, the higher risk of heart failure and the worse disease severity and prognosis of patients with heart failure [1, 8].

Twenty-three patients with NYHA FC II–IV class acute heart failure were declared to meet the inclusion and also the exclusion criteria. This is indicated in the tables which present the demographic data, therapeutic diagnosis obtained, and related laboratory data of all 23 patients. Based on the demographic data of 23 patients with heart failure, 61% of them were men, while the remaining 39% were women. This finding is in accordance with the previous study which revealed that the incidence of heart failure was more common in men than women. This is due to estrogen hormone which influences the general condition of women. There is a commonly accepted evidence that Estrogen plays an essential role for women. Before menopause, it can prevent the activation of RAAS. But, after a woman experiences menopause, ovarian estrogen will be lost, resulting in the pathogenesis of diastolic dysfunction, namely an increase in the levels of Angiotensin II and ROS (Reactive Oxygen Species and NOS).

It has been a previous evidence that heart failure prevalence increased with age. In the current study, the youngest age was 23 years, and the oldest age was 90 years. According to the age category, most of patients were involved in the age category of 50–59 years namely nine patients (39%). Meanwhile, only one patient (4%) was

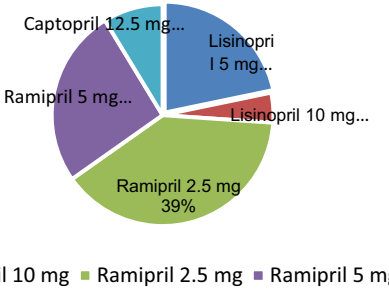


**Figure 1:** ACE inhibitors usage and dosage profile.

involved in the age category of >80 years. The study finding supported the previous study that the number of heart failure developed with age [12, 13].

The second cause of heart failure found in this study was diabetes mellitus by 27%. An increase in the levels of Advanced Glycosylation End Products (AGE products) and Reactive Oxygen Species in the endothelium of blood vessels can be due to an increase in the levels of plasma glucose, which in turn may cause endothelial damage. Further, such damage may cause blood vessels inflammation accompanied by nitric oxide synthesis inhibition and vasoconstriction hypertension will occur. Vasoconstriction hypertension is the third-leading cause of heart failure in this study, which was experienced by seven patients (23%). Chronic hypertension can cause stretching forces that can damage the endothelial lining of the arteries and arterioles. This stretching force may occur at sites which contains artery branches or bends and is typical for the coronary arteries, aorta, and cerebral arteries. Repeated damage of the endothelial lining may lead to a cycle of inflammation, accumulation of white blood cells and platelets, and clot formation. Any thrombus formed can escape from the artery and form an embolus in downstream [13–17].

In this study, the ACE inhibitor class therapy drug obtained by patients at inpatient department of Vascular and Cardiology Medicine of Dr. Soetomo Regional General Hospital Surabaya consisted of three types, namely ramipril, lisinopril, and captopril [1, 18–20]. The most widely used ACE inhibitor drug was ramipril for 15 patients (65%), and the least used ACE Inhibitor drug was captopril for two patients (9%). Based on the clinician decision, each patient received different type and dose of ACE therapy. In the current study, the available doses were ramipril 2.5 and 5 mg. The dose was adjusted according to the patient's condition. The difference in the dose of ramipril was based on the patient's blood pressure. Patients with lower blood pressure received a small initial dose of ramipril. The dose of ramipril administered to patients with heart failure was 1.25–2.5 mg, once a day. This could increase patient compliance in following the therapy prescription so that the outcome of treatment was achieved and more optimal. Lisinopril was also often used by patients with heart disease treated at Dr. Soetomo Regional General Hospital due to patient compliance factor. Administration of high doses of lisinopril has been known to improve the NYHA grade of heart failure due to hemodynamic improvement. In the ATLAS study, the administration of high dose of lisinopril was found to lower the risk of life-threatening events among heart failure patients. Meanwhile, captopril was rarely used in this study. The most excellent bioavailability of captopril is 60–70%, this is the reason to choose such drug. However, the half-life of captopril is quite short of two and a half hours and its duration is only 6–10 h, so it should be administered three times a day. The captopril dose used in this study was 12.5 mg. There are three kind of captopril dosages circulate in the market namely 25, 12.5 and 6.25 mg. The captopril dosage is adjusted to the patient's condition [8, 21–23].

There are several disease pathophysiology that can lead to heart failure. Thus, biomarkers in heart failure are classified according to the associated pathophysiological processes [6, 22]. MMP-9, while 15 patients experienced an increase in the ranks of MMP-9. The range of the levels of MMP-9 before therapy was 1,289–2,428 pg/mL; and the range of the levels of MMP-9 after therapy was 628.41–2,445 pg/mL. The mean MMP-9 levels before therapy (before the administration of ACE inhibitors) was 1,916.26 pg/mL ± 260.84, and the mean MMP-9 levels after therapy (after the administration of ACE inhibitors) was 1,916.93 pg/mL ± 383.

## Conclusions

Based on the results of the current study, it can be concluded that there was no significant change in the degrees of MMP-9 between before, after the administration of ACE inhibitors among 23 patients with NYHA FC II–IV class acute heart failure. This finding indicated that there was no worsening or progression of fibrosis in the myocardial infarction. Clinically, the patients involved in this study discharged with improved clinical conditions, characterized by vital signs within normal limits, no complaints of chest pain, no crackles, and no edema in the extremities. Limitation of the current study that can affect the results is that this study could not control confounding factors such as patient's age, weight, gender, and choice of Ace inhibitors type. Therefore, further study is expected to minimize the confounding factor in order to obtained more homogeneous samples.

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**Competing interests**: Authors state no conflict of interest.

**Informed consent**: Informed consent was obtained from all individuals included in this study.

**Ethical approval**: This study was designed to comply the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, with reference number No.1227/KEPK/V/2019.

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Elida Zairina*, Gesnita Nugraheni, Gusti Noorrizka Veronika Achmad, Arie Sulistyarini, Yunita Nita, Arief Bakhtiar and Muhammad Amin

# The correlation between self-related adherence, asthma-related quality of life and control of asthma in adult patients

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**Abstract**

**Objectives:** Medication non-adherence mostly occurs in patients with a wide range of disease severity, including asthma. The aim of the study was to assess the self reported adherence to asthma therapy and investigate the relationship between adherence, asthma control and asthma-related quality of life.

**Methods:** The study was a cross-sectional study in which participants were recruited from an outpatient department, in one hospital in Surabaya. Patients (aged≥18 years) with asthma who had used any regular asthma medications were included. Standardised questionnaires, including Juniper's Asthma Control Questionnaire (ACQ), Adherence to Refills and Medications Scales (ARMS) and Juniper's Asthma Quality of Life Questionnaire (AQLQ) were used.

**Results:** A total of 82 adults with asthma were recruited in the study. Male participants' mean age was 49.13 ± 14.10 years (n = 23). Approximately 59 participants (72.0%) were females, 30 participants (36.5%) were using Budesonide inhaler, and 73 participants (89.0%) never smoked. The mean of ACQ, AQLQ, and ARMS scores were 1.62 ± 1.19, 4.96 ± 1.24, and 16.98 ± 4.12, respectively. Of 82 patients studied 53 (64.6 %) had "uncontrolled asthma" and more than 85% participants both showed "non adherence" to asthma therapy and nearly 46% of them indicated that their quality of life was affected by asthma. There was a significant association between ACQ and AQLQ ($p<0.05$), whereas no statistically significant association was found between ACQ and ARMS.

**Conclusions:** The majority of patients reported non-adherence to asthma medications. Poor controlled asthma has been associated with lower asthma-related quality of life.

**Keywords:** adherence; asthma; asthma control; quality of life.

## Introduction

Asthma is defined as a chronic inflammatory disorder characterised by symptoms such as recurrent wheezing episodes, breathlessness, chest tightness, dyspnoea and coughing, especially at night or in the early morning [1, 2]. Asthma is one of the most severe public health problems among adolescents and children worldwide. According to World Health Organization (WHO) and Global Initiative for Asthma (GINA) asthma affects as many as 300 million people of all ages and ethnic backgrounds and it is estimated that the number is increased by 2025 [3]. The prevalence of Indonesian adolescents with asthma is poorly known. However, although the number may not be as large as other countries globally, asthma remains one of the public health problems affecting both adults and children in Indonesia.

When asthma is uncontrolled, it puts severe limitations on daily living and sometimes could even be fatal. Asthma affects people in specific ways, as the impact could impair in physical activity and educational lives. Asthma could also have an impact on social aspects, including the contribution to school absenteeism, loss of productivity and the limitation of participation in family and social life [3, 4].

The assessment of asthma based on morbidity and mortality outcomes is currently insufficient. Therefore, outcomes

***Corresponding author: Elida Zairina**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; Innovative Pharmacy Practice and Integrated Outcomes Research (INACORE) Group, Universitas Airlangga, Surabaya, Indonesia; and Center for Patient Safety Research, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 031 5933150, E-mail: elida-z@ff.unair.ac.id. https://orcid.org/0000-0003-0845-4640

**Gesnita Nugraheni, Gusti Noorrizka Veronika Achmad, Arie Sulistyarini and Yunita Nita,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Innovative Pharmacy Practice and Integrated Outcomes Research (INACORE) Group, Universitas Airlangga, Surabaya, Indonesia. https://orcid.org/0000-0002-8791-8556 (G. Nugraheni). https://orcid.org/0000-0003-2310-5211 (G.N.V. Achmad)

**Arief Bakhtiar and Muhammad Amin,** Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; and Department of Pulmonology, Universitas Airlangga Hospital, Surabaya, Indonesia

assessment based on patient-oriented assessment has been widely recognised in the last decades. Compliance in medication, asthma control and quality of life (QoL) have emerged as vital outcomes for improving asthma management and communication tools between patients and their healthcare professionals [5–8].

The aim of asthma management is to achieve and maintain adequate asthma control, which can be assessed by measuring asthma symptom severity, the frequency of asthma-related exacerbations and QoL-related asthma [3]. Adherence to medications is one of the main factors in the successful management of asthma [9]. The World Health Organization–Quality of Life Group (WHOQOL) has described as an "individuals" perception of their role in life in the context of the culture and values systems in which they live and of their goals, expectations, standards and concerns [5]. Therefore, QoL is additional outcomes in assessing and measuring health clinical outcomes and interventions, particularly in patients with asthma [10–12].

The study aimed to evaluate self-reported adherence to asthma therapy and to examine the association between adherence, asthma control and asthma-related QoL. This study also aimed to describe the characteristics of patients with poor asthma control and poor adherence based on socio-demographic factors.

## Materials and methods

The study was designed as a cross-sectional study with the study period was conducted from August 2017 to July 2019. The hospital's ethics committee approved the design and procedures of the study. Participants were recruited from the outpatient pulmonology department at a university-affiliated hospital in Surabaya, Indonesia. This study's eligibility requires asthma patients who have used any daily asthma medications in the previous 12 months, who were 18 years and older, and who speak Indonesian. Patients with chronic respiratory diseases other than asthma such as bronchiectasis or chronic bronchitis, emphysema or if they were unable or contraindicated to spirometer were excluded. Patients signed a written informed consent upon participated in the study.

The self-administered questionnaires contained questions about socio-economic and demography characteristics (i.e., age, gender, education and employment). Additional questions were asked about the history of asthma, smoking status, medicine used, and type of inhaler and comorbid conditions. Asthma control has been measured using the Juniper Asthma Control Questionnaire (ACQ) [13]. The ACQ consists of six questions about asthma symptoms and one question about lung function ($FEV_1$), with ACQ score 1.5 or higher is graded as "not-well controlled" [14]. The Juniper's Asthma Quality of Life Questionnaire (AQLQ) score [15] was used to measure the quality of life. The AQLQ is a 32-item disease-specific questionnaire designed to assess the functional impairments that are most troubling for adults with asthma [16]. Participants were asked to recall their experiences over the past two weeks and to rate each question on a 7-point scale (7 = not impaired at all, 1 = severely impaired), which the overall score of AQLQ (scores range 1–7) is the mean answer to all 32 questions with higher scores suggesting better quality of life [16]. The adherence to asthma treatment was assessed as self-reported using the Adherence to Refills and Medications Scales (ARMS) questionnaire [17]. ARMS consists of 12 questions designed for the response on a Likert Scale answer with the response of "none", "some", "most" or "all" of the time, which were given values from 1 to 4, with higher scores indicating higher "non-adherence" [17]. Lung function (pre-bronchodilator $FEV_1$) was tested using a computerised spirometer (Jaeger, v.4.31, Germany) by a trained respiratory nurse at the Department of Pulmonology.

Data were analysed using the Statistical Package for the Social Sciences, version SPSS version 22.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Categorical variables were presented as frequency and proportion. Means, standard deviations and frequencies are presented to explain the characteristics of the study sample. Descriptive statistics have been used to summarise the results. We used the chi-square test to determine the statistical significance between the categorical variables and to compare the means of the quantitative data used in the Student's t-test. We conducted stepwise multiple regression and path analysis to test the simultaneous and causal relationship between study variables. Logistic regression approaches with forwarding step-by-step conditional methods have also been performed. The odds ratio (OR) from this analysis is the OR for getting low QoL affected by asthma. Selected non-collinear variables with a $p<0.10$ have been introduced in the binary logistic regression. The significance level was set at $p<0.05$, with all probabilities reported were two-tailed.

## Results

The results of this study are based on baseline data of previous RCT study to evaluate the effectiveness of education management by the pharmacist in adults with asthma. In two years of study, 110 eligible subjects were screened in the study. Ten patients declined to participate, and 18 patients were excluded because they had another chronic pulmonary disease. Therefore, 82 adults with asthma were included in this study. Table 1 explains the characteristics of participants, including demographic and socioeconomic profile. Of the 82 enrolled participants enrolled, 72% (n = 59) were female. Mean age was 49.13 ± 14.10 for male and 52.58 ± 11.57 for female. As more participants were female, nearly 50% were housewives. The majority of the participants had basic or secondary school education, 64.6% (n = 53), and nearly 70% (n = 57) belonged to monthly gross household income less than three million rupiahs.

### Clinical characteristics of the subjects

About 73 (89.0%) patients were never smoked, and most of them had been diagnosed with asthma for more than five years. These data are included in Table 2, which indicates

**Table 1:** Demographic and socioeconomic characteristics of study participants (n = 82).

| Items | Categories | n, % |
|---|---|---|
| Gender | Male | 23 (28.0) |
| | Female | 59 (72.0) |
| Age, mean ± SD | Male | 49.13 ± 14.10 |
| | Female | 52.58 ± 11.57 |
| Height in cm, | Male | 166.89 ± 4.58 |
| mean ± SD | Female | 151.95 ± 11.57 |
| Weight in kg, | Male | 69.89 ± 12.66 |
| mean ± SD | Female | 59.73 ± 14.51 |
| Occupation | Public employee | 3 (3.7) |
| | Private | 26 (31.6) |
| | Self-employed | 9 (11.0) |
| | Housewives | 40 (48.8) |
| | Others | 4 (4.9) |
| Education | Basic or secondary school | 53 (64.6) |
| | Diploma | 6 (7.3) |
| | Higher education | 23 (28.1) |
| BPJS[a] membership | Yes | 81 (98.8) |
| Income | <IDR[b] 3,000,000 | 57 (69.5) |
| | IDR 3,000,000– | 20 (24.4) |
| | 6,000,000 | 2 (2.4) |
| | IDR 6,000,000– | 3 (3.7) |
| | 12,000,000 | |
| | Do not want to disclose | |

[a]BPJS, badan penyelenggara jaminan sosial; [b]Indonesian rupiah.

the clinical characteristics of the study. Regarding the level of asthma control, only 35.4% (n = 29) of the participants exhibit well-controlled asthma while the rest showed poorly controlled asthma. More than half of the participants (n = 45) experienced that their QoL was affected by asthma. At the same time, the medication adherence score showed low adherence in all participants (n = 82). Budesonide – Formoterol was asthma medication used by most of the patients. The chi-square analysis showed no significant difference in the control of asthma and adherence to medications was found based on the socio-demographic factors, including gender, educational level and age.

## The relationship between medication adherence, asthma control and asthma-related quality of life

We looked at the potential independent variables (overall ACQ and ARMS scores) and the dependent variable (overall AQLQ score) to analyse the variables' relationship. There was a direct association between AQLQ and ACQ scores, although no correlation was found between ARMS and AQLQ scores and between ARMS and ACQ scores. A linear

**Table 2:** Clinical characteristics of study participants (n=82).

| Items | Categories | n, % |
|---|---|---|
| Asthma medication used | Budesonide – Formoterol | 30 (36.5) |
| | Salmeterol – Fluticasone | 8 (9.7) |
| | Fenoterol | 3 (3.6) |
| | Budesonide – Formoterol & Salbutamol | 10 (12.1) |
| | Budesonide – Formoterol & Fenoterol | 14 (17.0) |
| | Budesonide – Formoterol, Tiotropium | 1 (1.2) |
| | Budesonide – Formoterol, Fenoterol, Tiotropium | 5 (6.0) |
| | Salmeterol – Fluticasone & Salbutamol | 8 (9.7) |
| | Others | 3 (3.6) |
| Asthma medication dosage form | MDI[e] | 3 (3.6) |
| | Turbuhaler | 30 (36.5) |
| | Turbuhaler & MDI | 24 (29.2) |
| | Turbuhaler & Handihaler | 1 (1.2) |
| | Turbuhaler, MDI, Handihaler | 5 (6.0) |
| | Accuhaler | 8 (9.7) |
| | Accuhaler & MDI | 8 (9.7) |
| | Others | 3 (3.6) |
| Changes in asthma medication in the last month | No | 55 (93.2) |
| Last visit to GPs/Primary care centres/Emergency Department | One week ago | 17 (20.7) |
| | Two weeks ago | 7 (8.5) |
| | Three weeks ago | 4 (4.8) |
| | One month ago | 54 (65.8) |
| Reasons for visiting primary care centres/Emergency Department | Regular visit for asthma control | 45 (54.8) |
| | Asthma episode | 15 (18.2) |
| | Other medical conditions | 22 (26.8) |

**Table 2:** (continued)

| Items | Categories | n, % |
|---|---|---|
| Last experience of asthma symptoms/period (breathless) | Never | 22 (26.8) |
| | One month ago | 7 (8.5) |
| | Two weeks ago | 3 (3.6) |
| | One week ago | 13 (15.8) |
| | Less than a week | 37 (45.1) |
| Smoking | Never | 73(89.0) |
| | Yes | 9 (11.0) |
| Level of asthma control | Well-controlled | 29 (35.4) |
| | Poor-controlled | 53 (64.6) |
| Level of adherence | High adherence | 0 (0.0) |
| | Low adherence | 82 (100.0) |
| QoL[a] related to asthma | QoL affected | 37 (45.1) |
| | QoL unaffected | 42 (51.2) |
| Comorbidities | Yes | 23 (30.7) |
| Other chronic diseases | Diabetes | 12 (14.6) |
| | Hypertension | 7 (8.5) |
| | Diabetes & Cardiovascular | 2 (2.4) |
| | Diabetes & Hypertension | 2 (2.4) |
| | None | 59 (71.9) |
| [b]$FEV_1$ (mL/s) – pre bronchodilator, mean ± SD | 1.52 ± 0.66 | |
| ACQ[c] score, mean ± SD | 1.62 ± 1.19 | |
| ARMS[d] score, mean ± SD | 16.98 ± 4.12 | |

[a]QoL, quality of life; [b]$FEV_1$, forced expiratory volume in 1 s; [c]ACQ, asthma control questionnaire; [d]ARMS, adherence to refill medication scale; [e]MDI, metered dose inhaler.

regression confirm that the AQLQ scores of adults with asthma was found to be significantly inversely correlated with their asthma control scores, r (n = 82) =−0.749, p<0.05, 95% CI [−0.978 to −0.596], AQLQ scores = 6.094−0.787 (ACQ scores). Logistic regression analysis identified that the model predicts the odds of having QoL affected with asthma is 0.075 lower (p<0.001) for those with well-controlled than poorly controlled asthma.

## Discussion

This cross-sectional study showed low medication adherence to in 82 patients with asthma (100.0%) who attended a large tertiary outpatient department at a university-affiliated hospital in Surabaya. While there was no statistically significant association between adherence to medications and asthma control in this study, most patients had poorly controlled asthma. This can be clarified as the consequence of having low adherence is affecting their asthma control. However, further study is required to confirm this association in a bigger population size. However, while age and income were considered as to be predictors for control of asthma, the demographic characteristics of participants such as level of education, gender and age were not significantly correlated with poorly controlled asthma. These findings were similar to those of the Latvian asthma population [8]. The Latvian study confirms that instead of the demographic characteristics of patients, patients' concerns about the side effects of medications were more significant to the level of asthma control and the adherence to medications [8].

In regards to the association between asthma control and asthma QoL, our study's findings are supported by other international studies in Brazilian and Latvian asthma patients [6, 7]. Our study had comparable findings with the research performed by Amaral et al. [6] in Brazil, which showed a significant correlation between asthma control and asthma-related QoL which was in accordance with other international studies [18]. QoL can be affected by multiple factors in asthma patients, which tend to play a crucial role in optimising asthma self-management in patients [19, 20].

There are several self-reported scales for determining adherence to medications in chronic diseases; however, they depend on the nature of the disease; there is no gold-standard scale for evaluating adherence to medicines used [21]. Measurement of adherence using ARMS has advantages in easy to use, is valid and reliable as a method to assess adherence in chronic disease populations and is suitable for patients with low literacy [22, 23]. As a result, we recommend

the use of ARMS in Indonesian asthma patients as a tool to assess adherence to medications.

Our study had several limitations that need to be considered. This study used self-reported scales for measuring adherence, asthma control and asthma-related QoL, although these scales were validated in previous studies, the findings might not be precise and still subject to recall bias or self-presentational that could overestimate the scores. A combination of clinically objective measurement for adherence, such as biomarker and spirometer lung function for asthma control could be more reliable and objective [24–25]. The small sample size could explain the insignificant correlation between adherence and asthma control and adherence to QoL-related asthma in our study. Therefore, we suggest that the same techniques be applied to a more significant population. Moreover, future research directions are warranted to validate different asthma adherence scales, asthma control scores and other related self-assessment tools in a larger sample of Indonesian with asthma. This is to improve their efficiency and optimise their asthma self-management.

## Conclusions

From this study, it can be concluded that poor asthma controlled was associated with lower asthma-related quality of life. Moreover, the majority of adults with asthma reported non-adherence to asthma medication in this study. Further study is needed to determine the effective method for improving adherence and optimising asthma management in adults with asthma.

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Rahmiyati Daud, Bambang Subakti Zulkarnain* and Ivan Virnanda Amu

# Providing counseling through home pharmacy care (HPC) for hemodialysis patients with hypertension in lowering blood pressure

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**Abstract**

**Objectives:** Hypertension is one of the main factors in increasing the risk of cardiovascular disease with 51% reported cause of death in chronic kidney disease (CKD) patients with end-stage renal disease (ESRD). It is a comorbid that needs to be managed properly and gets special attention from various health disciplines including a pharmacist.

**Methods:** This was a quasi experimental study with pretest–posttest intervention using home pharmacy care (HPC) counseling both on the counseling and the noncounseling group. Initial data collection and informed consent was done at the Hemodialysis Unit Aloei Saboe and Toto Kabila Hospital, Gorontalo. The parameters in the study were patients' compliance to their medication using the Medication Adherence Questionnaire (MAQ) and Pill Count Adherence (PCA) questionnaires and the patient's blood pressure.

**Results:** Fifty-eight patients met the inclusion criteria and were divided into two groups (the counseling group and the noncounseling group). Based on MAQ and PCA, the level of patient medication adherence increased significantly in the counseling group compared to the noncounseling group with a significance value of $p<0.05$. Increasing adherence was correlated with patients' outcome of lowering blood pressure. More patients in the counseling group showed decrease in systolic and diastolic blood pressure compared to the noncounseling group (86.2 vs. 17.2% for systolic BP and 69 vs. 10.3% for diastolic blood pressure (BP). Following adjusted confounding variables, counseling through HPC provided a chance of decreasing systolic blood pressure 32 times (95% CI: 7.198–144.550) and diastolic blood pressure 42 times (95% CI: 6.204–286.677).

**Conclusions:** HPC affects the improvement of patient medication adherence and reduction of blood pressure in hemodialysis patients with hypertension.

**Keywords:** blood pressure; hemodialysis patients with hypertension; home pharmacy care.

## Introduction

Home Pharmacy Care (HPC) is a concept of home pharmacy services to provide an understanding of treatment and ensure that patients who are already at home can use medicines properly [1, 2]. This kind of service cannot be applied to all patients considering the long and continuous service time [2]. However, this type of service is essential for some patients, especially for elderly patients receiving poly pharmaceutical therapy and patients with chronic diseases who require long-term therapy.

Chronic Kidney Disease (CKD) is a chronic disease that requires more attention from health workers, including pharmacist [3]. CKD patients who are at end-stage renal disease (ESRD) (GFR<15 mL/min/1.73 $m^2$) require renal replacement therapy, one of which is hemodialysis therapy [4].

Based on data obtained in 2017, the number of ESRD patients undergoing hemodialysis with comorbidities in the form of hypertension was 36% [5]. Hypertension and kidney failure can trigger each other. When kidney function is impaired, blood pressure will increase and cause hypertension. Hypertension also has a significant influence on other complications such as cardiovascular. Therefore, complications of hypertension in patients with kidney failure can be a significant factor causing cardiovascular disease. Currently, cardiovascular is reported as the leading cause of death by 51% [6]. This complication is often underdiagnosed and poorly treated. Therefore, this patient group should be recognized as having a high

***Corresponding author: Bambang Subakti Zulkarnain**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 811 3419355, E-mail: bambang-s-z@ff.unair.ac.id
**Rahmiyati Daud,** Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Ivan Virnanda Amu,** Department of Medicine, Faculty of Medicine, Gorontalo State University, Gorontalo, Indonesia

cardiovascular risk requiring special medical attention at the individual level [7].

Thus, pharmacists should be involved in managing blood pressure in hemodialysis patients by providing counseling through HPC related to pharmacological and nonpharmacological therapies because HPC is a basic promotive and preventive health service by a pharmacist in carrying out pharmaceutical practices. Pharmacists can improve patient therapy outcomes by optimizing the use of appropriate drugs through this service. Several studies related to HPC have been conducted before, including by Jeffrey et al. who concluded that HPC plays a role in resolving patient drug-related problems such as medication adherence and lack of information obtained by patients regarding medication [8]. Several years earlier in Indonesia, Utaminingrum had also conducted research related to providing home care to hypertensive patients to conclude that medication adherence in the group receiving pharmacist HPC had increased compared to the control group [9]. HPC is expected to increase the patient's adherence to therapy to achieve optimal therapeutic effects.

## Materials and methods

This quasi experimental study applied a pretest–posttest design on both the counseling and the noncounseling group. Measurement of blood pressure was carried out before and after HPC. Patient medication adherence was analyzed using the Medication Adherence Questionnaire (MAQ) and calculation of the number of drugs was through the Pill Count Adherence (PCA) method. MAQ consists of four questions that have been previously validated, covering the following aspects: forgetting to take medication, being careless in taking medication, stopping taking medication when they feel better, and stop taking medication when they feel worse. These four aspects were answered with a "yes" or "no." Each "yes" answer has a value of 1, and a "no" answer has a value of 0. The total score of 0 was included in the category of "High" level of adherence, 1–2 is in the "Moderate" category, and a score of 3–4 belongs to the "Low" category [10]. Meanwhile, the measurement of adherence through PCA was carried out by counting the number of medicine consumed by the patient, comparing it with the amount of medicine the patient should have taken according to the doctor's recommendation, then multiplying it by 100%. Patients are categorized as "Adherent" if the score is 80–100% and as "Non Adherent" if the score is less than 80% [11]. Researchers use the MAQ questionnaire in Indonesian which has been validated through testing on 20 research subjects and then analyzed using SPSS. PCA measurement in both groups was carried out at week 1 (pretest value) and week 6 (posttest value) because each group in those weeks (weeks 1 and 6) received daily pharmacist visits for seven days to measure blood pressure. On the first day of the visit (week 1), the pharmacist received information on the amount of medicine the patient had received from the hospital, and on the following days (for 7 days), the pharmacist could find out the amount of medicine left or the amount of medicine the patient had taken. From this information, the pretest value can be calculated. The same activities were also carried out at week 6.

This study was conducted on outpatients' clinic in the hemodialysis unit at Aloei Saboe Hospital and Toto Kabila Hospital during January–March 2020. The initial patient data collection was conducted in the Hemodialysis Unit followed by the provision of informed consent. The inclusion criteria of this study were as follows: 1) aged 19 years and over, 2) undergoing routine hemodialysis, 3) suffering from hypertension, 4) taking hypertension medicines, and 5) willing to take part in the study. Patients who met the inclusion criteria have then explained the study and obtained an informed consent sheet as a written form that the patient was willing to have a home visit by the pharmacist. Researchers divided patients into counseling and noncounseling groups based on hemodialysis schedules. This division is to minimize communication between groups. Patients included in the exclusion criteria were those who refused to be a subject in the study, while patients included in the dropout criteria were those who: 1) admitted as inpatients during the study; 2) died during the study.

All data obtained were processed using service solutions software (SPSS) version 21 with the dependent variable of HPC counseling and independent variables consisting of gender, age, education level, smoking habits, history of hemodialysis, frequency of hemodialysis, etiology, and comorbidities.

### Home pharmacy care (HPC)

At week 1 (pretest), the patient had daily home visits (7 days) for blood pressure measurements and adherence measurements on the last day. Furthermore, counseling through HPC was carried out for four visits with one-week interval visits. HPC was started at weeks 2, 3, 4, and 5. At week 6 (posttest), the patient had another daily visit to measure blood pressure and medication adherence. In every HPC visit, pharmacists always include leaflets as a medium to make it easier for patients and/or their families to understand counseling materials. Pharmacists also delivered different counseling materials each week. Broadly speaking, these materials consist of an introduction to high blood pressure and the importance of adherence to controlling blood pressure (HPC week 2), the importance of taking regular medication (HPC week 3), prevention and control of blood pressure through food and drink (HPC week 4), and strengthening and follow-up counseling at week 5. At each meeting, pharmacists always allowed patients to ask questions regarding any problems or complaints that patients often face regarding their drug use.

## Results

During the specified period, there were 84 active hemodialysis patients. However, some patients could not receive home visits because their homes were located outside the district. Meanwhile, other patients were unwilling to accept home visits, so they did not meet the inclusion criteria. Besides, several patients were included in the dropout criteria during the study. Thus, the total number of patients from the beginning until the end of the study was 58.

## Research sample characteristics

The results of the homogeneity test analysis on the data showed that the demographic and clinical characteristics of patients in each group were homogeneously distributed ($p>0.05$) (Table 1). Male gender characteristics exceeded half of the total sample in the study (72.4%), where the majority of the samples were in the age range of 39–59 years old (52.7%). Most patients (56.9%) were at the advanced education level based on education level (high school graduates or higher). Meanwhile, 98.3% of patients did not smoke/had stopped smoking based on smoking habits' demographic characteristics. There are only 1.7% of patients who still smoke. All patients in each group used Indonesian National Health Insurance.

In this study, more than half of the hemodialysis patients with hypertension had undergone hemodialysis therapy for more than six months with a constant hemodialysis frequency of 2 times/week. The underlying medical history of ESRD in these patients was hypertension (44.8%), diabetes mellitus, gout, and other diseases. Based on the comorbidity characteristics, some patients had more than one comorbid disease, where most comorbidities were diabetes mellitus by 31%. In this study, most patients in each

**Table 1:** Patient characteristics.

| Characteristic | Counseling (n = 29) | Noncounseling (n = 29) | Total | p-Value |
|---|---|---|---|---|
| Gender | | | | 0.557 |
| Male | 22 (75.9%) | 20 (69%) | 42 (72.4%) | |
| Female | 7 (24.1%) | 9 (31%) | 16 (27.6%) | |
| Age | | | | 0.141 |
| ≤19–39 years | 0 | 2 (6.9%) | 2 (3.4%) | |
| >39–59 years | 13 (44.8%) | 17 (58.6%) | 30 (52.7%) | |
| >59 years | 16 (55.2%) | 10 (34.5%) | 26 (44.8%) | |
| Level of education | | | | 0.426 |
| Basic | 14 (48.3%) | 11 (37.9%) | 25 (43.1%) | |
| Advanced | 15 (51.7%) | 18 (62.1%) | 33 (56.9%) | |
| Smoking habit | | | | 0.313 |
| Smoke | 1 (3.4%) | 0 | 1 (1.7%) | |
| Do not smoke | 28 (96.6%) | 29 (100%) | 57 (98.3%) | |
| Type of financing | | | | |
| BPJS | 29 (100%) | 29 (100%) | 58 (100%) | – |
| Length of undergone hemodialysis | | | | 0.788 |
| ≤6 months | 11 (37.9%) | 12 (41.4%) | 23 (39.7%) | |
| >6 months | 18 (62.1%) | 17 (58.6%) | 35 (60.3%) | |
| Frequency of hemodialysis | | | | – |
| 2 times a week | 29 (100%) | 29 (100%) | 58 (100%) | |
| Etiology | | | | 0.083 |
| Hypertension | 14 (48.3%) | 12 (41.8%) | 26 (44.8%) | |
| Diabetes mellitus | 4 (23.8%) | 8 (27.6%) | 12 (20.7%) | |
| Gout | 8 (27.6%) | 2 (6.9%) | 10 (17.2%) | |
| Others | 3 (10.3%) | 7 (24.2%) | 10 (17.2%) | |
| Comorbidities[a] | | | | |
| Diabetes mellitus | 8 (27.6%) | 10 (34.5%) | 18 (31%) | 0.570 |
| Cardiovascular | 6 (20.7%) | 8 (27.6%) | 14 (24.1%) | 0.539 |
| Hypercholesterol | 4 (13.8%) | 4 (13.8%) | 8 (13.8%) | 1.000 |
| Others | 10 (34.5%) | 7 (24.1%) | 17 (29.3%) | 0.387 |
| Total of hypertension drugs | | | | |
| One drug | 21 (72.4%) | 17 (58.6%) | 38 (65.5%) | |
| Two drugs | 8 (27.6%) | 11 (37.9%) | 19 (32.9%) | |
| Three drugs | – | 1 (3.4%) | 1 (1.7%) | |
| Types of hypertension drugs[b] | | | | |
| ARB | 14 (48.3%) | 19 (65.5%) | 33 (56.9%) | |
| CCB | 22 (75.9%) | 19 (65.5%) | 41 (70.7%) | |
| Diuretic Loop | 1 (3.4%) | 4 (13.8%) | 5 (8.6%) | |

[a]Patient can have more than one comorbidities; [b]Patients can use more than one type of antihypertension drugs.

group consumed a single antihypertensive drug. The CCB or calcium-channel blocker in the form of amlodipine was the most common type of antihypertensive drug used by hemodialysis patients with hypertension, amounting to 70.7%.

## Adherence to drug treatment

The patient therapy adherence level was measured using the MAQ and PCA questionnaires, as shown in Table 2. There was no difference baseline of adherence between groups with a significant value shown p>0.05 either by the MAQ or PCA methods. The pretest adherence measurement results showed that most of the patients in each group were at the adherence level of the "Moderate" category (72.4% counseling group and 65.5% noncounseling group). After measurement at the posttest, patient adherence on the counseling group increased to the "High" category (65.5%). The same thing did not happen in the noncounseling group (p<0.05). In the counseling group, patient adherence in the "high" category increased from 17.2 to 65.5%, while in the "moderate" category, it decreased. In the "Low" category, the level of patient adherence decreased to 0%. Thus, it can be assumed that the decrease in the number of patients in the "Moderate" and "Low" categories was due to increased adherence after counseling (change to a better category). Different results occurred in the measurement in the noncounseling group. Patient adherence decreased from 20.7 to 10.3% in the "High" category and increased in the "Moderate" and "Low" categories. Data in this noncounseling group may suggest that this group may not understand the purpose of antihypertensive drugs therapy (Table 2).

Measurement of adherence using PCA based on the number of medicines in the pretest–posttest showed an increase of 27.6% in the counseling group. Meanwhile, in the noncounseling group, there was a decrease in the level of adherence by 10.3%. The significance of this test result indicated a correlation between the provision of counseling through HPC and an increase in patient adherence based on the PCA method (p<0.05) (Table 3).

**Table 3:** Proportion of medication adherence based on PCA.

| Treatment group | | Medication adherence based on PCA | | p-Value |
|---|---|---|---|---|
| | | Adherent | Non adherent | |
| Counseling | Pretest | 16 (55.2%) | 13 (44.8%) | 0.421 |
| | Posttest | 24 (82.8%) | 5 (17.2%) | 0.023 |
| Noncounseling | Pretest | 19 (65.5%) | 10 (34.5%) | 0.421 |
| | Posttest | 16 (55.2%) | 13 (44.8%) | 0.023 |

## Average blood pressure

The patients' systolic and diastolic blood pressure values, based on the average measurement results of 2 times in the morning and two times in the afternoon for seven consecutive days at home visits week 1 (pretest) and week 6 (posttest) in both groups, showed a difference. The measurement of systolic average blood pressure in the counseling group tended to decrease after receiving counseling through HPC. Meanwhile, systolic blood pressure in the noncounseling group tended to increase, some of which were above 140 mmHg (Figures 1 and 2).

## The effect of counseling and confounding variables on the decline in average blood pressure

At the end of the analysis, an assessment was carried out on the effect of counseling by pharmacists and confounding variables in reducing blood pressure in hemodialysis patients with hypertension. Blood pressure values were categorized based on whether the blood pressure values decreased at the posttest. The decrease in blood pressure is defined when the blood pressure measured at the posttest is smaller than that measured at pretest.

Moreover, there is a difference in the proportion of patients who experienced decreased blood pressure in the counseling and noncounseling groups. After analysis in the counseling group, the proportion of patients who

**Table 2:** Proportion of medication adherence based on MAQ.

| Treatment group | | Medication adherence based on MAQ | | | p-Value |
|---|---|---|---|---|---|
| | | High | Moderate | Low | |
| Counseling | Pretest | 5 (17.2%) | 21 (72.4%) | 3 (10.3%) | 1.00 |
| | Posttest | 19 (65.5%) | 10 (34.5%) | 0 (0%) | 0.00 |
| Noncounseling | Pretest | 6 (20.7%) | 19 (65.5%) | 4 (13.8%) | 1.00 |
| | Posttest | 3 (10.3%) | 20 (69%) | 6 (20.7%) | 0.00 |

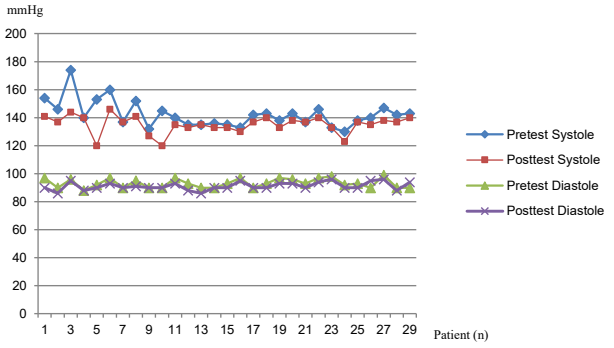


**Figure 1:** Mean blood pressure of patients in the counseling group.

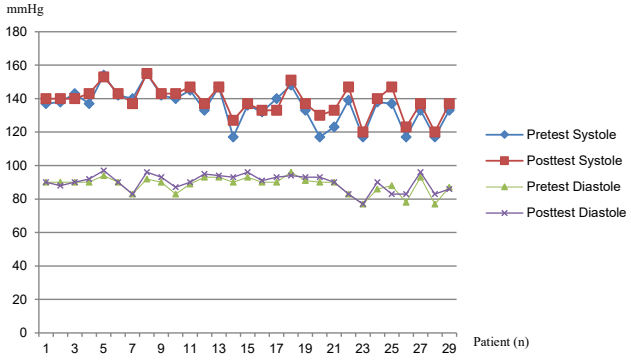


**Figure 2:** Mean blood pressure of patients in the noncounseling group.

experienced a decrease in systolic blood pressure was more than half of the total sample, 86.2%. On the other hand, the number of patients with decreased systolic blood pressure in the noncounseling group was only 17.2%. This decrease also occurred in the diastolic pressure value, where the largest proportion of the decline was in the counseling group, which was 69%, while in the noncounseling group, it was only 10.3%.

Multivariate logistic regression analysis showed that counseling by pharmacists had a significant effect on reducing systolic blood pressure. The OR values of single treatment and confounding variables were 30.000 (95% CI; 7.186–125.250) and 32.256 (95% CI; 7.198–144.550). This value indicated that patients who receive counseling by pharmacists through HPC have a 30 times greater chance of experiencing a decrease in systolic blood pressure than noncounseling patients. In addition, the chances of decreasing blood pressure in these patients were 32 times greater if controlled by the comorbid variables such as DM and other comorbidities. Meanwhile, diastolic blood pressure's effect showed that the OR value in a single treatment and confounding variables were 30.000 (95% CI; 7.186–125.250) and 42.173 (95% CI; 6.204–286.677), respectively. Based on the adjusted OR value, it can be concluded that patients tend to experience a decrease in diastolic blood pressure 30 times greater if counseled by a pharmacist and 40 times greater if controlled with confounding variables.

## Discussion

Any decrease in the patient's blood pressure significantly decreased the risk of cardiovascular [12]. However, the decrease in blood pressure cannot be separated from patient adherence related to therapy targets [13]. In this case, patient adherence to medication is an essential factor in controlling blood pressure [14]. Therefore, the provision of pharmaceutical care in the form of counseling by pharmacists through HPC is an effort to increase patient adherence to treatment.

In every HPC visit, leaflets were always included as a medium to make it easier for patients and their families to understand counseling materials. Each week, the counseling material presented was different. The outline consisted of introducing high blood pressure, patient adherence to controlling blood pressure, the importance of taking regular medication, prevention and control of blood pressure through food and drink, and reinforcement and follow-up at counseling in the last week. In addition to counseling materials, patients were always motivated to adhere to the recommended therapy and diet. They were also given information about several medicines that should be consumed in addition to hypertension therapy.

The predominance of sex characteristics in this study showed a similar trend to the data presented on Indonesia's hemodialysis patient population. Of 64,584 hemodialysis patients in Indonesia, 57% of them are male [6]. However, the age range in this study is different from that in several other studies. Studies from several countries involving 206,374 patients showed that hemodialysis patients' average age is over 60 years [15]. Elderly patients with decreased cognitive function were reported to be correlated with medication adherence [16]. However, age was not the only factor that affected cognitive function. Several other contributing factors included genetic factors, the use of certain medicines such as anticholinergic, differences in diet, and lifestyle [17, 18]. Therefore, it is necessary to conduct further studies concerning this issue.

The most widely used antihypertensive drugs are 1 type, 2 types, and 3 types of antihypertensive drugs. The type of antihypertensive drugs that is most often consumed is the CCB, namely amlodipine. A study showed that amlodipine is effective in lowering blood pressure and cardiovascular morbidity and mortality. It also has a beneficial effect in protecting kidney function [19]. Moreover, in hemodialysis

patients, amlodipine pharmacokinetics is not dialyzed, so it does not require dose adjustment [20].

In optimizing the targeted blood pressure achievement, hemodialysis patient adherence to therapy was carried out through MAQ and PCA questionnaires. Based on the use of MAQ and PCA, it appeared that patients in the counseling group or patients who received HPC experienced better changes in adherence to medication. This change did not occur in the non-counseling group. The results of the evaluation of the non-counseling group's answers to the MAQ questionnaire were that question 1 concerning "patients who often forget to take medication" and question three concerning "patients who often stop taking their medication when they feel better" showed a low level of adherence. Medication adherence is an essential factor that contributes to the success of blood pressure control [21]. Non-adherence the treatment increases resistant hypertension in hemodialysis patients. Thus, the provision of counseling by pharmacists through HPC is an effort that can be carried out to improve patient adherence to taking medication. As reported in several previous studies, pharmacists' provision of counseling has been proved to improve patient adherence to medication [22–24].

In addition to the subjective MAQ questionnaire, the PCA method can also be used to assess patient adherence to medication in a more objective way so that the two can complement each other. Measurement through these two methods is expected to provide better data on patient adherence [25]. Some nonadherence is intentional, and some are not. Intentional nonadherence occurs when a patient chooses to abandon a given therapy by delaying, changing, or skipping the prescribed medication schedule [26]. However, in this study, it was not further investigated whether the nonadherence to taking medication was on purpose or accidentally.

Increased adherence in the counseling group affected clinical outcomes in the form of a patient's targeted blood pressure that tends to decrease based on the JNC 8 recommendation below 140/90 mmHg. On the other hand, some patients' blood pressure in the noncounseling group tended to increase from the targeted blood pressure. These results are related to a study regarding patients' low adherence to antihypertensive therapy regimens due to poor blood pressure control [27]. Meanwhile, the reduction in blood pressure in the counseling group is in line with the results of a study that the provision of HPC to hypertensive patients can reduce the patient's blood pressure. Thus, counseling by pharmacists through HPC makes it easier for pharmacists to identify and solve problems faced by patients and influence patient nonadherence to medication [28]. Effective counseling by pharmacists can provide patients with an excellent understanding of being more adherent to medication and better controlling their blood pressure.

Home blood pressure measurement is the leading choice in this study because, based on international guidelines, the home blood pressure measurement can be widely applied for diagnosis and therapy in all general population [29]. Moreover, the hemodialysis unit's blood pressure measurement is inadequate for diagnostic management or assessment of the prognosis of hypertension for three reasons: low accuracy, high risk of hypotension on intradialytic measurements, and low correlation with the damage description of the target organ [30].

In this study, some patients' blood pressure tended to be higher when measured in the morning. This result is in line with the circadian rhythm that under normal circumstances, cortisol production increases at 06.00–09.00 am. However, the instability or alteration in cortisol production that usually occurs in CKD patients causes the suggestion of the stress hormone cortisol to increase and leads to an increase in blood pressure [31].

The decrease in blood pressure that was still not under the recommended target in some patients in the counseling group was not due to pharmacists' failure of counseling. Many other factors contribute to achieving the targeted blood pressure, including the choice of treatment regimen and the strength of the regimen received by the patient [32]. Therefore, health workers must collaborate so that therapy in patients can run successfully

## Conclusions

Providing pharmacist counseling through HPC can improve medication adherence and can clinically affect the reduction of blood pressure in hemodialysis patients with hypertension in the counseling group.

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Arina Dery Puspitasari*, Bindaria Mutmaina Prabawati and Alfian Nur Rosyid

# Community knowledge and attitude in recognizing asthma symptoms and using medication for asthma attacks: a cross-sectional study

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**Abstract**

**Objectives:** Uncontrolled asthma may be life-threatening. Poor understanding of disease process and appropriate medication use appears to influence community attitude in facing asthmatic patients in an emergency, thereby contributing to increasing the risk of mortality. This study aimed to analyze community-level knowledge about asthma and attitude towards asthma management.

**Methods:** This observational, cross-sectional study was conducted among the community in Gresik, Indonesia, from March to July 2019. Participants included in this study were adults, who could read, write, and communicate well. Data were collected through questionnaires to evaluate the level of knowledge and attitude towards asthma.

**Results:** In total, 100 respondents were selected with 91% of women, with a mean age of 49.11 ± 14.42 years and with various levels of education. The respondents had good knowledge by getting a score of 76%. Knowledge regarding recognition of asthma symptoms was scored the highest (83%). However, knowledge about medication use for asthma was lacking, especially in identifying the medicine choice (21%) and inhaler use (48%). The respondents also showed a 'positive' attitude with a score of 89%. Most respondents (72%) agreed that when inhaled drugs were unable to relieve the asthma attack, they need to bring the patient to a hospital.

**Conclusions:** The level of respondent's knowledge in recognizing asthma symptoms was good, but there were misconceptions about asthma medication, especially in inhaler use. Overall, the respondents had a positive attitude towards asthma perception and management.

**Keywords:** asthma; attitude; drug information; knowledge; medicine.

## Introduction

Asthma is a common, respiratory disease affecting 1–18% of people around the world. This disease is associated with airway hyperresponsiveness to stimuli or chronic airway inflammation. It is defined by the history of respiratory symptoms such as shortness of breath, wheezing, coughing, and chest tightness especially at night and morning [1, 2]. Symptoms and airway obstruction may resolve spontaneously or by taking medication, and may sometimes be absent for weeks or months [2].

The prevalence of asthma in Indonesia has been estimated at 4.5% with the highest age range of 25–34 years and has occurred more in women than in men [3]. Although its prevalence is relatively small, asthma may be life-threatening if not controlled. At the very least, it may lead patients to experience exacerbation (episodic flare-up). Exacerbation in asthma prognosis is the most significant factor causing asthma patients hospitalized, thereby carrying a significant burden to patients and the community [2, 4–6].

In Indonesia, the community knowledge of asthma signs, symptoms, and medication use has never been investigated. A study has showed the community contributed to the prevalence of asthma and the incidence of emergency related to asthma. The community social behaviors could likely adding the allergen exposure, thereby aggravating asthma unrecognizably [7].The community's poor understanding of the asthma process and its medication use, especially inhaled medication, may affect asthma control negatively and may increase

***Corresponding author: Arina Dery Puspitasari**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +6282132659919, E-mail: arinadery@ff.unair.ac.id
**Bindaria Mutmaina Prabawati,** Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Alfian Nur Rosyid,** Pulmonology and Respiratory Department, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

the risk of morbidity and mortality [8, 9]. Community awareness about asthma is equally important as they can lead to optimal management, for example by detecting early symptoms of exacerbation and helping the patients with their medication such as inhaled device [10].

From the background of the asthma prevalence, this study aimed to analyze the community knowledge and attitude in recognizing asthma symptoms and their ability of using medication for asthma attacks. Furthermore, this study also evaluated the relationship between community knowledge and attitude with their sociodemographic factors.

# Materials and methods

This study was an observational and cross-sectional study conducted in Gresik, East Java, Indonesia from March to July 2019. The study protocol was approved by the Health Research Ethics Committee, Faculty of Public Health, Universitas Airlangga, No. 217/EA/KEPK/2020. Eligible participants were adult, who could read, and communicate well, as well as have willingness to participate in this study. This study use Slovin's formula to determine sample size ($n=N/1 + Ne^2$), where N is number of population and e is margin of error. This formula is use when the population size is known but no information about the behavior of a population [11, 12]. The minimum sample size required was 83 participants.

Initially, the eligible participants were given information sheets regarding this study. Those who participated in this study also filled the written informed consent. After receiving the participants' informed consent, this study asked the participants to fill out questionnaires without any intervention from the researchers.

The questionnaires had the following sections:

(1) Sociodemographics: information on gender, age, education level, occupation, and history of asthma (e.g. family history).
(2) Knowledge of asthma disease: seven questions on 3-point Likert scale about knowledge of asthma symptoms, medication use for asthma, and triggers of asthma. Scores ranging from true (3 points), false (2 points), or do not know (1 point). Interval score range from 33 to 100%. Knowledge would be categorized as 'good' if scored ≥67%, while 'poor' knowledge would be scored <67%.
(3) Attitudes toward asthma management and control: five statements on 5-point Likert scale addressing the ability to manage and control asthma including asthma medication use. The score ranges from 1 for strongly disagree to 5 for strongly agree. Interval score range from 20 to 100%. Respondents indicate 'positive attitude' should achieve a score of ≥60%.

Validity and reliability tests on the questionnaire were done before the survey process began using the Cronbach's alfa method. Data collected though the questionnaires were then statistically analyzed using the SPSS. While descriptive statistics were used to analyze sociodemographic data. A Spearmen correlation test was employed to evaluate the relationship between variables with a p-value of ≤0.05 which would be considered statistically significant.

# Results

## Validity and reliability test of the questionnaire

Before conducting the survey, the validity of the questionnaires was tested to 30 respondents; the questionnaires were indeed valid if the significance level was <5%. Reliability testing was done upon receiving the validity score, and then the Cronbach's alfa method was used for the analysis. The results indicate that the questionnaires had a 'very high' reliability category (0.893) for knowledge section and a 'high' reliability category (0.790) for attitude section.

## Sociodemographics and its relationship to knowledge and attitudes

In total, 116 participants were recruited in this study. Sixteen questionnaires were incomplete, leaving 100 valid data to analyze. The samples consisted of 9% males and 91% females. Their ages ranged from 18 to 80 years (mean age of 49.11 ± 14.42). Most of the participants were housewives (60%) and had an elementary school degree (37%). Only 17% of the participants had a history of asthma. Additional characteristics are presented in Table 1.

**Table 1:** Sociodemographic subjects (n=100) and its relationship to knowledge and attitudes.

| Characteristics | (%) | Correlation with knowledge p-Value | Correlation with attitude p-Value |
|---|---|---|---|
| Gender | | | |
| Male | 9 | | |
| Female | 91 | | |
| Occupation | | 0.74 | 0.32 |
| Employee | 4 | | |
| Enterpriser | 8 | | |
| Housewife | 60 | | |
| Others | 28 | | |
| Level of education | | 0.46 | 0.07 |
| Drop out of primary school | 27 | | |
| Elementary school | 37 | | |
| Junior high school | 17 | | |
| Senior high school | 17 | | |
| College or higher | 18 | | |
| History of asthma | | 0.01[a] | 0.03[a] |
| Yes | 17 | | |
| No | 83 | | |

[a]p-value ≤0.05 was considered correlated.

## Asthma knowledge

Respondents' responses regarding knowledge of disease and medication are described in Figure 1. The majority knew about asthma symptoms (83%) and its triggering factors (73%), but they lacked knowledge about asthma medications. For instance, only 21% of the participants could identify that theophylline was an anti asthma medication labelled as over counter drug or purchased without any prescription, while 49% of them lacked understanding of the proper use of inhaled medication.

## Attitude towards asthma management

Attitude towards asthma attack was generally positive. More than half of the respondents agreed with some situation that could affect them. For example, controlling stress as one of asthma triggering factors was a positive attitude, they showed. Another positive attitude was done through routine control which was necessary although patients did not have symptoms. This is an alternative to identify what to do when asthma attacks happened. The only negative attitude of the respondents was their perception of using an inhaled medication without prescription. About 73% of the participants were unsure about this matter. The detailed responses are available in Figure 2.

Table 2 presents the number of respondents in 'good' or 'lacking' category based on total scores they received. The results show 76% of the respondents scored beyond the standard score in asthma knowledge, meaning they had been able to identify the asthma disease. Similar to their response to asthma self management and control,

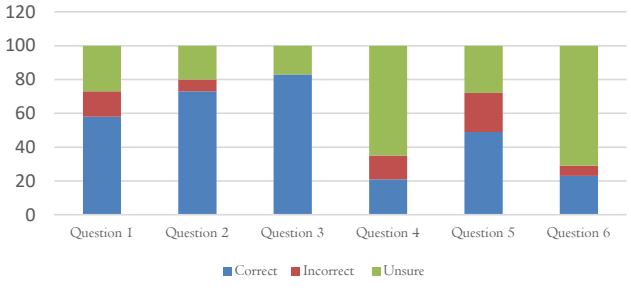


**Figure 1:** Respondents' knowledge about asthma. (Q1) Asthma is hereditary disease; (Q2) Dusts, smokes, and cold air are the causes of asthma; (Q3) Asthma symptoms are cough, shortness of breath, wheezing, and chest pain; (Q4) Theophylline is an asthma medicine and available over-the-counter; (Q5) Inhaled medications are used to treat asthma and use through the nose; (Q6) Inhaled medications can only be used when asthma attacks occurred.

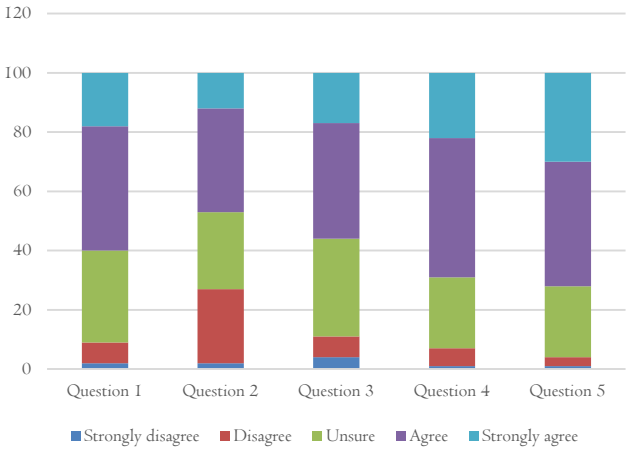


**Figure 2:** Respondents' attitude toward asthma management. (Q1) I can use inhaled medications properly; (Q2) If inhaled medication is running out, I can purchase it back at the nearest drug store without prescriptions; (Q3) Stress can triggers asthma attack; (Q4) Visiting the doctor regularly is important eventhough the asthma is well controlled; and (Q5) If inhaled medications can not relieve the asthma attack, we should bring the patient to nearest hospital immediately.

89% of the respondents received attitude scores passing the standard, showing positive attitudes.

# Discussion

This study aimed to identify the level of asthma knowledge and attitude towards asthma management in the community using validated questionnaires. Knowledge about asthma is a key factor in controlling the disease and determining the most effective response to an asthma emergency [2]. A previous study conducted in the high-risk population shows half of the participants believed asthma was a problem and their awareness of understanding more about asthma was lacking [13].

The results of this study indicate that the community knowledge in asthma was generally good with an overall

**Table 2:** Respondents' level of knowledge and attitude toward asthma disease.

| Section | Number of participants in each category, % | |
|---|---|---|
| | Good | Lack |
| Asthma knowledge | 76[a] | 24 |
| Attitude in asthma management | 89[b] | 11 |

[a]Number of participant with score ≥67%. [b]Number of participant with score ≥60%.

score of 76%. Knowledge of recognizing asthma symptoms was scored the highest (83%) among others, and the second highest-scored knowledge was about causes of asthma. Another study has investigated more specific information about gender and age in relation to knowledge about asthma. It shows older adults generally demonstrated high knowledge about asthma even without any history of the disease, and the female population had higher knowledge [14].

Community-based interventions such as giving basic information about asthma, its etiology and symptoms are important to educate the community and patients about the triggering factors and to make them understand the importance of using medications for better asthma control even in the absence of symptoms [9, 15]. Some previous studies in Indonesia and other countries have found the most common asthma risk factors included temperature changes, tobacco smoke, allergens, physical activity, emotional expression, and air pollution [16, 17]. The community awareness of these factors may depend on the intense information available through media [18]. Understanding the risk factors, the community may have appropriate actions to help patients in controlling the asthma effectively, especially in an emergency [19].

Despite strong knowledge of what is asthma, its common symptoms, and causes, the respondents still had poor understanding about medication use for asthma control. The results show that only a quarter of the respondents had properly applied theophylline, one of the asthma medications available in a drug store. Moreover, less than half of the respondents were unable to answer the techniques of using inhaled medicine correctly because they never got information about it before. Inhalation therapy is the effective foundation of asthma treatment if properly used [20] Inhaled medications such as a dry powder inhaler (DPI) or metered-dose inhaler (MDI) are the drugs suitable for the initial treatment when symptoms and signs exacerbate, such as breathlessness, inability to speak more than short phrases, use of accessory muscles, or drowsiness [4, 20].

Similar to our results, the Global Initiative for Asthma (GINA) also states most patients (up to 70–80%) are unable to use their inhaler correctly. Poor inhaler technique including overused device leads to poor asthma control, as well as increased risk of exacerbations and adverse effects [2, 20]. A high proportion of patients in the previous study did not have the competence to use the inhaler correctly because they forgot about how to use the inhaler, thereby resulting in an ineffective asthma treatment [18]. The previous finding shows the community had to know how to use the inhaled medicine as they might need it for helping people with asthma have better adherence to treatment.

Moreover, this study also explains most of the respondents (89%) had a positive attitude towards asthma self management. It was agreed that if the patients did not experience any improvement after taking a medicine for asthma, the respondents would bring them to the emergency unit immediately. The respondents also considered stress as a triggering factor of asthma. Even, when asthma symptoms are absent, they agreed that the patients still need to take a medication routinely and visit health professionals to maintain their conditions.

However, the respondents showed a negative attitude towards medication use. More than half perceived that they could buy an inhaler without a prescription. Given its high risk to worsen disease if not used properly, such as incorrect doses, people need a prescription to buy inhaled medications. On the other hand, it is still debated whether the Food and Drug Administration make inhaled medicines available over the drug counters. If asthma medications including theophylline, albuterol, and inhaled corticosteroid are available for nonprescription use, it will be more likely to decrease the emergency unit visits. However, it is concerned that there is still a gap of knowledge on understanding the medication use that may influence the effectivity of drugs [21–23].

Preparing the community with disease mitigation measures is important for asthma self management. Other psychosocial characteristics, such as motivation, favorable expectations of outcomes, and social support may be associated with a more positive attitude towards asthma. Actualizing knowledge into behavior depends on beliefs or confidence that will yield the desired outcomes [24]. Many studies have demonstrated that individuals who received a belief-based asthma management education improved preventive behavior towards asthma episode and good asthma control [25]. Community including local organizations must be aware of the prevalence of asthma in their environment and of providing services and assistance to reduce the disease burden in families with asthma patients [8].

Many factors may contribute to individuals' asthma knowledge and attitude towards asthma management; these include education level, occupation, age, experience in taking care of an asthmatic patient, and personal history of asthma [13, 26]. In this present study, notable knowledge and awareness of asthma were correlated with the history of asthma ($p<0.05$). It has shown the participants with a history of asthma, including their experience in taking care of asthma patients, were significantly more knowledgeable

about asthma signs, symptoms, and medications, and this finding is consistent with other studies [26, 27].

However, many studies also have demonstrated that patients with higher education had better asthma knowledge and a more positive attitude towards asthma management. In this study, only 35% of the respondents had a high school (or higher) degree and possessed a good level of knowledge regarding asthma. It may occur due to the accessibility of health information in contemporary society; the respondents live in a digital era where they can access any information, including health information on the Internet easily. Indeed, a previous study has reported only half of their participants used the Internet as a source of information about asthma, due to the minimum access to health education in their areas [24]. These findings may give insights to health providers to enhance trusted health service information including drug information through online services consistently and globally.

Experience and history of asthma contribute to someone's self efficacy and belief. This present study points out more favorable and general satisfaction with asthma status was correlated with greater self efficacy. Beliefs about asthma can motivate different behaviors [28]. On the positive side, the community can motivate patients to live with their asthma and fight back against the disease, controlled through medications to ultimately achieve a quality everyday life and to be devoid of further attacks.

## Conclusions

The respondents showed good knowledge about asthma signs and symptoms, as well as a positive attitude towards asthma management. The participants who had asthma experience had better knowledge about asthma. However, misconceptions about asthma medications and inhaled drugs still become visible concerns, which attests that the community needs to improve their knowledge of asthma medications to ensure better asthma control and lessen the community burden regarding asthma attacks.

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Arina D. Puspitasari*, Daniel Dwi Christiananta Salean, Didik Hasmono, Rudy Hartono and Meity Ardiana

# A study of anticoagulant therapy in patients with coronary artery disease

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**Abstract**

**Objectives:** One of the methods used to treat coronary artery disease (CAD) is anticoagulant therapy, which involves administering anticoagulants to patients that inhibit the arrangement and actuation of clotting factors. Anticoagulant therapy in patients with CAD must be monitored and evaluated because its greatest side effect is the risk of bleeding. The research aimed to analyze anticoagulants used in therapy for CAD patients and identify potential adverse drug reactions and adverse drug interactions.

**Methods:** This was an observational study which collected data retrospectively at Bhayangkara Hospital Surabaya. Patient data had to meet the requirements for inclusion, which were patients treated for a diagnosis of CAD with anticoagulant therapy and were in conditions with or without complications and comorbid diseases. Data were obtained from 40 patient medical records. The data were then processed descriptively.

**Results:** Most patients were male (80%) and aged 61–70 years old (37.5%). Fondaparinux was administered to 18 patients at a dose of 1 × 2.5 mg SC. Furthermore, enoxaparin was administered to 15 patients at a dose of 2 × 60 mg SC, and seven patients received warfarin at a dose of 1 × 2–4 mg per oral.

**Conclusions:** The anticoagulants used in this study were fondaparinux 1 × 2.5 mg SC (45%), enoxaparin 2 × 60 mg SC (37.5%), and warfarin 1 × 2–4 mg PO (17.5%). Side effects of the anticoagulants were absent. However, drug interactions with aspirin, clopidogrel, and allopurinol increased the risk of bleeding.

**Keywords:** anticoagulants; coronary artery disease; drug-related problems; enoxaparin; fondaparinux; warfarin.

## Introduction

Coronary artery disease (CAD) is an abnormality of coronary arteries that occurs when they are narrowed or obstructed. It causes an imbalance between blood supply and oxygen, which can cause myocardial ischemia. Coronary artery disease is classified into acute coronary syndrome (ACS) and chronic stable angina pectoris [1]. Blockages in blood vessels can cause various diseases depending on the location of the blockages [2].

ACS, which usually consists of myocardial infarction and unstable angina, is the form of CAD causing the majority of deaths. ACS occurs due to the rupture or erosion of atherosclerotic plaque in the coronary arteries with the continuing activation and aggregation of extrinsic blood clots [3].

According to data from the World Health Organization (WHO), 31% or 17.9 million deaths worldwide in 2017 were caused by cardiovascular disease. CAD has caused 42.3% of deaths (7.4 million) with a prevalence of 1.5% in Indonesia according to Basic Health Research (2018). According to the WHO, in 2018, deaths due to CAD in Indonesia reached 318,820 or 18.73% of total deaths. The mortality rate based on age was 181.43 per 100,000 population.

The use of anticoagulants can reduce the occurrence of myocardial ischemia, but anticoagulants can also cause bleeding in CAD patients, thus its administration and effects on patients must be monitored [4]. Bleeding can increase the risk of death. Anticoagulant therapy should be done to minimize the risk of bleeding [5]. Several types of anticoagulants used include vitamin K antagonists, low-molecular-weight-heparin (LMWH), unfractionated heparin (UFH), direct thrombin inhibitors, and factor Xa inhibitors [6]. There are many pharmacological advantages

***Corresponding author: Arina D. Puspitasari**, Clinical Pharmacy Department, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Universitas Airlangga Hospital, Surabaya, Indonesia, E-mail: arinadery@ff.unair.ac.id
**Daniel Dwi Christiananta Salean,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Didik Hasmono,** Clinical Pharmacy Department, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Rudy Hartono and Meity Ardiana,** Bhayangkara Hospital, Surabaya, Indonesia

of LMWH, such as reduced monitoring, ease of use, and a lower risk of thrombocytopenia [7]. During the administration of LMWH and UFH anticoagulants, minor bleeding occurred in 53% of the 230 hospitalized patients, moderate bleeding occurred in 32%, and 15% suffered major bleeding, such as intracranial bleeding. Major gastrointestinal bleeding occurred in 52% of the 56 patients, 34% suffered intracranial bleeding, and 14% experienced bleeding in other areas with the use of factor Xa inhibitors [8]. The use of the vitamin K antagonists' class of anticoagulants had a major bleeding incidence rate of 1,729 events, 338 incidences of intracranial bleeding, and 649 incidences of major gastrointestinal bleeding [9]. With the use of bivalirudin, major bleeding occurred in 1% of patients and minor bleeding occurred in 2–4% of patients [10].

Anticoagulant therapy in patients with CAD must be monitored and evaluated since it may result in bleeding. Problems related to anticoagulant drugs occur due to the selection of anticoagulant types, their side effects, their dosage, and the possibility of interactions between anticoagulants and other drugs. This study aimed to analyze anticoagulants used and identify potential adverse drug reactions and drug interactions in patients with CAD at Bhayangkara Hospital Surabaya in order to improve pharmaceutical services.

## Materials and methods

This observational study collected data retrospectively from January 1st to December 31st, 2019 at Bhayangkara Hospital Surabaya. Inclusion criteria of the data included all medical records of the patients treated for a diagnosis of CAD with anticoagulant therapy at the Inpatient Cardiac Installation of Bhayangkara Hospital Surabaya and in conditions with or without complications and comorbid diseases. Data analysis was carried out descriptively. The data analyzed involved patient profiles (name, age, weight, and height), patient history, patient treatments such as anticoagulant therapy and other drugs (dose, duration of use, route, and time of administration), diagnosis, clinical data, and laboratory data. The minimum number of samples needed was 35 based on the Lemeshow formula.

$$\text{Lemeshow Formula}: n = \frac{Z\alpha^2 \times p \times q}{d^2}$$

Description:

$n$=number of minimum sample; $Z\alpha$=standarize normal deviate; $p$=prevalence outcome; $q$=1 − $p$ and $d$=clinically expected variation (precision) [11].

Medical record data, which usually included the patient's condition on that day, were used for identifying adverse drug reactions. Drug interactions were identified from patients' therapeutic profiles and searched in the literature for potential interactions from Stockley [12].

## Results

There were 40 medical records reviewed for this study. Table 1 shows that 80% of the patients were male, and most patients with CAD were 60–69 years old (42.5%).

Table 2 shows that most of the patients with CAD were treated for four days (52.5%). Most patients were diagnosed with ST-elevation myocardial infarction (STEMI) (37.5%) as shown under the CAD classification in Figure 1.

The highest prevalence of comorbid disease that the patients had was diabetes mellitus (37.5%) as shown in Figure 2. Table 3 shows that the various kinds of anticoagulants found included fondaparinux, enoxaparin, and warfarin. Table 4 shows the potential drug interactions due to the polypharmacy patients experienced during treatment.

Potential drug interactions in this study, especially involving anticoagulants, are of great importance. Anticoagulants may cause bleeding as a side effect, and interactions with other drugs may increase the occurrence of bleeding. Potential anticoagulant drug interactions in this study with other drugs, namely aspirin, clopidogrel, and allopurinol. Interaction between fondaparinux and aspirin can result in increasing the incidence of bleeding. Table 5 shows the use of other therapies in patients with CAD.

## Discussions

Table 1 illustrates 32 (80%) patients were male, while 8 (20%) were female. Men had a greater risk of developing CAD than women (American Heart Association, 2015). The morbidity of men with CAD is greater than in women due to the combination of estrogen and progesterone present in women that may act as secondary prevention of CAD.

Susceptibility toward CAD increases with age, especially in patients aged over 45 years old, while the

**Table 1:** Patient demographics.

| Profile | Total, % |
|---|---|
| **Gender:** | |
| Men | 32 (80%) |
| Women | 8 (20%) |
| **Age:** | |
| 40–49 years old | 1 (2.5%) |
| 50–59 years old | 16 (40%) |
| 60–69 years old | 17 (42.5%) |
| 70–79 years old | 5 (12.5%) |
| ≥80 years old | 1 (2.5%) |

**Table 2:** Length of stay (LOS) CAD patients.

| LOS, days | Total patients | Percentage, % |
|---|---|---|
| 2 | 1 | 2.5 |
| 3 | 10 | 25 |
| 4 | 21 | 52.5 |
| 5 | 7 | 17.5 |
| 6 | 1 | 2.5 |

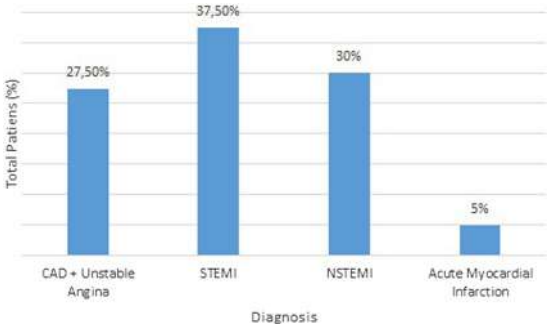


**Figure 1:** Distribution of CAD patients by diagnosis.

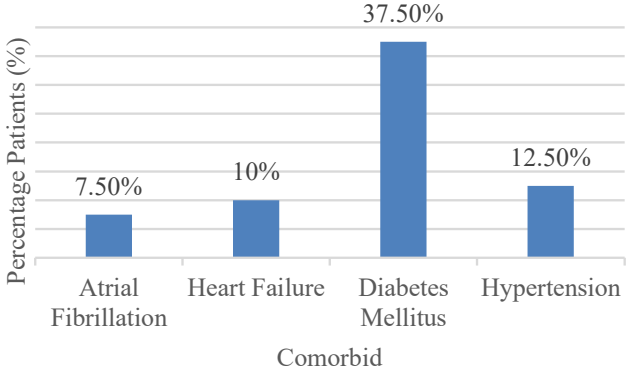


**Figure 2:** Distribution of comorbidity by diagnosis.

**Table 3:** Use of anticoagulant therapy.

| Drug classification | Type of medicine | Dosage | Route | Total |
|---|---|---|---|---|
| Anticoagulant | Warfarin | 1 × 2–4 mg | po | 7 |
| | Enoxaparin | 2 × 60 mg | sc | 15 |
| | Fondaparinux | 1 × 2.5 mg | sc | 18 |

incidence of CAD is very rare in patients under 40 years old. As a person grows older, changes in the physiology of the heart and blood vessels will occur despite the absence of disease. The myocardium of the aging heart sometimes rests imperfectly between heartbeats, thus the heart's pumping chamber will become stiffer and work less efficiently (American Heart Association, 2015).

All patients with CAD examined in this study received inpatient care for less than seven days (Table 2). A total of 21 patients (52.5%) were treated for four days. One study with 119,398 samples showed that patients with CAD received a mean length of stay of 5.5 days and with a median of four days. The length of care for CAD patients may depend on heart care procedures. The shorter period of treatment indicates good treatment procedures [13].

## CAD classification based on diagnosis

CAD is categorized into non-ST-elevation myocardial infarction (NSTEMI), STEMI, unstable angina, and stable angina pectoris. Acute myocardial infarction is classified into STEMI and NSTEMI. Figure 1 shows 15 patients were diagnosed with STEMI, 12 patients with NSTEMI, 11 patients with unstable angina, and two patients with acute myocardial infarction.

Patients experienced several comorbid diseases, with the most prevalent being diabetes mellitus (Figure 2) with a total of 15 patients (37.5%). A study has shown that diabetes mellitus was a risk factor that could worsen the condition of patients with CAD. This comorbid disease occurs due to the interaction of metabolic changes in the pre-diabetic level, such as the presence of atherogenic dyslipidemia, the endothelial function no longer functioning properly, increased free fatty acids, subclinical inflammation, changes in the adipokine layers and the thrombosis, and fibrinolysis systems [14].

## Therapy in patients with CAD

Table 3 shows that, seven patients (17.5%) used warfarin, 18 patients (45%) were given fondaparinux, and 15 patients (37.5%) used enoxaparin. Fondaparinux is a drug that catalyzes the inhibition of factor Xa by antithrombin and does not increase the inhibition of thrombin [15]. Enoxaparin is an anticoagulant in the LMWH group, and it has a mechanism of action similar to heparin, which affects the activity of antithrombin (AT III). What distinguishes heparin from enoxaparin is the more specific degradation of factor Xa that enoxaparin inhibits, while heparin tends to focus on the inhibition of thrombin by antithrombin. Fondaparinux in doses of 2.5 mg may be given to all patients once a day, with a half-life of 15–17 h by the subcutaneous route [16].

This present study shows the patients received fondaparinux within 2–3 days, enoxaparin within 2–5 days, and warfarin in 2–3 days. Several types of anticoagulants,

**Table 4:** Potential drug interactions (n=40).

| Drug interactions | Mechanisms and effects of drug interactions | Total | Troubleshooting |
|---|---|---|---|
| Fondaparinux + aspirin and NSAIDs | In general, fondaparinux as an anticoagulant can cause bleeding because of its mechanism of action. The use of antiplatelet and NSAIDs can also increase the incidence of bleeding → combined use with fondaparinux can increase the risk of bleeding and the severity of bleeding. | 16 (40%) | More closer monitoring is warranted when using fondaparinux with antiplatelet or NSAIDs. The time of drug administration can be spaced. |
| Enoxaparin + clopidogrel | Clopidogrel inhibits platelet aggregation thereby prolonging bleeding time → increases the risk of bleeding when used concurrently. | 8 (20%) | The administration of drugs has the potential for bleeding, so it can be overcome by administering different times for the two drugs. |
| Warfarin + allopurinol | Allopurinol can increase the half-life and work of the anticoagulant → the longer the half-life can increase the duration of warfarin action so that possible side effects of warfarin. | 2 (5%) | Concomitant use of allopurinol with the anticoagulant warfarin can reduce the side effects of warfarin. |
| Warfarin + aspirin + clopidogrel | Aspirin and clopidogrel are antiplatelet agents that work to inhibit platelet aggregation → the use of aspirin and clopidogrel in combination with warfarin can increase the risk of bleeding | 6 (15%) | The use of anticoagulants can be given an interval of administration so that they are not simultaneously used. |

**Table 5:** Use of other therapies in patients with CAD (n=40).

| Drug classification | Type of medicine | Dosage | Route | | Total |
|---|---|---|---|---|---|
| Vasodilator nitrat | ISDN | 2.5–5 mg | po | 26 | (65%) |
| Fibrinolitic | Streptokinase | 1,500,000 IU | iv | 4 | (10%) |
| | Alteplase | 15 mg | iv | 2 | (5%) |
| Antiplatelet | Aspirin | 100 mg | po | 34 | (85%) |
| | Clopidogrel | 75 mg | po | 34 | (85%) |
| | Ticagrelor | 90 mg | po | 3 | (7.5%) |
| β-blocker | Bisoprolol | 1.25–5 mg | po | 24 | (60%) |
| ACE inhibitor | Lisinopril | 5–10 mg | po | 9 | (22.5%) |
| | Ramipril | 2.5–5 mg | po | 7 | (17.5%) |
| Antidyslipid | Atorvastatin | 20–40 mg | po | 39 | (97.5%) |
| | Fenofibrate | 300 mg | po | 1 | (2.5%) |
| ARB | Candesartan | 8–16 mg | po | 2 | (5%) |
| Diuretic | Furosemide | 40 mg | po, iv | 6 | (15%) |
| | Spironolakton | 25–50 mg | po | 7 | (17.5%) |
| Sedative | Alprazolam | 0.5–1 mg | po | 19 | (47.5%) |
| Other drug | Allupurinol | 100–300 mg | po | 11 | (27.5%) |
| | Digoxin | 0.25 mg | po | 5 | (12.5%) |
| | Glimepiride | 2–4 mg | po | 4 | (10%) |
| | Metformin | 500 mg | po | 2 | (5%) |
| | Lantus | 4–20 IU | sc | 5 | (12.5%) |
| | Apidra | 3 × 4–12 IU | sc | 4 | (10%) |

such as warfarin, require monitoring using laboratory international normalized ratio data, whereas fondaparinux and enoxaparin anticoagulants can use partial thromboplastin time. This study found that the patients with complications caused by other diseases, such as atrial fibrillation (AF) and heart failure, were given more warfarin as an effective anticoagulant to prevent ischemic stroke according to the Indonesian Heart Association (2015). During AF, there is blood stasis, atrial hypercontractility, and remodeling of the atrial structures, platelet activation, and the coagulation cascade. These conditions will increase the risk of thrombus formation and the occurrence of ischemic stroke. The use of fondaparinux anticoagulants is more common for several reasons. First, anticoagulant

administration of this type is recommended for all patients receiving antiplatelet therapy (Indonesian Heart Association, 2015), which does not violate religious rules and drug prices. The use of enoxaparin in patients who had undergone percutaneous coronary intervention therapy was more effective than fondaparinux, as thrombus could be formed more easily when fondaparinux was used. However, the risk of bleeding when using enoxaparin was greater than when using fondaparinux [17].

### Drug-related problems (DRPs)

Potential drug interactions in this study, especially involving anticoagulants, were of great importance. The administration of anticoagulants often resulted in bleeding as a side effect. Interactions with other drugs increased the occurrence of bleeding. This study points out potential anticoagulant drugs could interact with other drugs, such as aspirin, clopidogrel, and allopurinol. This research can allow pharmacists to improve pharmaceutical services by monitoring and evaluating the effects caused by potential drug interactions.

## Conclusions

The anticoagulants used in this study were fondaparinux $1 \times 2.5$ mg SC (45%), enoxaparin $2 \times 60$ mg SC (37.5%), and warfarin $1 \times 2$–4 mg PO (17.5%). There were no adverse effects found from using fondaparinux, enoxaparin, and warfarin, however, potential drug interactions with aspirin, clopidogrel, and allopurinol were found to increase the risk of bleeding.

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Sura F. Alsaffar*, Haider A. Rasheed, Jabbar H. Yenzeel and Haider F. Ghazi

# The association of FKBP5 polymorphism with asthma susceptibility in asthmatic patients

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**Abstract**

**Objectives:** Inhaled corticosteroids are the most effective controllers of asthma, although asthmatics vary in their response. FKBP51 is a major component of the glucocorticoid receptor which regulates its responses to corticosteroids. Therefore, the present study aims to identify the role of FKBP5 gene polymorphism in asthma susceptibility and corticosteroid resistance.

**Methods:** DNA was extracted from the blood of 68 asthmatic and 40 control subjects. FKBP5 gene fragments were amplified by PCR and sequenced by the Sanger method. The sequencing results were aligned by mapping on the reference sequences of National center of Biotechnology Information (NCBI) and single nucleotide polymorphisms (SNPs) which were checked. Finally, the genotype, allele frequency and odds ratio (OR) were calculated.

**Results:** The FKBP5 fragment sequencing revealed the presence of rs1360780 and one novel SNP found in 17 samples taken from asthmatic patients as compared to db SNP data in the NCBI database. The FKBP5 variant (rs1360780) indicated that the allele frequency of risk allele T was 41.18% in patients and 20% in control group members $p<0.001$ and OR=2.8 when compared to a wild C allele frequency of 58.82% in patients and 64% in the control group members. The novel SNP FKBP5 was compared to the SNP database in the NCBI database in which wild T allele was substituted with G. The novel SNP was submitted to the ClinVar Submission Portal at NCBI with accession number: rs1581842283 and confirmed an asthma susceptibility risk factor with allele G frequency of 11.76% in asthmatics and 2.5% in the control group members (OR=5.2, $p<0.05$), as compared to a wild T allele frequency of 88.24% in asthmatics and 97.5% in the control group members.

**Conclusions:** The risk allele T of rs1360780 and the novel SNP rs1581842283 risk allele G predict asthma susceptibility but show no association with corticosteroid resistant.

**Keywords:** asthma; corticosteroid resistance; FKBP5 polymorphism.

## Introduction

Asthma is a chronic inflammatory disease affecting the lower respiratory tract characterized by airway narrowing due to bronchospasm, coughing, wheezing, shortness of breath, tightness of the chest, and excessive production of mucus [1]. Asthma exhibits a T-helper-2 type inflammatory response with the up-regulation of the IL-4 gene cluster linked to atopy (allergen exposure). Moreover, pathological features of nonallergic asthma characterized with inflammation and remodeling can occur independently of atopy [2]. The inflammatory changes are driven by various immune cells, especially the T cells subset playing an important role, they are main source of cytokines include IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [3], while polymorphism in the gene encodes cytokines such as IL-13 [4] and IL-4 [5] demonstrating correlation with the occurrence of asthma. Inhaled glucocorticoids reduce inflammation and swelling of the bronchial airways, thereby controlling asthma symptoms, preventing asthma attacks, and reducing the need for hospitalization [6].

FKBP51 (FK506 binding protein 51) constitutes a major component of the corticosteroid receptor NRC3, its co-chaperone with HSP90, which regulates corticosteroid (Glucocorticoid) sensitivity and lowers its affinity with the cortisol hormone. FKBP5 gene overexpression of FKBP5 reduces cortisol binding affinity and GR nuclear translocation by stimulating the nuclear translocation of GR Beta (nonactive isoform of GR) with FKBP51. FKBP51 encoded by the FKBP5 gene consists of 13 exons, located on chromosome 6 p21.31, extended for 155 kb [7].

FKBP5 expression increases eightfold within 3 h of the administration of 1.5 mg dexamethasone orally. The rapid induction of FKBP5 and its inhibitory effect on

***Corresponding author: Sura F. Alsaffar**, Department of Biology, College of Science, University of Baghdad, Al-Jaderyia Campus, Baghdad, Iraq, E-mail: suraa.alsaffar@sc.uobaghdad.edu.iq. https://orcid.org/0000-0002-4657-9404
**Haider A. Rasheed and Jabbar H. Yenzeel,** Department of Internal Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq
**Haider F. Ghazi,** Department of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq. https://orcid.org/0000-0002-1282-2146

glucocorticoid receptor creates intercellular ultrashort negative feedback that regulates GR sensitivity. FKBP5 mRNA stimulation by dexamethasone induction produces an extremely sensitive read out of GR function [8]. Consequently, elevated plasma cortisol fails to suppress after the administration of 1 mg dexamethasone with a normal or slightly elevated adrenocorticotropic hormone (ACTH) value which is a sign of corticosteroid resistance [9].

Glucocorticoid receptor (GR) induces FKBP51 expression via the glucocorticoid response element (GRE) located in an area that spans over 100 kb and induces it upstream of the promotor region as well as in intron 2, 5, and 7 of the FKBP5 gene [10].

The FKBP5 gene polymorphism associated with mood disorder, anxiety, and psychiatric disease increases susceptibility to a major depression [11], post-traumatic stress disorder [12] also increases the risk of suicide [13]. As no previous study on FKBP5 gene polymorphism correlation with asthma susceptibility has been conducted, the present one aims to investigate the association between FKBP5 gene fragment polymorphism leading to susceptibility to asthma; corticosteroid resistance in asthmatic patients via the measurement of cortisol; and ACTH levels, FKBP51, and GR immunocytochemical expression in lymphocyte and induced sputum cells.

## Materials and methods

A case control study was designed and conducted between March 2017 and April 2018, with 68 asthmatic patients and 40 control subjects being randomly enrolled. The patients, ranging in age from 17 to 79 years old, were diagnosed by a pulmonologist at the Al-Immamain Alkadimain Medical City Outpatient Clinic on the basis of their symptoms and asthma control test (ACT) results. They were treated with different types of corticosteroids (inhaled, orally administered, or injected) depending on their disease status and the diagnosis of a pulmonologist. Written informed consent was obtained from all asthmatics participating in the study which was approved by the Ethical Committee of the Department of Biology, College of Science, at the University of Baghdad (Reference No. 1856).

Smokers (defined as individuals consuming more than ten pack-year), asthmatics with a Forced Expiratory volume in 1 s of FEV1<1 L or FEV1<50%, those having experienced a respiratory infection in the previous four weeks and individuals with life-threating asthma were excluded from the study. The blood and sputum samples were taken from both patients and control subjects; immunocytochemical FKBP51 protein expression and GR expression in sputum cells and blood lymphocytes were studied [14]; and cortisol ACTH hormones were measured in the authors' previous studies [14, 15].

In the current study, corticosteroid resistance (insensitivity) was studied from other perspectives including calculating the mean cortisol, ACTH, FKBP51, and GR expression ± standard error in T risk allele carrier and C wild allele carrier asthmatic patients.

DNA was extracted from the whole blood samples that had been taken from both the asthmatics and healthy control subjects by using a DNA extraction kit GS100 (Geneaid, Korea). The FKBP5 gene fragment which spans 35,639,343–35,640,046 on chromosome 6 21.31 is located in intron 2. This fragment is rich in glucocorticoid response elements (GRE) which are activated by GR binding and increase the expression of glucocorticoid sensitive genes. The primers were designed by online basic local aligment search tool (BLAST) contained in the National center of Biotechnology Information (NCBI) website, and supplied by Integrated DNA Technology (IDT, USA) (see Table 1).

Gene amplification was carried out by the, conventional polymerase chain reaction PCR in pre-mix 20 µL reaction volume from Bioneer, Korea. Three microliter template DNA, 1.5 µL forward primer, and 1.5 µL reverse primer were added to the pre-mix with the volume being increased to 20 mL following the addition of DNase/RNase free D.W. The tube was subsequently transferred to a previously programed thermocycler (see Table 2).

The PCR product was loaded onto agarose gel to ensure its exact size (704 bp). These amplicons were subsequently sequenced using the Sanger method (BigDye terminator method) at the Macrogen company, Korea, to detect the exact sequence of nucleotides [16] and to identify the variation of this gene fragment from its reference sequence.

Sequences were analyzed by means of online geneious prime software version 2019, 1.1 and aligned by mapping on reference sequence on NCBI and single nucleotide polymorphisms (SNPs) which were checked. Genotype, allele frequency, and odds ratio were calculated by using SPSS 20 SPSS Inc., Chicago, Illinois, USA.

## Results

The current study showed no significant difference between asthmatic patients and those of healthy control group in terms of age, body mass index, and gender distribution (see Table 3). The asthmatics demonstrated mean disease duration of 12.43 ± 2.03 years, while the majority of patients were of severe uncontrolled status. The mean Asthma control test (ACT) result was 13.37 ± 0.77.

The FKBP5 fragment sequence revealed the presence of rs1360780 and one novel SNP found in 17 samples of asthmatic patients as compared to db SNP at NCBI.

The FKBP5 SNP (rs1360780) is located at intron 2 according to HGVS, at NC_000006.12:g 35,639,794T>C in which the wild allele C while the polymorphic T. The prevalence of the polymorphic genotype TT of the FKBP5 variant (rs1360780) in asthmatic patients (17.65%) when compared to healthy control (10%), odds ratio OR=3.75,

**Table 1:** Primers sequences and their properties.

| | Sequence (5–3) | TM °C | GC% |
|---|---|---|---|
| Forward primer | AGCCTCATGGTGAAATCCGA | 59.18 | 50 |
| Reverse primer | TGTGGTGAGACAATCAGAGCAT | 59.89 | 40 |

**Table 2:** PCR program.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Pre-denaturation | 95 °C | 5 min | One cycle |
| Denaturation | 95 °C | 20 s | 35 cycle |
| Annealing | 59 °C | 30 s | |
| Extension | 72 °C | 45 s | |
| Final extension | 72 °C | 10 min | One cycle |

**Table 3:** Demographic data of asthmatic patients and control.

| | | Asthmatic patients | Control | p-Value |
|---|---|---|---|---|
| Age, years | | 41.65 ± 3.21 | 43.53 ± 1.9 | (p>0.05) |
| BMI, kg/M$^2$ | | 31.32 ± 0.72 | 29.03 ± 0.99 | (p>0.05) |
| Gender distribution no., % | Female | 45(66.67%) | 24(60%) | (p>0.05) |
| | Male | 23(33.33%) | 16(40%) | |

p<0.05 while Heterozygous TC appeared as (47.06%) in asthmatics, and (15%) in control individuals p<0.05, OR=6.67 whereas the wildtype genotype CC frequency was (35.29%) in patients and (75%) in control subjects.

The allele frequency of risk allele T was significantly different in patients (41.18%) compared to members of the control group (20%) p<0.001 and OR=2.8 when compared to wild C allele frequency in patients (58.82%) and control group members (64%), (see Table 4 and Figure 1 respectively).

Immunocytochemical evaluation of FKBP51 in the lymphocyte and sputum cells of asthmatics and control group members appears in previous studies conducted by the authors [14]. The current research revealed that no significant differences exist between polymorphic T allele and wild C allele carriers with regard to ACT, Cortisol, ACTH, GR nuclear, and cytoplasmic expression in lymphocyte and sputum cells, while sputum FKBP51 nuclear expression showed a significant increase (41.16 ± 5.69%) p<0.05 in risk allele T carrier when compared to C allele carrier (34 ± 5.46%). The cytoplasmic expression rose significantly in Wild C allele carrier (68.64 ± 3.7%) p<0.05 more than the T allele carrier (55.11 ± 5.91%), whereas the lymphocyte FKBP51 expression showed no significant differences between them (see Table 5).

Sequence result alignment shows novel SNP FKBP5 as compared to db SNP at NCBI located at NC 000006.12:g 35,639,490 where wild T allele was substituted with G found in 17 patients. Their sequences were submitted to the GenBank at the National Center for Biotechnology Information, (accession number Bankit: MN0129050, MN012951 to MN012966), while the new SNP was also submitted to the ClinVar submission portal with the accession number: rs1581842283.

All 17 samples (patients) were heterozygous, (see Figure 2). Genotype GT frequency amounted to 23.53% in asthmatic subjects as compared to 5% in control group members (OR=5.85, p<0.05). Wild genotype TT was present in (76.47%) of patients and (95%) of the control group members as illustrated in Table 6.

The risk factor with allele G frequency was 11.76% in the asthmatic group and 2.5% in the control group (OR=5.2, p<0.05) when compared to wild T allele frequency of (88.24%) and (97.5%) in each group, respectively.

## Discussion

Uncontrolled severe asthma negatively affects patients, possibly resulting in a poor quality of life and potential life-threatening attacks, despite their strict adherence to a prescribed course of treatment (e.g., corticosteroid). This situation led the authors to study corticosteroid

**Table 4:** Genotype and allele frequency of FKBP5 variant rs1360780.

| | | Patient | Control | p-Value | OR (CI: 95%) |
|---|---|---|---|---|---|
| Genotype | Polymorphic TT | 12 (17.65%) | 4 (10%) | 0.046 | 3.75 (1.09–11.49) |
| | Heterozygous TC/CT | 32 (47.06%) | 6 (15%) | 0.002 | 6.67 (2.34–18.49) |
| | Wild CC | 24 (35.29%) | 30 (75%) | 1 | 1 |
| Total | | 68 | 40 | | |
| | | **Patient** | **Control** | | |
| Allele | Risk allele T | 56 (41.18%) | 16 (20%) | 0.0016 | 2.8 (1.46–5.49) |
| | Wild C | 80 (58.82%) | 64 (80%) | 1 | 1 |
| Total | | 136 | 80 | | |

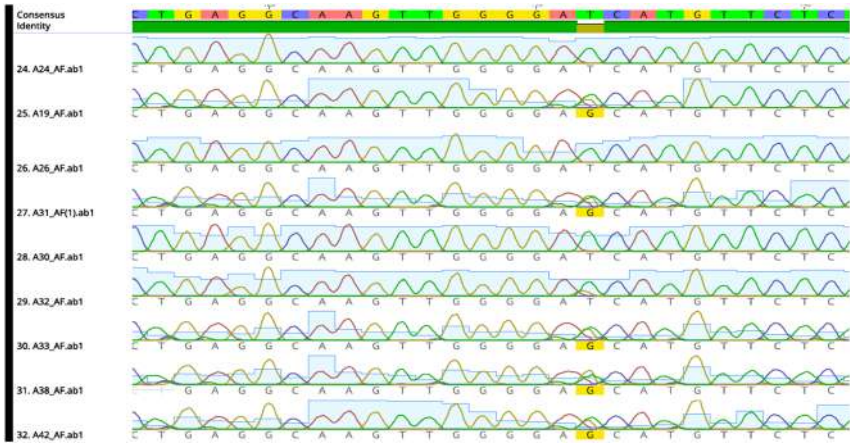


**Figure 1:** FKBP5 sequence alignment showing rs1360780.

**Table 5:** Immunocytochemical FKBP51 expression in lymphocyte and sputum %.

| | rs1360780 genotype | | | rs1360780 allele | |
|---|---|---|---|---|---|
| | CC | CT | TT | C | T |
| Lymphocyte FKBP51 nuclear % | 55 ± 3 | 56 ± 3 | 53 ± 3 | 56 ± 2 | 53 ± 3 |
| Lymphocyte FKBP51 cytoplasmic % | 22 ± 3 | 20 ± 3 | 21 ± 3 | 22 ± 2 | 21 ± 3 |
| Sputum FKBP51 nuclear % | 26 ± 3.18 | 43.6 ± 6.88 | 40.29 ± 6.99** | 34 ± 5.46 | 41.16 ± 5.69** |
| Sputum FKBP51 cytoplasmic % | 75.67 ± 2.79** | 60.2 ± 3.71 | 53.29 ± 7.83 | 68.64 ± 3.7** | 55.11 ± 5.91 |

*p<0.05, **p<0.001.

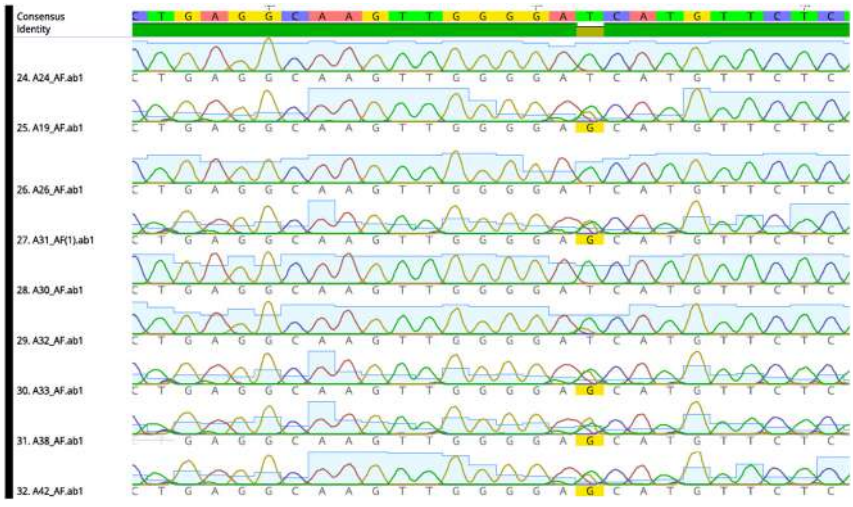


**Figure 2:** FKBP5 sequence alignment showing novel SNP.

insensitivity which remains a challenging clinical problem, as yet unaffected by the availability of modern forms of treatment.

Thomas [17] summarized the genetic and environmental factors affecting asthma corticosteroid insensitivity. Moreover, he explained the mechanism to which the alteration of GR subtypes; binding and nuclear translocation; increase in pro-inflammatory transcription factors; and defective histone acetylation are attributed.

Glucocorticoid (GC) sensitivity is associated with GR activation, an increase in FKBP5 mRNA expression and cortisol suppression (negative feedback) after exposure to stress [18].

The authors' previous studies [14, 15, 19] showed that an increase in Nuclear GR expression together with one in FKBP51 nuclear expression in lymphocyte and induced sputum cells also causes a rise in cortisol. However, the ACTH level still remained within the normal level. All of

**Table 6:** Genotype allele frequency of FKBP5 new variant rs1581842283.

| | | Patient | Control | p-Value | OR (CI: 95%) |
|---|---|---|---|---|---|
| Genotype | Polymorphic GG | 0 (0%) | 0 (0%) | | |
| | Heterozygous TG/GT | 16 (23.53%) | 2 (5%) | 0.015* | 5.85 (1.42–26.58) |
| | Wild TT | 52 (76.47%) | 38 (95%) | 1 | |
| Total | | 68 | 40 | | |
| | | **Patient** | **Control** | | |
| Allele | Risk allele G | 16 (11.76%) | 2 (2.5%) | 0.020* | 5.2 (1.38–23.23) |
| | Wild T | 120 (88.24%) | 78 (97.5%) | 1 | |
| Total | | 136 | 80 | | |

*p<0.05, **p<0.001.

these signs of corticosteroid insensitivity led them to complete a study of the molecular level. FKBP5 gene fragment sequencing revealed the presence of single nucleotide polymorphism rs1360780 indicating a significant increase in nuclear FKBP51 in the sputum cells of risk T allele carriers when compared to wild C allele carriers ($p<0.05$). In contrast, lymphocyte cells possibly play a role in corticosteroid insensitivity, although the conducting of an extensive study featuring a large sample size is required to confirm the results.

Malteset et al. [20] referred to increased nuclear expression primarily associated with GC insensitivity and elucidated the mechanism to find that variant rs1360780 demonstrates nonsignificant correlation with a GC response for Crohn's disease and ulcerative colitis. Lao et al. [21] reported that the rs1360780 risk allele did not affect the GC response in idiopathic thrombocytopenic purpura.

The present study indicated that FKBP5 variant (rs1360780) risk T allele is associated with asthma susceptibility (OR=2.8). It is first seeking to establish the association of FKBP5 polymorphism rs1360780 with asthma susceptibility. In addition, the novel SNP approved by resequencing and submitted to the NCBI bankit and ClinVar database rs1581842283 [15] demonstrated a strong association with asthma susceptibility. The risk allele presented at a level five times higher in the patients than in the control subjects (OR=5.2).

Previous studies demonstrated that FKBP5 was associated with asthma susceptibility and increased after inhaled corticosteroid treatment in the bronchial brushing and bronchial biopsy specimens of asthmatic subjects [22]. Hawkins et al. [23] studied six SNPs of FKBP5 other than rs1360780, reporting no correlation with the baseline lung function measures of asthmatics and FEV1 improvement post-corticosteroid treatment, while another FKBP5 variant, rs4713916, was strongly associated with elevated lung function after inhaled corticosteroid treatment in chronic obstructive pulmonary disease (COPD) patients [24]. Therefore, other studies are required to approve the association of these SNPs with asthma susceptibility follow up after ICS treatment for at least eight weeks to measure improvement in lung function on an asthma control test.

# Conclusion

rs1360780 and the novel variant rs1581842283 showed association with asthma susceptibility but no association with corticosteroid resistance.

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**Competing interests:** Authors state no conflict of interest.
**Informed consent:** Written informed consent was obtained from all patients and control individuals.
**Ethical approval:** Study was approved by the Ethical Committees of the Department of Biology, College of Science, University of Baghdad (Reference No. 1856).

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Mahardian Rahmadi*, Nily Su'aida, Pratiwi Yustisari, Wahyu Agung Dewaandika, Elma Oktavia Hanaratri, Mareta Rindang Andarsari, Sumarno and Toetik Aryani

# Gastroprotective effect of fluvoxamine and ondansetron on stress-induced gastric ulcers in mice

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**Abstract**

**Objectives:** The association between stress and gastric ulcers has been well reported. This study is divided into two parts: the first part of this study is consisted of analyzing the effect of fluvoxamine administration by intracerebroventricular (ICV) and intraperitoneal (IP) injections on stress-induced gastric ulcers. The second part investigates the effect of ondansetron in influencing the protection of the gastric mucous by giving fluvoxamine to the mice before being induced with stress.

**Methods:** Water immersion restraint stress (WIRS) was used to induce stress. Fluvoxamine 50 and 100 mg/kg by IP injection, fluvoxamine 9.3 μg, and 18.6 μg by ICV injection 30 min before the induction of stress. Meanwhile, single drug and in combination administered to the mice, ondansetron 3 mg/kg was given by IP at 60 min, and fluvoxamine 50, 100 mg/kg orally at 30 min before stress induction.

**Results:** The obtained results show fluvoxamine 50 and 100 mg/kg by IP, and fluvoxamine 18.6 μg by ICV had significantly reduced ulcer index with $p<0.005$, $p<0.001$, and $p<0.005$ while fluvoxamine 9.3 μg showed the insignificant result. Fluvoxamine 50 mg/kg, fluvoxamine 100 mg/kg, and ondansetron 3 mg/kg monotherapy have a significant reduction in ulcers with $p<0.005$, $p<0.001$, and $p<0.05$, while the combination drugs showed an insignificant reduction in ulcers.

**Conclusions:** Fluvoxamine with different administration routes and ondansetron monotherapy before stress reduce the occurrence of gastric ulcers, while the combination drugs did not increase the protective effect of the gastric mucosa.

**Keywords:** fluvoxamine; gastric ulcers; health risk; ondansetron; stress.

***Corresponding author: Mahardian Rahmadi**, Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +62 81224656516, E-mail: mahardianr@ff.unair.ac.id
**Nily Su'aida, Pratiwi Yustisari, Wahyu Agung Dewaandika, Elma Oktavia Hanaratri, Mareta Rindang Andarsari, Sumarno and Toetik Aryani,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

## Introduction

Ulcers are described as open sores cut through the thickness of gastrointestinal mucosal [1]. Peptic ulcer is a disorder in the digestive tract characterized by mucosal damage that extends to the submucosa or muscularis propria due to the secretion of pepsin and stomach acid. Peptic ulcer occurs most often in proximal duodenum (duodenal ulcers) and in the stomach (gastric ulcers), and rarely occurs in distal duodenum, jejunum, and in the lower esophagus [2, 3], approximately four million gastric ulcers cases recorded in the world per year [4].

The pathophysiology of gastric ulcers is characterized as an imbalance between aggressive factors (e.g., leukotriene, stomach acid, pepsin, and *Reactive Oxygen Species* [ROS]) with defensive factors (e.g., prostaglandins [PG], mucosal perfusion, and bicarbonates) [5, 6]. *Helicobacter pylori* and prolonged use of nonsteroidal anti inflammatory drug (NSAID) are commonly known as associated with gastric ulcers [7]. Apart from *H. pylori* infection and the use of NSAIDs, gastric ulcers also occur due to stress [8].

Physiological stress triggers the *Hypothalamus–Pituitary–Adrenal* (HPA) axis activation and leads to secrete *Corticotropin-Releasing Factor* (CRF) and affects the secretion *of Adrenocorticotropic Hormone* (ACTH), which stimulates cortisol secretion [9]. Cortisol inhibit phospholipase A2 and lead to a reduction of prostaglandin synthesis [10] and decrease blood flow to the gastric mucosa due to vasoconstriction on the vessels [11].

It has been well documented that the *Selective Serotonin Reuptake Inhibitor* (SSRI) such as fluvoxamine has a mechanism to inhibit the formation of ulcers in the stomach as indicated by a decrease in the value of the *Bax/Bcl-2* ratio in gastric tissue, escalate the expression of Hsp70 protein (one of the markers of gastric defends), reduction of

ulcer index scores and intraluminal bleeding scores [4, 12–14]. Administration of fluvoxamine increases serotonin levels and activates its receptors, for instance, 5-$HT_1$-5-$HT_7$. Activation of the 5-HT3 receptor increase gastric acid secretion and affect ulcer formation [15, 16]. The mechanism of the anti ulcer effect of SSRIs may influence by its effect on the brain, so it is necessary to research to see the effect of the direct route of SSRI on the brain by intracerebroventricular (ICV) and compare it with the systemic route by intraperitoneal (IP) and whether the administration of 5-HT3 receptor antagonist (ondansetron) affect the gastric mucosa protection by SSRIs on stress-induced gastric ulcers.

# Materials and methods

### Materials

The materials used were fluvoxamine maleate 5 g (Wako Pure Chemical Industries, Osaka, Japan), ondansetron, normal saline 0.9% (PT. Widarta Bhakti, Pandaan, Pasuruan, Indonesia), tween 80 (Wako Pure Chemical Industries, Osaka, Japan). 1% tween were freshly prepared for solutions and drug suspensions.

### Experimental animals and treatments

Male 6–8 week-old mice were used in the experiments. All mice were maintained under the same treatment and freely accessed water and standard chow, and subjected to a 12/12 h light-dark cycle. Experiments were performed under "The Guiding Principles for the Care and Use of Animal Research of Universitas Airlangga No. 683-KE".

After an adaptation period of 2 weeks, 71 mice were randomly divided into two main groups: 36 mice into the comparative groups where fluvoxamine 50 & 100 mg/kg given by IP, and fluvoxamine 9.3 and 18.6 µg given by ICV 30 min before the induction of stress. A total of 35 mice divided into seven groups were treated with a single drug (monotherapy), and in combination, drugs are given to the mice, ondansetron 3 mg/kg given by IP at 60 min and fluvoxamine 50, 100 mg/kg orally at 30 min before stress induction.

### Gastric ulcers induction

Induction of stress was conducted following the method of Ji et al. [17]. Stress induction was started 30 min after treated with fluvoxamine or 60 min after treated with ondansetron. All mice were immobilized in a restraint tube and immersed in a water bath to the depth of the xiphoid process at 23 °C for 6 h.

### Assessment of gastric mucosal injury

Immediately after 6 h of stress induction, all mice were sacrificed, and the stomachs were removed, opened along the greater curve, and washed with the normal saline. The stomachs were assessed for the severity of intraluminal bleeding, as explained by the following arbitrary scale described by Chiu et al. [18]. Ulcer index (UI) was indicated in terms of gastric mucosal lesions. Gastric tissues were pinned out flat on a corkboard and photographed for lesion assessment. The area with lesion was calculated in square millimeters, and the accumulative area of all sores assessed out of the severity of ulcers [17].

### Statistical analysis

All results obtained are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA followed by *Tukey's post-hoc* tests for UI and *Kruskal–Wallis* method followed by *Dunn's post-hoc* test for intraluminal bleeding score were used to evaluate the difference between the treated and control group. p-value <0.05 was claimed statistically significant.

# Results

## Effect of fluvoxamine given by ICV and IP on stress-induced gastric ulcers

The treatment of mice with *Water Immersion Restraint Stress* (WIRS) model to induce stress on mice produced gastric lesions. The ulcer index in treated groups of fluvoxamine 50, 100 mg/kg (IP) and 18.6 µg (ICV) showed significantly prevented the gastric ulcers as compared to the stress group (Figure 1A). The results for the severity of intraluminal bleeding show only fluvoxamine 100 mg/kg (IP) significantly decreased intraluminal bleeding as compared to the control group (Figure 1B). Meanwhile, the representative photograph of the stomach in both fluvoxamine (IP and ICV) shows that both administrations' route can reduce the occurrence of gastric ulcers. The lesions in the gastric lumen were depicted by the dark spots on the representative photograph (Figure 2). Fluvoxamine given by IP has slightly lesser dark spots' appearance than fluvoxamine given by ICV.

## Effect of fluvoxamine and ondansetron on stress-induced gastric ulcers

The ulcer index in treated groups of fluvoxamine 50, 100 mg/kg and ondansetron 3 m/kg (monotherapy) and in combination (fluvoxamine 50 mg/kg + ondansetron 3 mg/kg; fluvoxamine 100 mg/kg + ondansetron 3 mg/kg) showed significantly decreased as compared to the stress group (Figure 3A). Pretreatment mice with high dose of fluvoxamine 100 mg/kg monotherapy and in combination with ondansetron 3 mg/kg significantly reduced the

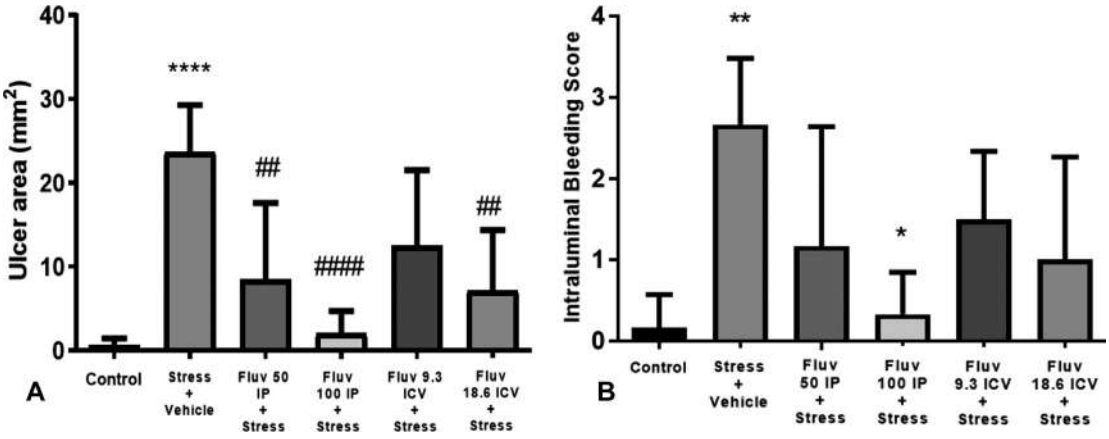


**Figure 1:** Effect of fluvoxamine given by ICV and IP on stress-induced gastric ulcers.
Effect of fluvoxamine given by ICV and IP decrease gastric ulcers. Ulcer index (A), stress group + vehicle vs. control group; stress group + fluvoxamine 50 mg/kg (IP); stress group + fluvoxamine 100 mg/kg (IP); stress + fluvoxamine 18.6 µg (ICV) group; ****p<0.0001; ##p<0.005; ####p<0.0001; ##p<0.005 vs. stress group + vehicle. Intraluminal bleeding score (B), stress group + vehicle vs. control group, stress group + fluvoxamine IP 100 mg/kg; **p<0.005; *p<0.05 vs. stress group + vehicle. Each column represents mean ± SEM of six mice.

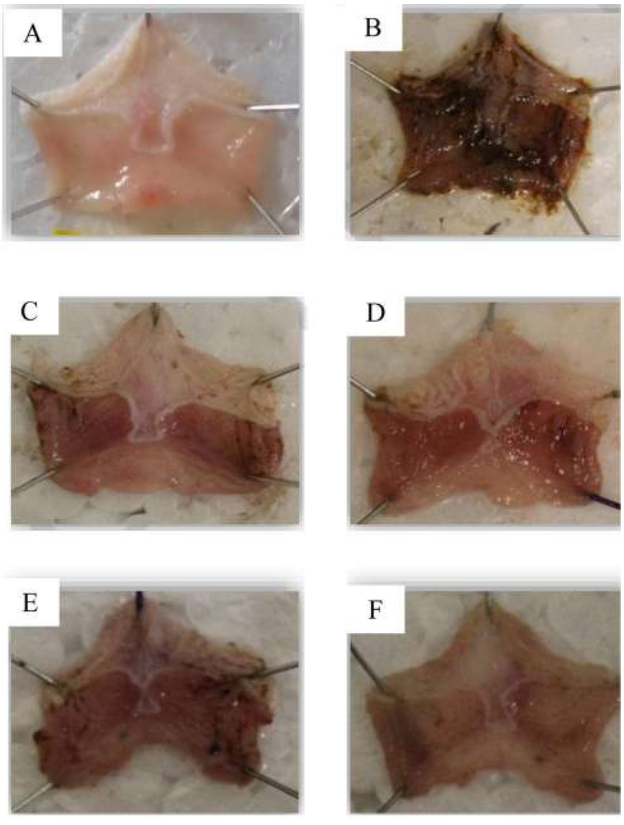


**Figure 2:** Representative photograph of the gastric lumen of mice. Control group (A), stress + vehicle group (B), fluvoxamine 50 mg/kg (IP) group (C), fluvoxamine 100 mg/kg (IP) group (D), fluvoxamine 9.3 µg ICV group (E), and fluvoxamine 18.6 µg ICV group (F). The blue line indicates the position of the ulcer.

intraluminal bleeding score as compared to the stress group (Figure 3B). The representative photograph of the stomach in both groups of fluvoxamine (monotherapy and combination) reduced the presence of dark spots in the lumen (Figure 4). A high dose of fluvoxamine with and without combination with ondansetron has lesser dark spots' appearance than fluvoxamine low dose and ondansetron monotherapy.

## Discussion

The preventive effect of fluvoxamine was analyzing in mice using the WIRS model to induced stress. In the present study, the obtained results show administration by IP and ICV injection can reduce the occurrence of gastric ulcers. The ICV route's administration goal is to bypass the *Blood–Brain Barrier* (BBB) and other mechanisms that restrict the distribution of drugs to the brain [18]. The IP administration route is included as a parenteral route. However, the pharmacokinetics of drugs that are given by IP is comparable to the oral administration due to the primary route of absorption towards the mesenteric vessels, depleted within the portal vein, and traversed the liver [19]. Our previous study showed fluvoxamine 50 and 100 mg/kg have a protective effect against the gastric mucosa [4, 14]. Based on previous research conducted by Schreiber et al. [20], it was found that the IP dose of 50 mg/kg was equivalent to a dose of 9.3 µg given by ICV, and

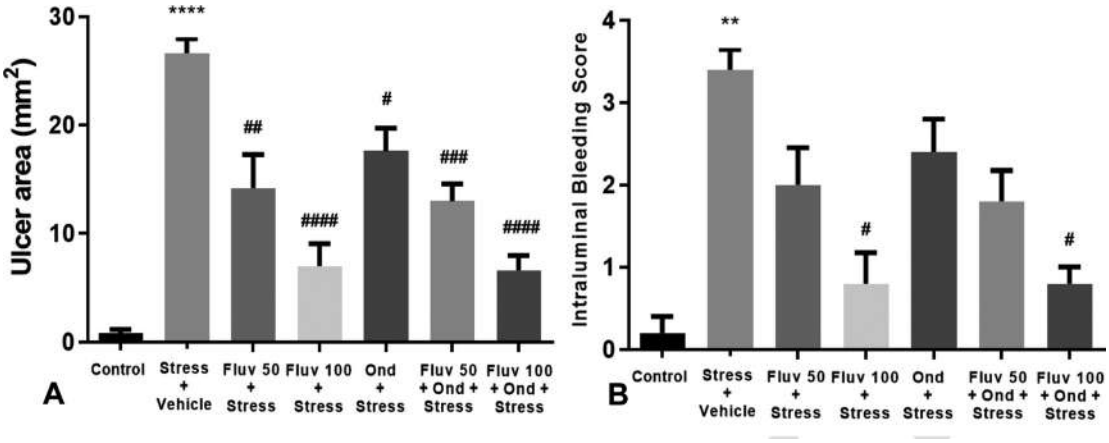


**Figure 3:** Effect of fluvoxamine and ondansetron on stress-induced gastric ulcers.
Effect fluvoxamine and ondansetron decrease gastric ulcers. Ulcer index (A) stress + vehicle, stress + fluvoxamine 50 mg/kg, stress + fluvoxamine 100 mg/kg, stress + ondansetron 3 mg/kg, stress + fluvoxamine 50 mg/kg + ondansetron 3 mg/kg, stress + fluvoxamine 100 mg/kg + ondansetron 3 mg/kg; ****p<0.0001 vs. the control group; ####p<0.0001, ###p<0.0005, ##p<0.005, #p<0.05 vs. stress group. Intraluminal bleeding score (B) **p<0.005 vs. control group; #p<0.05 vs. stress group. Each column represents the mean ± SEM of five mice.

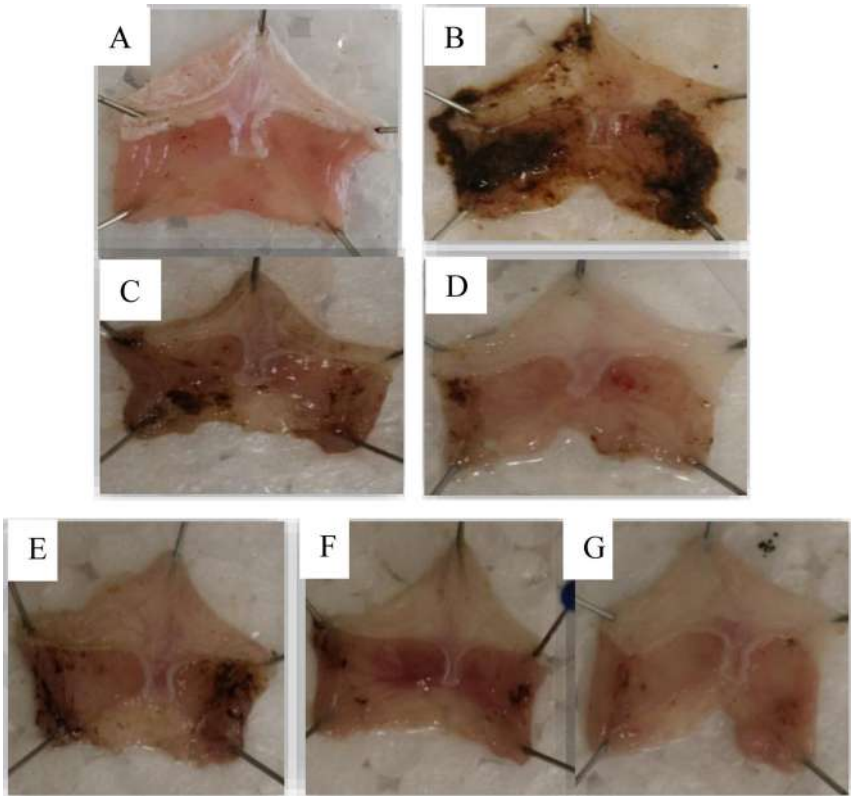


**Figure 4:** Representative photograph of the gastric lumen of mice.
Control group (A), stress + vehicle group (B), fluvoxamine 50 mg/kg group (C), fluvoxamine 100 mg/kg group (D), ondansetron 3 mg/kg group (E), fluvoxamine group 50 mg/kg + ondansetron 3 mg/kg group (F), and fluvoxamine group 100 mg/kg + ondansetron 3 mg/kg (G).

100 mg/kg IP was equivalent to 18.6 μg ICV. The results in present study showed the IP administration greater than ICV. This can be due to serotonin levels in the gastrointestinal (GI) tract higher than in the brain. Serotonin is found about 80% in the GI track and the remnant being divided between the central nervous system (CNS) and the platelets [21].

Fluvoxamine works by blocking the reuptake of serotonin transporter (SERT), subsequently increase extracellular serotonin (5-HT) levels [22]. Serotonin administration has pharmacological effects, which inhibit gastric acid secretion, stimulate mucus discharge, stimulate the intestinal secretion, and stimulate ionic and mucus secretion [23, 24]. Serotonin on the brain may play a role in activating

the vagus nerve, and releasing serotonin in the brain due to stress increase gastric acid secretion, leading to an increased risk of gastric ulcers. Serotonin causes activation of 5-HT3 receptors, which increase the activity of the vagus nerve [25]. The vagus nerve is a nerve that allocates a two-way affinity between the stomach and brain, consisting of afferent nerve fibers that send messages from the stomach to the CNS, and laden efferent that convey motor information from the dorsal motor nucleus to the stomach muscles [26].

The second part of this research examines the effect of 5-HT3 receptor antagonist (ondansetron) in influencing the protection of the gastric mucous by giving fluvoxamine to the mice before being induced with stress. 5-HT3 receptor antagonist prevent serotonin to bind with 5-HT3 receptor on GI vagus nerves and the chemoreceptor trigger zone (CTZ) on the brain [27] The administration of fluvoxamine (monotherapy) at a dose of 50 and 100 mg/kg protect the gastric mucosa marked by reducing intraluminal bleeding score and UI. The results support the previous studies [4, 14–16]. Administration of ondansetron 3 mg/kg monotherapy to stress-induced gastric ulcers in a representation shows a reduction in the occurrence of ulcers. Monotherapy of ondansetron provides this gastroprotective effect according to the results of previous studies [28, 29]. Ondansetron reduce gastric ulcers in several gastric ulcer induction methods, and it is thought that through several mechanisms. Ondansetron reduce gastric acid secretion, reduce total acidity, and increase mucus secretion [28].

The combination drug by adding ondansetron 3 mg/kg showed mucosa's protection against the stress group. The protection was characterized by a substantial reduction in the ulcer index and a significant reduction in intraluminal. However, these results show less significant protection when compared with the fluvoxamine monotherapy group. This can emerge from 5-HT receptor competition. Based on previous study showed co administration of ondansetron with SSRI significantly increase levels of extracellular 5-HT and partially blocked the suppressant effect on dorsal raphe nucleus [30]. SSRI decrease the potentiation of 5-HT3 receptor via pharmacodynamic competition. This combination increase the potential accumulation of 5-HT because of receptors competition [31].

Previous studies have shown a pro ulcerogenic effect via 5-HT3 serotonin receptors while an anti ulcerogenic effect via 5-HT4 serotonin receptors [30]. In this study, the results show that blocking or inhibiting the 5-HT3 serotonin receptor with ondansetron can act as an anti ulcer. It supports the results of previous studies, which state that the 5-HT3 receptor is a pro ulcer. However, since the actual mechanism of SSRI's antidepressant effect on stress-induced gastric ulcers is not known to involve serotonin and its receptors or it may have other mechanisms, it is necessary to carry out further research to examine with other receptors besides 5-HT3 to determine the antidepressant effect of SSRIs on stress-induced gastric ulcers.

## Conclusions

The SSRI (fluvoxamine) with different administration routes and 5-HT receptor antagonist (ondansetron) as monotherapy before stress can reduce the occurrence of gastric ulcers, while the combination drugs did not increase the protective effect of the gastric mucosa. Moreover, further research on the actual mechanism of SSRI's antidepressant effect on stress-induced gastric involving serotonin and its receptors is needed.

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**Ethical approval:** All experiments were conducted at the Animal Research Laboratory of Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, in accordance with the Guidelines for the Care and Use of Laboratory Animal issued by approved by by National Institutes of Health revised in 1985. The Ethics Committee of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, approved the study protocol.

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Prihartini Widiyanti*, Hartmut Kuehn and Soetjipto Soetjipto

# Osteoblast iron genes: real time PCR and microarray hybridization approach under hyperoxia

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**Abstract**

**Objectives:** Iron is essential for cell growth, differentiation, electron transfer, and oxygen transport. Hyperoxia may increase the turnover of bone matrix components with a net effect of accelerated bone growth. Although hyperoxia was claimed could increase osteoblast activity, but expression level in possible genes which play role in proliferation is still unclear. This research aims to prove the differences of expression level of transferrin receptor gene and iron regulated transporter and other genes of 7F2 under 24 h normoxia, 24 h hyperoxia, and 48 h hyperoxia and the effect of hyperoxia by using osteoblast cell culture $7F_2$.

**Methods:** Reverse transcriptase, real time Polymerase Chain Reaction (PCR), and microarray is used to qualitatively detect gene expression. The computer softwares such as National Center for Biotechnology Information (NCBI) data base, Software Affymetrix, DNA Strider program, Genomatix – DiAlign program, Oligo 5.0 program (Software primer design) from Wojciech & Piotr Rychlik, and Genetyx-Mac version 8.0 have been used to analyze the PCR result.

**Results:** Under 24 h hyperoxia, there were 3,884 copies of transferrin receptor mRNA per 1,000,000 copies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. After 24 h hyperoxia, 8,325 copies of transferrin receptor mRNA per 1,000,000 GAPDH mRNA copies were found showing 2.1-fold up regulation. After 48 h hyperoxia, there was no significant increase at the level of expression of transferrin receptor mRNA, 8,079 mRNA copies per 1,000,000 copies of mRNA were found (2.0-fold up regulation compared with 24 h normoxia).

**Conclusions:** It can be concluded that hyperoxia might have an effect on upregulating the expression of some osteoblast genes which might have an impact on osteoblast activity.

**Keywords:** hyperoxia; osteoblast iron-regulated transporter gene; osteoblast transferrin receptor gene.

## Introduction

Iron is essential for many physiological process including cell growth, differentiation, apoptosis, electron transfer, oxygen transport, and detoxification [1].

Hyperoxia *in vitro* may increase the turnover of bone matrix components with a net effect of an accelerated bone growth. Although in some literatures, hyperoxia was claimed to being able to increase osteoblast activity as bone forming cell, but the level of expression of both transferrin receptor gene and iron-regulated transporter (IRT) gene of mouse osteoblas cell-line (7F2) as the possible genes which play a role in proliferation is still unclear.

Transferrin is the dominant iron which binds protein in the blood. It is an 80 kDa glycoprotein protein, which migrates in serum electrophoresis in the β-globulin fraction and is capable of binding two ferric ions ($Fe^{3+}$) with high affinity. It is one of the major serum proteins which contributes to about 5% to the protein mixture in human serum.

Transferrin is an essential component of all serum-free cell culture media [2]. Transferrin is a carrier molecule that plays a role in iron transport cycle related to absorption, storage, utilization, and secretion and their interaction with tissue target [3].

IRT plays role in iron circulation and transportation [4]. According to Steinbrech and coworkers' research in 1999 and 2000, osteoblast cell under normoxia exposure ($O_2$ 20%) just showed a tiny increase in cellular proliferation. In contrast, ender hyperoxia ($O_2$ 90%) showed reverse phenomena. Hyperoxia has been suggested to improve the activity of osteoblasts to synthesize and secrete bone matrix components. However, the mechanistic reasons for this effect have not been investigated in detail. There is no

***Corresponding author: Prihartini Widiyanti**, Biomedical Engineering, Faculty of Science and Technology, Universitas Airlangga, Campus C, Mulyorejo, 60115, Surabaya, Indonesia; and Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, E-mail: pwidiyanti@fst.unair.ac.id
**Hartmut Kuehn,** Institute of Biochemistry, Charite University of Clinics, Humboldt University, Berlin, Germany
**Soetjipto Soetjipto,** Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Department of Biochemistry, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

comprehensive characterization of the gene expression pattern for osteoblasts available in the literature and the impact of hyperoxia on isolated osteoblasts in cell culture has neither been studied.

# Materials and methods

### Mouse osteoblast cell culture

Mouse osteoblast cell line 7F2 was obtained from the American Tissue Cell Culture. The cell culture was grown in hyperoxic (80% $O_2$, 5% $CO_2$, and 15% $N_2$) and normoxic (20% $O_2$, 5% $CO_2$, and 75% $N_2$) condition, Minimal Essential Medium M 4526 from SIGMA, trypsine, Phosphate Buffer saline, β- Mercaptoethanol, ethanol 70%, cell incubator, RNAse free syringe, buffer RPE, Coomassie Brilliant Blue G250, dimethyl sulfoxide (DMSO), buffer RLT 3507600.

### RT-PCR

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA and to detect iron gene iron ion transporter, transferrin receptor. The computer softwares such as National Center for Biotechnology Information (NCBI) data base, Software Affymetrix, DNA Strider program, Genomatix – DiAlign program, Oligo 5.0 program (Software primer design) from Wojciech & Piotr Rychlik, and Genetyx-Mac version 8.0 have been used to analyze the Polymerase Chain Reaction (PCR) result.

### Electrophoresis

Electrophoresis was applied for the separation and characterization of proteins, nucleic acids, and subcellular-sized particles like viruses and small organelles. The materials were electrophoresis chambers, TE buffer, agarose LE, and marker 100 bp DNA Ladder from Biolabs.

### Microarray studies

Microarray was used to determine sequence or to detect variations in a gene sequence or expression or for gene mapping (MeSH). The materials were Oligo-dT, dNTPs, primer, Qiaquick PCR purification – Qiagen system, microarray reader, hybridization chamber, deionized formamide array chip, and PCR. The growth curve (cell number, protein, and DNA concentration) under normoxia and hyperoxia (after the cell proliferation experiment is performed) of the mouse's osteoblast cell line 7F2 culture was recorded. Then, mouse osteoblast cell line 7F2 culture was put into an incubator under three conditions which were 24 h normoxia (21% $O_2$), 24 h hyperoxia (95% $O_2$), and 48 h hyperoxia (95% $O_2$). The next procedure was total RNA isolation. M*icroarray* hybridization produces data such as spot densities (relative fluorescent unit). *Reverse Transcriptase*-PCR produced band appearance of certain genes in electrophoresis gel (confirmed method of microarray data) and *DNA sequencing* is performed in target gene. Real Time-PCR produced quantification of gene expression level. We performed DNA sequencing to determine the nucleotide sequence of DNA. The forward and reverse primers used were (Table 1):

# Results

## Qualitative evaluation of the microarray data

To obtain experimental information on these effects, we quantified the expression levels of iron genes in 7F2 cells cultured under hyperoxic conditions and compared them with the corresponding values of cells grown under normoxia. In order to perform these experiments, preconfluent cells were kept for different time periods (24, 48 h) under normoxic and hyperoxic conditions. These intensities mirror the expression levels of the different gene products. In Figure 1, the fluorescence intensity pattern of a microchip hybridized with the labeled cDNAs prepared from 7F2 cells grown under normoxic conditions and hyperoxic conditions was shown.

On each microarray bride spots represents the expression level of high-level expressed genes. On the other hand, low-level expressed genes were indicated by a lack of fluorescence. Since there are some tens of thousands genes, genes on the array and each gene was represented by at least eight different matching and eight mismatching oligonucleotides. There were more than 300,000 spots (including all control oligonucleotides) on one microarray chip.

## Enzymes involved in iron metabolism

To obtain more detailed information on the iron homeostasis of 7F2 cells under normoxic and hyperoxic conditions, we extracted from our database genes related to the iron turnover using the Affimetrix software looking at the go-class of iron metabolism.

In Table 2, the most strongly up regulated gene products are summarized. The transferrin receptor, which is represented on the microarray by several oligonucleotides, was most strongly up regulated (3.73-fold).

In addition, we found up regulation of an iron ion transporter (2.55-fold) and of ferroportin-1 (2.35-fold). It should, however, have been stressed that there was no uniform tendency for expression regulation of iron-related gene products. In fact, the majority of genes related to iron metabolism were not regulated in response to hyperoxia. Thus, it cannot be predicted by evaluation of the gene expression data whether or not there were functional alterations in the iron metabolism.

**Table 1:** The forward and reverse primers.

| Name of gene | Sense | Primer length | Antisense | Primer length | Length of expected amplification product |
|---|---|---|---|---|---|
| Iron-regulated transporter primer (Slc40A1) | 5′-CCG AGA TGG ATG GGT CTC CTA C-3′ | 22 | 5′-GGA CCA AAG ACC GAT TCT AGC AG-3′ | 23 | 526 bp |
| Transferrin receptor primer (CD71) | 5′-TTT CCT TGC ATA TTC TGG AAT CCC-3′ | 24 | 5′-CGT CAC CAG AGA GGG CAT TTG C-3′ | 22 | 629 bp |
| Growth arrest DNA damage inducible 45 alpha primer (Gadd45a) as housekeeping genes | 5′ -GGA TGC CCT GGA GGA AGT GCT -3′ | 21 | 5′ -AGG ATG TTG ATG TCG TTC TCG CA-3′ | 23 | 210 bp |

Real Time-PCR was used to amplified-DNA which was identified as real time reaction.

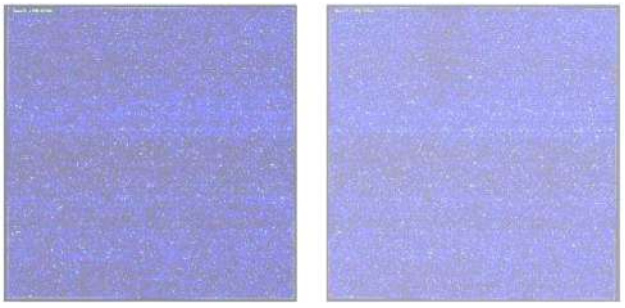

**Figure 1:** Fluorescence pattern obtained by microarray hybridization. 7F2 cells were grown for 24 h under normoxic (A) and hyperoxic (B) conditions.

**Table 2:** Gene products involved in iron metabolism.

| No. | Affimetrix no. | Database entry | Gene | Change fold |
|---|---|---|---|---|
| 1 | 1417061_at | BC00343 | Solute carrier family 39 (iron-regulated transporter), member 1 | 2.55 |
| 2 | 1448566_at | AF226613 | Musmusculus ferroportin1 (Fpn1) mRNA, complete cds. | 2.35 |
| 3 | AFFX-TransRecMur/X 57349_3_at | X57349 | Transferrin receptor mRNA | 3.73 |

## Reverse transcriptase-polymerase chain reaction (RT-PCR)

a. Semi-quantitative RT-PCR

### Iron-related proteins

From Figure 2, it can be seen that expression of the transferrin receptor (upper panel) was higher under hyperoxic conditions when compared to normoxia.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – This observation was made regardless of whether the cells were incubated for 24 h (traces a and b) or 48 h (traces c and d). In both cases, the PCR bands obtained with the hyperoxic samples (traces b and d) were more intense than those of the normoxic cells (traces a and c). Similar results were obtained for the iron ion transporter (middle B). These data are consistent with our observations of the microarray studies. Here we observed a 3.7-fold increase in expression of the transferrin receptor and a 2.5-fold up regulation of the iron ion transporter.

## Real-time RT-PCR

Under 24 h hyperoxia, there were 3,884 copies of transferrin receptor mRNA per 1,000,000 copies of GAPDH mRNA. After 24 h hyperoxia, 8,325 copies of transferrin receptor mRNA per 1,000,000 GAPDH mRNA copies were found showing 2.1-fold up regulation. After 48 h hyperoxia, there was no significant increase at level of expression of transferrin receptor mRNA, 8,079 mRNA copies per 1,000,000 copies mRNA were found (2.0-fold up regulation compared with 24 h normoxia).

Under 24 h normoxia, there were 359 copies IRT mRNA per 1,000,000 copies GAPDH mRNA. After 24 h hyperoxia, there were 555 copies IRT mRNA per 1,000,000 GAPDH mRNA copies indicating a 1.5-fold up regulation. After 48 h hyperoxia, a significant increase of the level of expression of IRT mRNA, that is 1,655 mRNA copies per 1,000,000 copies GAPDH mRNA (4.6-fold up regulation compared with 24 h normoxia).

These data confirm the results obtained by microarray hybridization and semi-quantitative RT-PCR. However, the

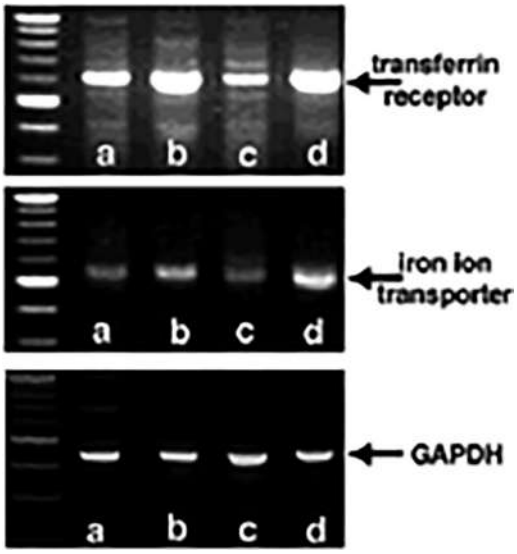


**Figure 2:** Semi-quantitative RT-PCR of transferrin receptor and iron ion transporter expressed in 7F2 cells grown under normoxic and hyperoxic conditions. 7F2 cells were grown for 24 h undernormoxic and hyperoxic conditions. After harvesting the cells, total RNA was extracted and RT-PCR was carried out as described in the methods section. Upper panel: transferrin receptor, a) normoxia 24 h, b) hyperoxia 24 h, c) normoxia 48 h, and d) hyperoxia 48 h. Middle panel: iron ion transporter, a) normoxia 24 h, b) hyperoxia 24 h, c) normoxia 48 h, and d) hyperoxia 48 h. lower panel: a) normoxia 24 h, b) hyperoxia 24 h, c) normoxia 48 h, and d) hyperoxia 48 h.

extent of up regulation was somewhat different from the three methods. In microarray hybridization, we observed a 3.2-fold increase in transferring receptor mRNA concentration when the cells were cultured for 24 h under hyperoxic conditions and a similar value was obtained in semi-quantitative RT-PCR. However, exact quantification by real-time PCR indicated a lower level of up regulation (1.8-fold).

## DNA sequencing

DNA sequencing and homology checking using Genetyx-Mac version 8.0 was performed with the transferrin receptor gene (CD71) and iron-regulated transporter gene (Slc40A1). The data are 100% identic of the sequence data obtained in this research sequencing with the sequence of transferrin receptor (CD71) retrieved from the database. There was 98.5% sequence identity. The mismatching nucleotides might represent mutations in this gene but they might be introduced as sequencing artifacts. Repeated sequencing may be performed to exclude sequencing artifacts.

# Discussion

Iron plays a vital role in the maturation, growth, and division of cells [5]. Under normal conditions, approximately 1–2 mg of iron per day enters the body via the cells of the proximal small intestine. This newly absorbed dietary iron is released into the bloodstream and binds to the serum transferrin (Tf). Approximately 3 mg of iron circulates bind to this protein, and as compared with 4 g of total body iron, it is a small portion. Iron ions bound to Tf are taken up by cells via transferrin receptor-1 (TfR1)-mediated endocytosis [6]. Thereafter, old or damaged red blood cells are removed from the bloodstream by macrophages of the reticulo-endothelial system and iron is recycled back to plasma Tf. The total iron content of Tf corresponds to less than 0.1% of the body iron, but it is highly dynamic. Iron can be used to synthesize many proteins, such as cytochromes containing heme and proteins containing iron-sulfur (Fe-S) clusters. Approximately 10–15% of body iron is present in such proteins, with up to 80% of it found in muscle cell myoglobin. The remaining 20% of body iron is present as the storage iron, predominantly located in the macrophages of the reticulo-endothelial system and the hepatocytes of the liver [7]. The extracellular transport and dynamic storage of iron within the body is accomplished by its binding to a specific carrier protein, transferrin [8].Transferrin receptor is composed of two 760-amino-acid glycoprotein subunits of ~95 kDa [9, 10].Transferrin is required for subsequent proliferation and terminal differentiation [11]. Transferrin receptor is the most important gene for iron cellular homeostasis [12]. The transferrin occupancy reflects the balance of iron entering the serum (from intestinal absorption, macrophage iron release, and hepatic iron mobilization) and iron leaving the serum (primarily for utilization in erythropoiesis) [13]. Transferrin receptor numbers at cell surface are increased in rapid proliferating-cell [14]. The improvement level of transferrin in the proliferating cell is required for DNA synthesis [15]. Transferrin receptor reflected the potency to perform cell proliferation. The proliferative status of the cell is a factor which is able to affect transferrin receptor per second. A high proliferative status is associated with a high density of transferrin receptors [16]. The number of transferrin receptor is related with cell density and proliferation rate *in vitro*. Physiological level of transferrin could increase DNA synthesis and the inclination could improve osteoblast proliferation [17]. This gene is responsible for cellular iron uptake and its deficiencies will lead to severe imbalance of iron supplies. Transferrin receptor is important for the control of cell growth. The blockade of transferrin receptor

will inhibit DNA synthesis [18]. The time for G1 arrest related to initial level of surface transferrin receptors. When the expression is low, the possibility of G1 arrest is bigger. In contrast, if the expression is high, the possibility of G1 arrest remains smaller. The expression of transferrin receptor during cell cycle varies in each type of cell but most of them is high expressed at the initial phase of G1 [19]. According to our microarray data, the transferrin receptor clearly belongs to the class of hyperoxia-inducible genes. It is low-level expressed in normal 7F2 cells (relative fluorescence units 483), but is 4-fold up regulated under hyperoxic conditions. It should, however, have been stressed that there was no uniform tendency for expression regulation of iron-related gene products. In fact, the majority of genes related to iron metabolism were regulated in response to hyperoxia.

IRT is the main transporter that is responsible for iron uptake [20]. According to our microarray data, the IRT clearly belongs to the class of hyperoxia inducible genes. This gene is expressed in low level under normoxia (relative fluorescence units 624), but this gene then was regulated for three fold under hyperoxia. It seems that up regulation of IRT 2.55-fold. Thus, it cannot be predicted by evaluating the gene expression data whether or not there are functional alterations in the iron metabolism.

Ferroportin (SLC40A1) is an iron transporter postulated to play roles in intestinal iron absorption and cellular iron release. Iron is exported from the cell by ferroportin, located in the cellular membranes capable of regulated of iron export such as enterocytes, reticuloendothelial macrophages, hepatocytes, and placental cells [21]. A study in which the murine ferroportin gene, Slc40a1, was inactivated globally and selectively showed that ferroportin is essential for iron export in enterocytes as well as in macrophages and hepatocytes. Ferroportin-deficient animals' accumulated iron in enterocytes, macrophages, and hepatocytes, is consistent with a key role for ferroportin in those cell types. Intestine-specific inactivation of ferroportin confirmed that it is critical for intestinal iron absorption. These observations define the major sites of ferroportin activity and give insight into hemochromatosis. Iron efflux from the enterocyte basolateral membrane is performed by membrane-bound ferrous iron transporter – FPN-1, also known as iron-regulated transporter- 1 (IREG-1), metal transporter protein-1 (MTP-1), or the product of solute carrier family 40, member one gene (SCL40A1) previously called as solute carrier family 11, member three gene (SCL11A3) [22]. This protein has been independently identified by three groups of researchers who used different approaches [21–23]. FPN-1 is synthesized in many iron-exporting cells, including hepatocytes, macrophages – where FPN-1 exports iron from the cytosol into the bloodstream (for iron recycling) and placental syncytiotrophoblasts. Thus, it does not only play a fundamental role in the release of iron from tissues but also in maternal iron transfer to the fetus [23, 24]. There is evidence that FPN-1 is also synthesized in the renal cells located in the glomerulus and proximal tubular cells of this tissue but its role remains obscure [25]. The FPN-mediated efflux of $Fe^{2+}$ from enterocytes and macrophages, which is responsible for clearing senescent red blood cells into the plasma, is critical for the systemic iron homoeostasis. Afterward, iron is oxidized by a membrane bound ferroxidase–hephaestin (HEPH), that physically interacts with FPN-1 yielding ferric ions that are then bound by plasma Tf and distributed around the body via blood [26, 27].

The expression of transferrin receptor and ferroportin were high under hyperoxia. Consistently, hypoxia might have induced loss of this transferrin receptor expression. These alterations of the transferrin receptor happen in response to oxygen. The intracellular mechanism(s) to sense iron and/or oxygen is required for these actions [28, 29].

## Conclusions

The expression studies presented herein are reveral to many genes of potential interest under hyperoxia and normoxia conditions, most of which are poorly analyzed and previously unknown. The relation of oxygen role, proliferation, and iron metabolism should be further explored. The movement of iron in the human organism is a tightly-regulated process, which can be modulated according to the iron requirements. The recent advancements in the knowledge of the regulation of iron metabolism have been made possible to understand better the pathophysiology of some human disorders. This work aimed to present the current state of knowledge of the regulation of the human systemic iron homeostasis, without focusing on the diseases derived from the disturbances of this process.

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Aniek Setiya Budiatin, Ilham Bagus Sagitaras, Ika Putri Nurhayati, Nismatun Khairah, Khoirotin Nisak, Imam Susilo and Junaidi Khotib*

# Attenuation of hyperplasia in lung parenchymal and colonic epithelial cells in DMBA-induced cancer by administering *Andrographis paniculata* Nees extract using animal model

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**Abstract**

**Objectives:** This study was designed to evaluate the potential of *Andrographis paniculata* ethanolic extract to inhibit the increase in proliferation and induction of abnormal cell death.

**Methods:** The hyperplasia stage as an early stage of cancer development was induced by oral administration of 20 mg/Kg BW DMBA to SD rats twice a week for 5 weeks. There were five groups in this study include negative control, positive control, and treatment groups of DMBA induction followed by administration of *A. paniculata* ethanolic extract in doses equivalent to 10, 30 or 100 mg/Kg BW andrographolide once per day for 6 consecutive weeks. On the last day, rats were sacrificed, lung and colon tissues were collected. Histological examination by HE staining and immunohistochemistry using p53, telomerase, and caspase-3 antibodies were aimed at observing hyperplasia state in these tissues.

**Results:** DMBA induction to SD rats was able to produce hyperplasia in lung parenchymal and colon epithelial tissue. This can be showed by the increasing number of proliferated cells and as indicated by the number of brown-colored nuclei with sharper intensity. As well telomerase appears to be overexpressed strongly, while p53 and caspase-3 show low intensity. The administration of *A. paniculata* extract for 6 weeks showed a decrease in the number of cells that actively proliferate, a decrease in telomerase activity, and an increase in caspase-3 levels which indicate cellular death activity.

**Conclusions:** *A. paniculata* ethanolic extract can inhibit the development of cancer at the hyperplasia stage by reducing telomerase activity and increasing apoptosis, marked by an increase of caspase-3 expressions.

**Keywords:** *Andrographis paniculata*; caspase-3; DMBA-induced cancer in rats; hyperplasia; telomerase.

## Introduction

Cancer is a group of disorders characterized by uncontrolled cell growth, local tissue invasion, and distant metastases. Abnormal cell proliferation leads to hyperplasia, an increase in the number of cells in an organ, and can spread to other organs through the metastasis process where metastasis is the main cause of cancer death. The formation of cancer cells is believed as the result of physical, biological, or chemical carcinogen damage and alters genetic and epigenetic [1, 2].

According to the report, cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or one in six deaths, in 2018. Lung and colorectal cancer are the most common type of cancer in both men and women, and the most common cause of cancer death. Its high mortality rate is due to the absence early diagnosis and the symptoms in the early stage, so it can be detected and treated after reaching an advanced stage [3]. For this reason, inhibition of abnormal cell growth at the hyperplasia stage becomes critical in reducing the rate of cancer development and the incidence of death.

The current paradigm of cancer research is focused on therapy that has a more selective target so as to minimize the toxic effects on normal cells. Therapies that selectively act directly on target proteins, growth receptors or small

***Corresponding author: Junaidi Khotib**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Jl Mulyorejo, Kampus C Unair, 60286, Surabaya, East Java, Indonesia, E-mail: junaidi-k@ff.unair.ac.id
**Aniek Setiya Budiatin, Ilham Bagus Sagitaras, Ika Putri Nurhayati, Nismatun Khairah and Khoirotin Nisak,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, East Java, Indonesia
**Imam Susilo,** Department of Pathological Anatomy, Faculty of Medicine, Airlangga University, Surabaya, East Java, Indonesia

molecule inhibitors that have overexpression on cancer cells. With an understanding of the genetic changes and cell cycle in cancer cells compared to normal cells, it will encourage the discovery of new drugs that are selective in cancer cells. The expected effects of anticancer therapy include decreased cell proliferation and increased apoptosis.

The components that play a role in cell proliferation are telomere and telomerase. Telomeres are repeated DNA complexes (TTAGGG) that have an important role in the life of cancer cells. They are maintained by an enzyme called telomerase in the vast majority of tumors. The mechanisms underlying the maintenance of telomere length (TL) and telomerase expression are related to transcription, post-transcription, and epigenetic regulation, and an in-depth understanding of these mechanisms can provide new biomarkers and targets for early detection of disease, determination of disease prognosis, and development of therapy [4].

Inhibition of telomerase activity can induce apoptosis, which is programmed cell death that genetically regulates the development and homeostasis of an organism. Apoptotic activation occurs in two pathways, intrinsic and extrinsic, each of which can activate the effector caspases, caspase-3 and 7. If the effector caspase is activated, the cell will experience apoptosis [5]. In cancer patients, where cell proliferation cannot be controlled, apoptosis is important as a mechanism to control cancer cell growth. Drugs that inhibit telomerase activity and induce apoptosis can be used as an effective and selective alternative to colon cancer therapy so as to minimize side effects.

Cancer treatment depends on the stage of the cancer. in the early stages (I, II, III) cancer can be cured, but in the late stage (IV) treatment is aimed at palliative therapy to reduce symptoms, prevent complications, and prolong life [1]. Cancer is used to be treated with surgery, radiation, and systemic anticancer agent where the choice of treatment is based on the type and stage of cancer. Systemic anticancer agents such as chemotherapy, targeted therapy, and immunotherapy, aimed to kill cancer cells and minimizing side effects on healthy cells [1]. Therefore, it is necessary to develop new anticancer agents that can increase the effectiveness and tolerance.

One of the plants that have been suspected of having an anticancer effect is *Andrographis paniculata* Ness, with andrographolide as a chemical substance that is responsible for its anticancer activity. *A. paniculata* extracts and their major diterpenoid component has been reported that able to suppress cell proliferation, induce cell cycle arrest, and induce cell apoptosis of cancer cells [6]. Combination of andrographolide and fluorourasil (5-FU) is used for treatment 5-FU resistance in colorectal cancer. Andrographolide in combination with fluorouracil (5-FU) is used for the treatment of 5-FU resistance in colorectal cancer. Andrographolide can induce apoptotic cell death so that 5-FU sensitivity in cancer cells increases [7]. *A. paniculata* ethanolic extract played a role in the intervention of cancer formation. Administration of *A. paniculata* extract in colon cancer can reduce the number of abberant cancer cells and frequencies of aberration per cell [8]. *A. paniculata* extract can reduce telomerase activity and increase apoptosis by activating the PI3K/AKT/AP-1 signaling pathway [9]. Therefore, this study was designed to evaluate the potential of andrographolide in *A. paniculata* ethanolic extract to inhibit the increase in proliferation and induction of abnormal cell death.

## Materials and methods

*A. paniculata* Ness ethanolic extract obtained from the Pharmacognosy and Phytochemical Laboratory of Faculty of Pharmacy Airlangga University. The extract was suspended with CMC Na 0.1% (w/v) in aquadest. The preparations were made in three different doses that equivalent to 10, 30 and 100 mg andrographolide/kgBW. Andrographolide content was determined using Thin Layer Chromatography (TLC) and Densitometer Shimadzu CS-930. Mixture of chloroform and methanol (9:1) was used as an eluent in TLC method.

Female Sprague Dawley (SD) rats, 7–10 weeks of age, were acquired from Integrated Research and Testing Laboratory of Gadjah Mada University. In this study, SD rats were divided into five groups. Group 1 (negative control) was fed orally with Oleum Maydis twice a week for 5 weeks (at induction phase) and CMC-Na everyday (at treatment phase). Group 2 (positive control) was induced by oral administration of 20 mg/Kg BW DMBA to SD rats twice a week for 5 weeks (induction phase) and CMC-Na everyday (at treatment phase). Group 3, 4, and 5 (treatment groups), were induced by DMBA and followed with administration of doses that equivalent to 10, 30 or 100 mg/Kg BW andrographolide in ethanolic extract once per day for 6 consecutive weeks. On the last day, rats were sacrificed, lung and colon tissues were collected. Doses of 10, 30 or 100 mg/kg BW andrographolide was adopted as low, medium, and high dosage that altered the expression of genes in cellular compromise, cell cycle, and "DNA recombination, replication, and repair" [10].

Telomerase and caspase-3 were detected using affinity purified rabbit anti-rat caspase-3 antibody (Bioworld Technology Inc.) and rabbit anti-rat telomerase antibody (Bioworld Technology Inc.). Tissue sections were rehydrated using alcohol solution with a decrease in concentration every 2 min (99, 99, 90, 80, 70%) and subjected to specific antigen retrieval treatment. Endogenous peroxidase activity was blocked with H2O2 0.3%/methanol. Background was blocked using Block Serum Free X 0909, dropped in "marking pen immunologic" area at room temperature for 5 min, then washed with TRIS buffer/Tween 20 for 5 min and swap the tissue around "marking pen immunologic" area.

For immunohistochemistry (IHC), Primary antibodies were dropped into the "Marking pen immunologic" area and then incubated in

closed magnetic immunostaining at room temperature for 1 h and incubate 4 °C overnight with the tissue, washed with TRIS buffer/Tween 20 for 5 min and swap the tissue around "marking pen immunologic" area. Secondary antibodies (Biotin-labeled Link) was dropped on "marking pen immunologic" area at room temperature for 10 min, washed then dropped HRP (Streptavidin Peroxidase Conjugate) and incubate at room temperature for 10 min. Washed then dropped DAB at room temperature for 5–10 min and washed with water for 5 min. Then, tissue sections were counterstained with hematoxylin and dehydrated. The results of immunohistochemistry staining were analyzed qualitatively on the expression of telomerase and caspase-3 proteins by observing using a 1000x magnification light microscope.

## Results

Andrographolide content in *A. paniculata* ethanolic extract had been shown in Table 1. The result show that *A. paniculata* ethanolic extract that used in this research contain 11.65% w/v andrographolide.

Administration of *A. paniculata* extract to SD rats that had been induced by DMBA was able to improve the lung structure of rats as shown in Figure 1. In this figure, it has shown the histology of the lungs in rats that have been given *A. paniculata* extract with doses equivalent to 10, 30 and 100 mg/kg BW andrographolide. Compared with the histology of the lungs in rats that were only given DMBA (A), the alveolar structure began to appear with narrower space between the alveoli and the wider alveolar cavity on the lung histology after administration of *A. paniculata* extracts (B), (C), and (D).

From Figure 1, it can be seen that giving the extract with andrographolide dose of 10 mg/kg BW suppresses the rate of cell proliferation not as much as the extract with andrographolide dose of 30 and 100 mg/kg BW. This can be seen from the number of cells that are still quite large in the alveolar space compared to the other two doses. Meanwhile, the extract with andrographolide doses of 30 and 100 mg/kg BW resulted in almost the same histological picture of lung structure improvement.

The ability of *A. paniculata* extract in suppressing the rate of cell proliferation is associated with decreased telomerase activity in cells, shown in Figure 2. From the results of observations of telomerase using a light microscope qualitatively, it was found that *A. paniculata* extract could

**Table 1:** Andrographolide content in *A. paniculata* ethanolic extract.

| Samples | Total andrographolide applied in TLC, 2 µL | Total andrographolide measured, 5.0 mL | Total weighed sample | Andrographolide content in sample |
|---|---|---|---|---|
| 1 | 922.20 ng | 2.305 mg | 20.4 mg | 11.32% |
| 2 | 973.74 ng | 2.434 mg | 20.3 mg | 11.99% |
| **Mean andrographolide concentration in sample** | | | | 11.65% |

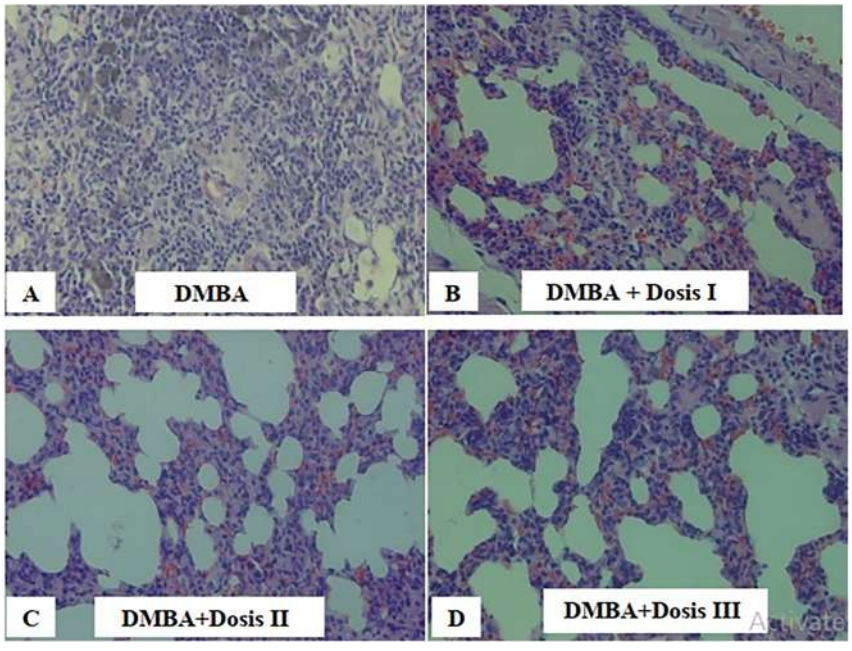


**Figure 1:** Histopathology of SD rats lung given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide using Hematoxylin-Eosin staining at 400x Magnification.

reduce the expression of telomerase protein. The decrease in telomerase protein expression at the extract with dose of andrographolide 10 mg/kg BW was lower than the other doses (Figure 2B), it appears that the number of cells that absorb brown is still quite a lot. Whereas at the extract with andrographolide dose of 100 mg/kg BW (Figure 2D), it appears that number of cells that absorb the brown color less than the other doses. However, from these observations it was not certain that the dose that was able to suppress telomerase activity was the best among the three doses given.

In observing caspase-3 activity, the results showed that there was an increase in the expression of caspase-3 after giving treatment as seen in Figure 3 where cells absorbed more brown in the treatment group compared to the DMBA group. The extract with andrographolide dose of 10 mg/kg BW showed the lowest caspase-3 expression among other doses.

Changes in colonic tissue morphology after treatment with *A. paniculata* extract can be observed in Figure 4. When compared with DMBA group (Figure A), where the epithelial cells appear thicker due to increased proliferation and hyperchromatic nuclei, the three treatment groups showed improvement in decreasing cell proliferation. In treatment group I extract with a dose of andrographolide 10 mg/kg BW (Figure 4B) showed that the enlargement of the nucleus was reduced, but the epithelial cells that proliferated and showed hyperchromatic nuclei were still present. Treatment group II extract with a dose of andrographolide 30 mg/kg BW (Figure C) showed a more pronounced decrease in proliferation and cell boundaries. Treatment group III extract with a dose of andrographolide 100 mg/kg BW (Figure D) also showed a decrease in proliferation, but the difference with treatment group II was not clear.

Telomerase expression after treatment with *A. paniculata* extract in the three treatment groups can be observed in Figure 5. When compared with DMBA group, it can be clearly observed that the telomerase expression in the treatment group decreased. This can be seen from the reduced number of cells whose nuclei are brown. The color intensity in the treatment group appeared sharper and varied. In treatment group I (Figure 5B), cells expressing nuclear telomerase appear larger and elongated, indicating that the cell is in a mitotic state. The difference in telomerase expression between treatment groups could not be known because scoring could not be done due to cutting the tissue that was too thick. From immunohistochemistry, it is known that treatment using *A. paniculata* extract can reduce the proliferation of colonic epithelial cells exposed to DMBA which is characterized by a decrease in telomerase expression.

The expression of caspase-3 in the group that had received *A. paniculata* extract treatment can be seen in Figure 6. The difference in the immunohistochemistry between DMBA group and the treatment group that received *A. paniculata* extract treatment was that the number of caspase-3 expressions was more often found in the treatment group. When compared with the test control group (Figure 6A), the color intensity of epithelial cells expressing caspase-3 in treatment groups I (Figure 6B) and II (Figure 6C) was sharper. In the treatment group III

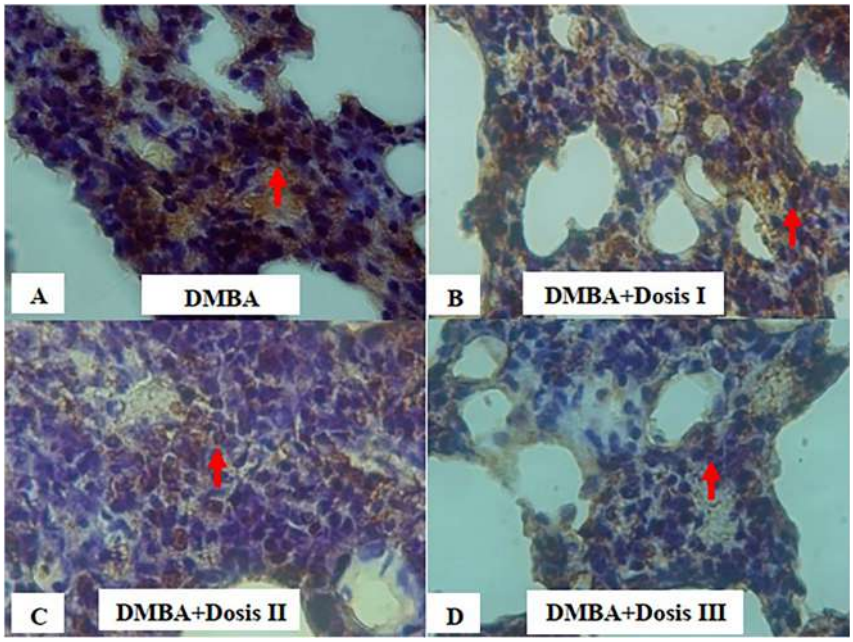


**Figure 2:** Immunohistochemistry staining using telomerase antibodies in SD rats lung given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide using Hematoxylin-Eosin staining at 400x magnification. Red arrows indicate cells that express telomerase.

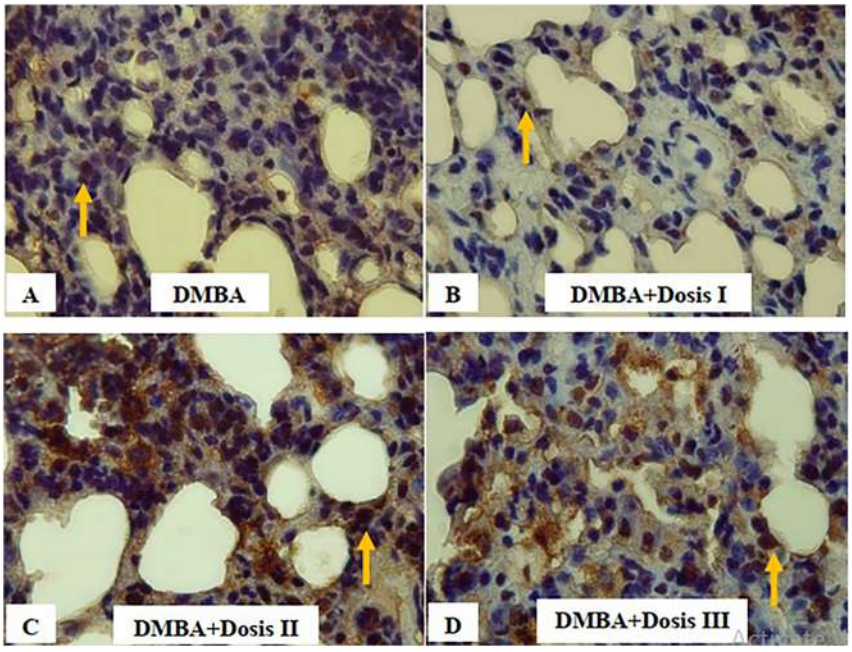


**Figure 3:** Immunohistochemistry staining using caspase-3 antibodies in SD rats lung given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide using Hematoxylin-Eosin staining at 400x magnification. Yellow arrows indicate cells that express caspase-3.

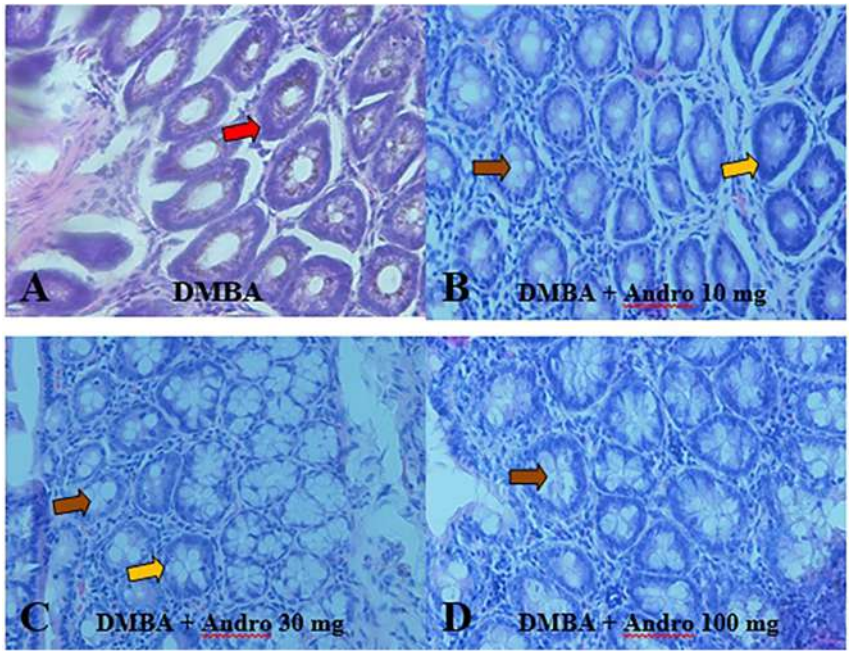


**Figure 4:** Histopathology of SD rats colon given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide Hematoxylin-Eosin staining at 400x magnification. Red arrow indicates epithelial cells without treatment, yellow arrow indicates epithelial cells after treatment, brown color indicates goblet cells.

(Figure 6D), the apoptosis also increased with a more diverse color intensity.

## Discussion

Lung and colon cancer is one of the leading causes of cancer death. Several studies on the causes of this cancer state that the development of these cancers is related to dietary factors, environment, lifestyle, and comorbid conditions as well as genetic factors due to genetic mutations and physical weakness [1]. Experimental models showing complex cancer-causing interaction equations are needed to test various mechanisms and determine the carcinogenic potential of a chemical compound. In this study, the effect of telomerase and caspase-3 expression on lung and colon cancer that given *A. paniculata* extract was carried out using experimental animals Sprague-Dawley rats with induction of 7,12-Dimethylbenz(a)anthracene (DMBA).

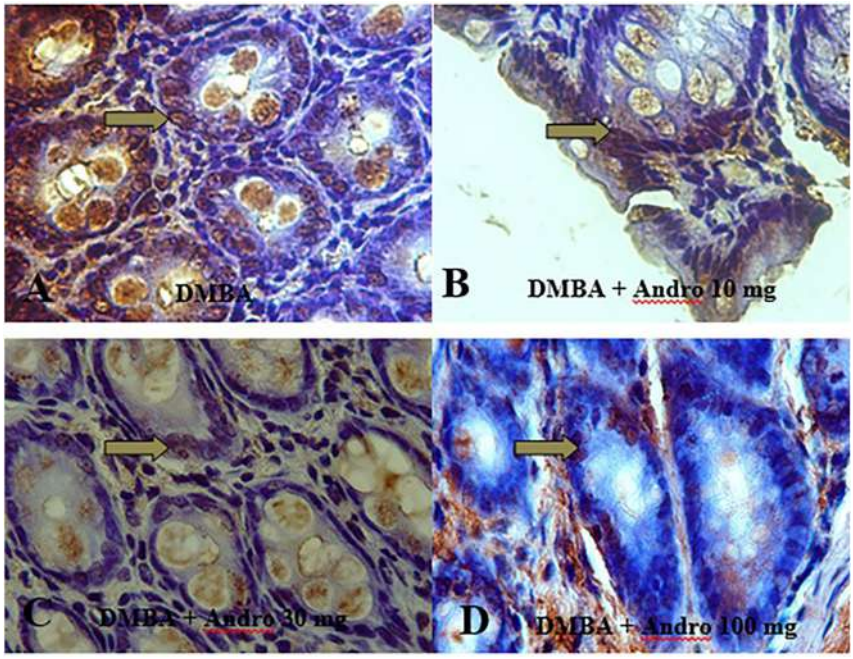


**Figure 5:** Immunohistochemistry staining using telomerase antibodies in SD rats colon given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide using Hematoxylin-Eosin staining at 400x magnification. Gray arrows indicate cells that express telomerase.

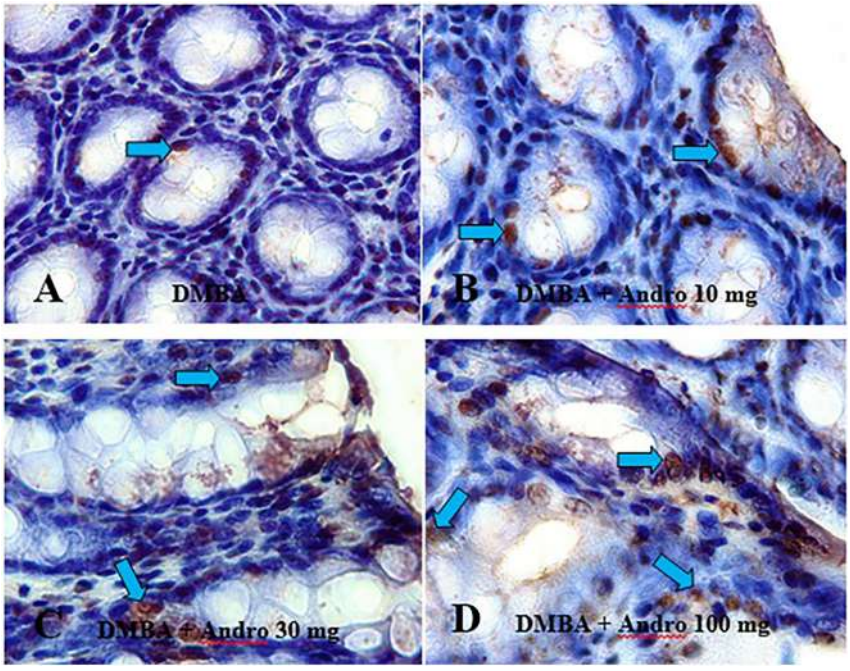


**Figure 6:** Immunohistochemistry staining using caspase-3 antibodies in SD rats colon given DMBA (A) and given *A. paniculata* extract with doses equivalent to 10 mg/kg BW (B), 30 mg/kg BW (C), and 100 mg/kg BW (D) andrographolide using Hematoxylin-Eosin staining at 400x magnification. Blue arrows indicates cells that express caspase-3.

The use of experimental animals in cancer research is supported by the fact that experimental animals have in common with humans in terms of response to exposure to carcinogens. Some of the protooncogenes and gene suppressor tumors in humans are homologous to rats. The difference is that in rats, fewer genetic changes are required [11]. Long-term studies on rat models prove that this model is useful for knowing the stages of initiation, promotion, and progress of carcinogenesis.

DMBA is a polycyclic aromatic hydrocarbon (PAH) carcinogen. Chronic exposure to PAH class carcinogens can lead to cancer, especially in cells that have a fast replicative cycle, for example bone marrow, skin, and lungs. Previous research conducted by Harris et al. [12] stated that the induction of benzo (a) pyrene can cause adenoma in the colon. In other studies, it is demonstrated that oral administration of DMBA can induce lung cancer [13].

Evaluation of the effect of *A. paniculata* extract on lung and colon carcinogenesis in rats was carried out by histological observation using Immunohistochemistry. Cell proliferation can be determined by observing protein expression telomerase in cancer [14]. In addition, an observation of the expression of caspase-3 which was an effector caspase in the apoptosis process with immunohistochemistry staining using caspase-3 antibodies. In lung carcinogenesis, pulmonary septal interalveolar cells of rats given DMBA, whereas in normal rats, there was no expression of telomerase. In normal cells, the expression of telomerase is very low due to the activation of checkpoints in the cell cycle which causes telomere shortening. The shortening of the telomere causes the cell to enter the senescence phase, which was the phase where the cell does not divide anymore [2].

Observations of caspase-3 activity were also carried out to determine the presence of apoptosis in cells. From the observations, it was found that no caspase-3 expression was seen in normal cells, while cells that had been exposed to DMBA appeared to express caspase-3. An increase in caspase-3 expression indicated apoptosis due to DMBA administration which could cause DNA damage that triggered apoptosis.

Efforts that can be made to inhibit carcinogenic processes are by suppressing telomerase activity so as to suppress cell proliferation and induce apoptosis. *A. paniculata* is a plant known to have anticancer properties. Therefore, the observation of the effect of *A. paniculata* extract on proliferation and apoptosis in lung cells of SD rats that had been induced by DMBA was conducted. To determine the effect of *A. paniculata* extract on rats that have been induced by DMBA, histological microscopic observation using immunohistochemistry was carried out using telomerase and caspase-3 antibodies.

Observation of telomerase activity was carried out qualitatively by looking at the cells that expressed telomerase, cells whose nuclei were brown due to the application of chromogen DAB on immunohistochemistry staining. Observations were made using a light microscope with 400x and 1000x magnifications. The decrease in telomerase protein expression was carried out by observing interalveolar septal cells in several fields of view and then compared between treatment groups. From these observations, it was found that there was a decrease in telomerase expression in rat lung cells after giving *A. paniculata* extract. Meanwhile, on the caspase-3 activity examination to determine the presence of apoptosis, the results showed that there was an increase in caspase-3 expression in the lung cells of rats given *A. paniculata* extract which was shown in brown on the cells. However, from the qualitative analysis conducted, it was not known the optimal dose that could significantly suppress telomerase activity and induce apoptosis.

In colon carcinogenesis, the difference in cell morphology between the DMBA-given group and the treatment group (*A. paniculata* extract) can be seen in Figure 4. With an increase in the dose of *A. paniculata* extract given, it was seen that there was an improvement in the form of decreased epithelial cell proliferation and goblet cell formation began to clear. Although hyperchromatic nuclei were still observed in the treatment group, its intensity was not as big as the control group. *A. paniculata* extract plays a role in reducing the initiation and promotion of carcinogens against colon cancer but does not inhibit its progress. This mechanism is often obtained in the anticancer of plant extracts [15].

Furthermore, an immunohistochemistry examination was carried out to determine how changes in cell proliferation and apoptosis after DMBA exposure and treatment with *A. paniculata* extract. In this study, an increase in cell proliferation due to chronic DMBA exposure to rats colonic epithelial cells was observed with an increase in telomerase enzyme expression. Telomerase acts as a major positive regulator of telomere length and can bind to telomeres which facilitate the elongation of chromosomes and prevent their shortening. Progressive shortening occurs during cell division so that increased telomerase activity can be found in cells that are actively proliferating. Cells that express telomerase positive will appear brown in the nucleus region. The immunohistochemistry features using telomerase antibodies can be seen in Figure 5.

In Figure 5, it is clear that the expression of the telomerase in the DMBA-given group is greater than that in the normal control group. This suggests that exposure to DMBA can increase the proliferation of colonic epithelial cells. The DNA adducts binding between DMBA metabolites and DNA causes mutations. In the treatment group that received *A. paniculata* extract treatment equivalent to 10, 30, and 100 mg andrographolide/kg BW of rats showed a decrease in telomerase enzyme expression. This shows that *A. paniculata* extract can reduce the expression of the telomerase enzyme so that it can limit uncontrolled cell growth as the beginning of carcinogenesis.

In addition to reducing cell proliferation, apoptosis is indispensable to prevent carcinogenesis. Apoptosis as an important mechanism in controlling cancer cell growth was observed in this study using immunohistochemistry examination with caspase-3 antibodies. In cancer conditions, the apoptosis process cannot run properly, so that cell growth is not controlled. Cells that are positive to express caspase-3 will show a brown color in the cytoplasm and over time it can be observed in the nucleus [16].

Immunohistochemistry results of colonic tissue with caspase-3 antibodies can be seen in Figure 6. Both treatment groups I, II, and III, colonic epithelial cells express caspase-3. Observation of the color intensity shows that the intensity in the treatment group is greater and clearer than the control test. The number of cells expressing caspase-3 in the treatment group was also higher.

The use of caspase-3 antibodies to detect apoptosis is a sensitive, accurate, and independent method of DNA fragmentation [16]. Previously, it was known that treatment with *A. paniculata* extract could reduce the activity of the telomerase enzyme. Inhibition of telomerase activity can induce apoptosis, namely programmed cell death which genetically regulates the development and homeostasis of an organism [5]. Because hyperplasia is an early stage of cancer development, it is hoped that *A. paniculata* extract can be used as an alternative to more effective colon cancer therapy.

## Conclusions

From this study, it can be seen that *A. paniculata* extract is able to suppress telomerase activity and increase the occurrence of apoptosis, this can be used as an early stage in developing the use of *A. paniculata* as an anticancer. Moreover, *A. paniculata* extract has the ability to inhibit carcinogenesis in colonic epithelial cells due to chronic DMBA exposure through inhibition of telomerase enzyme activity, thereby reducing proliferation and inducing apoptosis which is marked by an increase in caspase-3.

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**Ethical approval:** All experiments were conducted at the Animal Research Laboratory of the Faculty of Pharmacy University of Airlangga, Surabaya, Indonesia in accordance with the Guideline for the Care and Use of Laboratory Animals issued by the National Institutes of Health revised in 1985. The Ethic Committee of Faculty of Veterinary Medicine Universitas Airlangga, Surabaya, Indonesia, approved the study protocol.

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Devy Maulidya Cahyani, Andang Miatmoko*, Berlian Sarasitha Hariawan, Kusuma Eko Purwantari and Retno Sari

# N-nitrosodiethylamine induces inflammation of liver in mice

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**Abstract**

**Objectives:** For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for five weeks induction.

**Methods:** The BALB-c mice were induced with NDEA 25 mg/kg of body weight once a week for five weeks intraperitonially and it was then evaluated for the body weight during study periods. The mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances and histopathology evaluations.

**Results:** The results showed a significant decrease of body weight of mice after five times induction of 25 mg NDEA/kgBW per week intraperitonially. Different morphological appearances and weight of mice organs specifically for liver and spleen had also been observed. The histopathology examination showed that there were hepatic lipidosis and steatohepatitis observed in liver and spleen, respectively that might indicate the hepatocellular injury.

**Conclusions:** It can be concluded that inducing mice with NDEA intraperitonially resulted in fatty liver disease leading to progress of cancer disease.

***Corresponding author: Andang Miatmoko**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, 60115, Surabaya, Indonesia, Phone: +6231 5933150, E-mail: andang-m@ff.unair.ac.id

**Devy Maulidya Cahyani, Berlian Sarasitha Hariawan and Retno Sari,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

**Kusuma Eko Purwantari,** Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

**Keywords:** cancer; inflammation; liver; mice; n-nitrosodiethylamine.

## Introduction

Cancer is the world's leading health problem and the second leading cause of death in United States [1]. Cancer continues to increase worldwide, primary liver cancer is the leading cause of cancer with case about 841,000 new patients and causing 782,000 deaths in 2018 [2, 3]. There are two types of liver cancer, first *Hepatocellular carcinoma* (HCC) which causes 75% of all liver cancer cases and *Intrahepatic Cholangiocarcinoma* (ICC) which causes 12–15% of incidence [4]. HCC comes from hepatocytes, in which it is caused due to oxidative stress, inflammation, and is based on liver disease. On the other hand, ICC appears on c*holangiocyte* which is an intrahepatic bile duct [4, 5]. The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumor initiation, growth, development, and metastasis [6]. Inflammation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte/macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].

In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic

immune system, allowing cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].

Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a "secretome" that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].

The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC.

Making animal models provides a great opportunity to study a disease as well as designing strategies for the treatment, whether it is preventive or curative actions [10]. Preventive care could highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice are often used for animal model, especially for cancer research [11]. This is because animals, especially rodents, have biological similarities both genetically and physiologically to humans. Therefore, the use of mice as experimental animal models is very suitable to identify the dangers caused by a xenobiotic or study the pathogenesis of a disease [12, 13].

The most common animal models of cancer are *xenograft* models [14]. However, the animals models using the *xenograft* model has a weakness, such as it can harm the immune system so it cannot represent cancer that occurs naturally in humans [11]. Another method of using mice as the inflammation disease model is the induction of hepatocarcinogen. Chemically, hepatocarcinogen can cause changes in the DNA structures and instability including N-Nitrosodiethylamine (NDEA), aflatoxine, carbon tetrachloride, dimethylnitrosamine, and thioacetamide. Inducing hepatocarcinogens using NDEA is a commonly used method for producing HCC animal model [11, 12].

In liver, NDEA can induce progressive, proliferative, and mutagenic metabolism of tumors, so it can cause a wide variety of tumors in all animal models by intraperitoneal injection for about 8 weeks or more [15]. NDEA can produce pro-mutagenic products namely $O^6$-ethyl deoxy guanosine and $O^4$ and $O^6$-ethyl dioxy thymidine in the liver which are responsible for its carcinogenic effects [16]. NDEA, which is a chemical hepatocarcinogen, is also known to induce the Transforming Growth Factor Alpha (TGF-α) expression, which is closely involved in hepatocarcinogenesis and transformation in humans and animals [17]. NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy. Thus, this study aimed to evaluate the liver disease model observed in mice induced with hepatocarcinogenic NDEA for five weeks intraperitonal injection.

# Materials and methods

## Materials

N-Nitrosodiethylamine was purchased from Sigma-Aldrich (Tokyo, Japan). Normal saline was the product of PT. Widathra Bhakti (Pasuruan, Indonesia). This study used male Balb/c mice aged six weeks obtained from the animal laboratory, Faculty of Pharmacy, Universitas Airlangga.

## Induction of NDEA in mice

All of the experimental procedures using animals had been approved by the Ethics Commission of Faculty of Veterinary, Universitas Airlangga. The mice were induced for liver disease by using NDEA diluted in normal saline. Mice were given NDEA intraperitonially at a dose of 25 mg/kgBW. The NDEA injection was given five times every seven days for five weeks. The disease progress induced by NDEA was evaluated by weighing the mice body weight every week.

## Preparation of mice organs

At the end of NDEA induction, the mice were then sacrificed and excised for evaluating their organs (heart, lungs, liver, spleen, and kidneys) including physical and morphological appearances. The organs including liver and spleen were excised and stored at −20 °C for further analysis. The organs were evaluated for the weight and morphological appearances. Moreover, the histopathology evaluations were also performed by hematoxylin-eosin staining for liver and spleen tissues.

### Data analysis

The results were presented as the mean ± SD. To determine the significant differences between data, a statistical analysis was carried out using the Oneway Analysis of Variance (ANOVA) method which was followed with the Honestly Significant Difference (HSD) post hoc test. The difference was statistically significant if the p-value was <0.05.

## Results

### Body weight evaluation of mice induced with NDEA

To evaluate the results of NDEA induction, the mice induced by NDEA 25 mg/kg per week were weighed every week and compared with mice injected with normal saline used as the control. The presence of weight loss in mice induced by hepatocarcinogens is one of parameters for cancer progress. The evaluation results of mice body weight can be seen in Figure 1. The NDEA-induced mice experienced weight loss while normal mice gained weight continuously. The results showed that there was a significant weight loss on the 29th day after five times NDEA induction. On the 31st day, the mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances.

### Physical appearances of mice organs

Based on observation of excised organs shown in Figure 2A–C, there were differences between organs specifically for liver and spleen of mice induced with normal saline and with NDEA for five weeks. In the control group, the morphological appearances of liver were shiny and bright red (Figure 2A). However, mice induced with NDEA had liver appearances with nodules and discoloration (Figure 2C). This suggests that NDEA induction for five weeks affects the liver cells, causes liver damage, and changes the external morphology of the liver of mice.

### Evaluation weight of mice organ

The organ weights of mice in the control and NDEA-induction groups were evaluated to determine whether there were any significant differences on the physical weight during the induction. As it can be seen in Table 1, the liver in mice induced with NDEA was significantly relatively smaller than the control group ($p<0.01$), while the spleen were slightly smaller but no significant differences was observed ($p>0.05$).

### Histopathological evaluations of liver tissue

According to the results as shown in Figure 3, the normal liver and spleen had regular architecture and cellular integrity with no fibrosis. After induction of NDEA, there were no malignancies observed in liver on spleen tissues in mice; however, there were single large fat droplets, alongside nuclei dislocation to the cell periphery that seemed to be macrovesicular steatosis. According to these results, there were lipidosis in liver and steatohepatitis observed for spleen tissue.

## Discussion

Making the ideal of animal models of liver disease with pathological analogous to liver disease in humans,

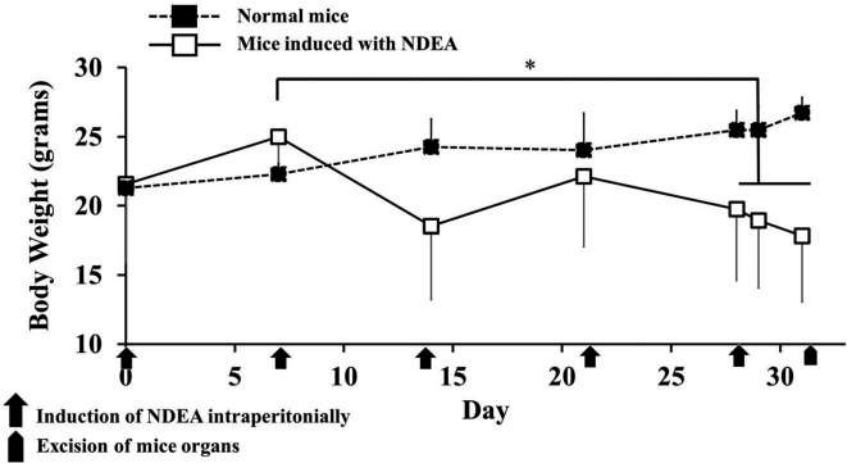


**Figure 1:** The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25 mg/kgBW intraperitonially once a week for five times and mice were then sacrificed at day 31 (n=7). **p<0.05.

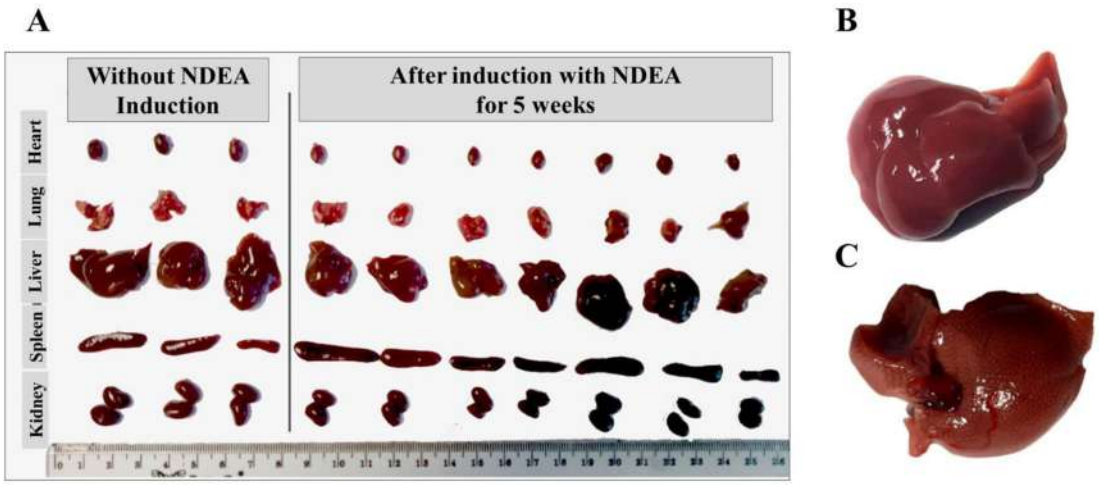


**Figure 2:** The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for five times, n=7.
(A) The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice.

**Table 1:** Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25 mg/kg five times then mice were sacrificed and excised for evaluating their organ (n=7).

| Organ | Organ weights (mean ± SD) | |
|---|---|---|
| | Control | After NDEA induction |
| Heart | 0.11 ± 0.01 g | 0.08 ± 0.03 g |
| Lungs | 0.20 ± 0.04 g | 0.32 ± 0.05 g |
| Liver | 1.86 ± 0.13 g | 0.97 ± 0.27 g |
| Spleen | 0.23 ± 0.12 g | 0.20 ± 0.12 g |
| Kidneys | 0.40 ± 0.05 g | 0.25 ± 0.06 g |

especially for HCC cancer formation model both pathologically and biochemically is a challenge for researchers [18]. NDEA is a compound that is generally known to be mutagenic, teratogenic, and carcinogenic. Recent study reports that the use of NDEA as a hepatocarcinogen is known to have a strong ability and is able to induce primary liver cancer such as HCC which is at various stages of liver cirrhosis, besides that it can greatly simulate the histopathological evolution of clinical liver cancer [19].

It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered

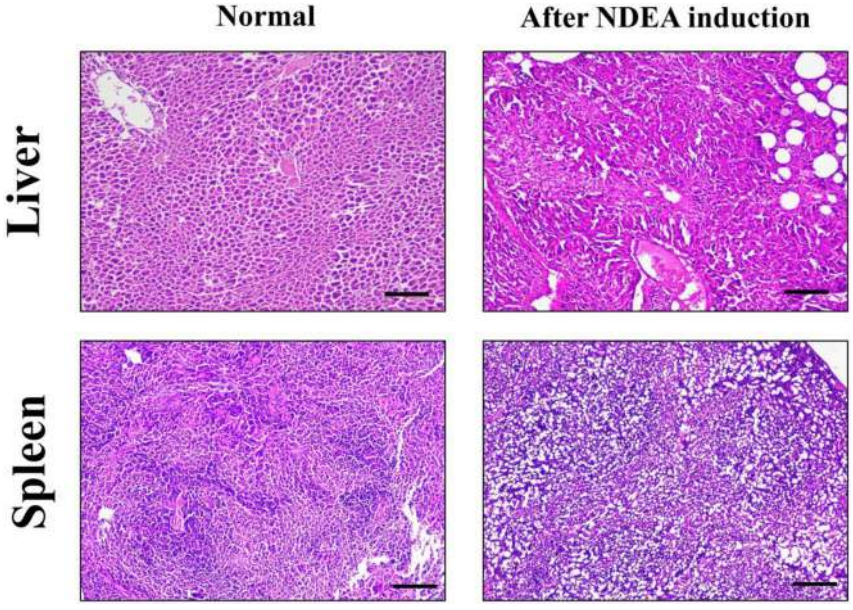


**Figure 3:** The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for five times. Scale bar=100 µm.

mitosis in liver tissue [20]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents. NDEA induction at a dose of 25 mg/kgBW for five weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [21]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [22]. However, in this study, there was no evaluation of food consumed by the mice during the experiments.

Based on the weight data for each organ shown in Table 1, it was known that the weight of liver organs in the treatment group decreased compared to control group. NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [23]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [24]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group.

NDEA induction for five weeks affects liver cells, causes liver damage, and changes the external morphology of the liver of mice. Previous studies report NDEA induction in mice causes a change in the structure of the liver in mice which is characterized by a reduction in size, discoloration, bleeding, scarring, and formation of nodule-like structures [25]. This is because NDEA is a toxic agent against the liver that can cause liver fibrosis [25, 26]. Fibrosis is formation of excess connective tissue, causing hardening and scar formation, in which about 20% of cancer cases are associated with chronic inflammation due to fibrosis, as found in liver cancer [27]. However, in this study, instead of malignancies, hepatic lipidosis and steatohepatitis were observed in mice liver and spleen after five weeks induction of NDEA. This indicates that the disease progress is still in the early stage of liver cancer diseases. It has been known that hepatic lipidosis is an early manifestation of some other underlying conditions related to cancer, pancreatitis, and other liver problems [28]. Another study reports that rats induced with NDEA will show the appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis after eight weeks of NDEA induction [20]. This early disease stage can be used for exploring preventive therapy of some drug compounds, such as for comparing the efficacy of drug delivery system. Lipid peroxidation and oxidative stress are dangerous to cells resulting in liver injury, which leads to liver fibrosis and cirrhosis or cancer. However, further biochemical investigation is required to definitely score the stage of liver disease after five weeks induction of NDEA.

## Conclusion

Induction of NDEA in mice for five weeks results in hepatic lipidosis or fatty liver and steatohepatitis confirmed as the liver inflammation which may indicate the early stage of liver cancer disease, thus providing the potential use of NDEA for making animal models for the preventive cure of liver disease.

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Angelica Kresnamurti*, Dita Nurlita Rakhma, Amitasari Damayanti, Septiyan Dwi Santoso, Enggar Restryarto, Wifqi Hadinata and Iwan Sahrial Hamid

# AST/ALT levels, MDA, and liver histopathology of *Echinometra mathaei* ethanol extract on paracetamol-induced hepatotoxicity in rats

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**Abstract**

**Objectives:** *Echinometra mathaei* was known to have potential antioxidant activities because it contains of *polyhydroxy-naphthoquinone* (*echinochrome* and *spinochromes*). The antioxidant properties contributed to the hepatoprotective effect by binding to free radicals compound that causes oxidative stress and necrosis in the hepatocytes. The research aimed to determine the hepatorepair effects of the *E. mathaei* ethanol extract on high-dose paracetamol-induced hepatic damage in Wistar rats.

**Methods:** This research used a true experimental method. Thirty white male rats were divided into sixth groups, i.e., normal control group, group II–VI was induced paracetamol 2,000 mg/kg BW for three days. After paracetamol-induced, group III–VI was treated with curcumin 800 mg/kg BW, *E. mathaei* extract 400, 800, and 1,200 mg/kg BW for seven days. The hepatorepair parameter was obtained from AST/ALT, MDA tissue levels, and the number of hepatocyte necrosis cells. The data results were analyzed using the ANOVA test, followed by the LSD test to determine the difference between each treatment.

**Results:** The results showed that *E. mathaei* significantly ($p<0.05$) decreased the AST levels, MDA levels and the number of hepatocyte necrosis cells at a dose of 800 mg/kg BW per orally treatment.

**Conclusions:** *The E. mathaei* ethanol extract repaired the hepatic damage induced by paracetamol.

**Keywords:** AST/ALT; *Echinometra mathaei*; hepatocyte necrotic cell; hepatorepair; MDA; paracetamol-induced; sea urchin; Wistar rat.

***Corresponding author: Angelica Kresnamurti**, Pharmacy Study Program, Faculty of Medicine, Hang Tuah University, Surabaya, Indonesia, Phone: +62-82123341711,
E-mail: angelica.kresnamurti@hangtuah.ac.id. https://orcid.org/0000-0001-5094-6767
**Dita Nurlita Rakhma, Amitasari Damayanti, Septiyan Dwi Santoso, Enggar Restryarto and Wifqi Hadinata,** Pharmacy Study Program, Faculty of Medicine, Hang Tuah University, Surabaya, Indonesia
**Iwan Sahrial Hamid,** Pharmacology, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia

## Introduction

The liver has a big role in maintaining nutrient metabolism and xenobiotic detoxification, susceptible to damage [1]. Indications of liver damage shown by increasing levels of hepatic enzymes in the blood [i.e., alanine transaminase (ALT) and aspartate transaminase (AST)], and high lipid peroxidation [2]. The lipid peroxidation process increased MDA levels and decreased the number of endogenous antioxidants [3]. MDA acts as an indicator of repeated lipid peroxidation processes. MDA levels' elevation occurs due to oxidative stress in hepatocyte tissue after long-term or overused hepatotoxic drugs [4]. Drug-induced hepatotoxic (DIH) can be triggered by the use of drugs metabolized in the liver with long-term use or excessive doses. The drug is metabolized in the liver into an active metabolite. If the endogenous antioxidants are lower than active metabolites, the drug turns into free radicals that damage cells [5].

Paracetamol is widely used as an analgesic and antipyretic drug at its therapeutic doses [6]. However, excessive use of paracetamol leads to liver damage due to the formation of N-acetyl-p-benzoquinone (NAPQI) and free radicals through the biotransformation process by the cytochrome P450 enzyme [7, 8]. The long-term use of paracetamol causes hepatocyte necrosis due to damage from NAPQI elevation [9–11]. This condition requires a hepatorepair agent, a compound that protects cells and repairs liver tissue damaged by exposure to toxic substances. The hepatorepair agent plays its role through antioxidant mechanisms.

In several countries with marine areas, several studies on the antioxidant potential of marine biota have begun. Kuwahara et al. [12] have researched the antioxidant

activity of *polyhydroxy naphthoquinone* (PHNQ) pigment, isolated from *Anthocidaris crassispina* sea urchins. The results showed potent free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide radicals, anions, and hydrogen peroxide [12, 13]. Other studies have shown the potential antioxidant activity of PHNQ pigment from *Scaphechinus mirabilis*, one type of sea urchin [13]. *Echinometra mathaei* from the Persian water is known to have antioxidant and anti-inflammatory potential [14], and the echinocrome, a pigment that isolated from sea urchin was proved a hepatorepair activity in septic rats [15].

The natural agents against free radicals, such as antioxidants, may be useful therapeutics for repairing hepatotoxicity induced by drugs. Therefore, this study was conducted to observe the effect of *E. mathaei* ethanol extract against paracetamol-induced liver damage in rats. Curcuma was chosen as a positive control because it has been used clinically as a hepatorepair and hepatoprotective agent for TB patients [16] and is one of the most commonly used indigenous molecules endowed by various shielding functionalities that protects the liver [17].

# Materials and methods

### Preparation the extract

Purple Sea urchin (*E. mathaei*) was collected from Weh Island, Sabang, Nangroe Aceh Darussalam, Indonesia, under the sea level in October 2018. The Biology Service Unit from the Faculty of Sains and Technology, Airlangga University Surabaya, has authenticated the sample.

The shell and gonad were not separated but mixed, shaded, into a dried coarse powder. The sample powder was then extracted with 70% ethanol using three cycles of solvent replacements (1:10) to maximize the extraction through maceration, concentrated by rotary evaporator, and then dried.

### Animals handling

White male Wistar rats (*Rattus norvegicus*) weighing 150–250 g were obtained from The Surabaya Government Veterinary Research Center. The rats were housed in individual cage, at room temperature (28 ± 2 °C) and a 12 h light/dark cycle.

### Experimental design

Thirty white male Wistar rats were divided into six groups: normal control (aquadest was given daily without paracetamol induction); negative control (only given paracetamol 2,000 mg/kg BW per oral once daily for three days and aquadest at day 4–10), and four treatment groups (paracetamol 2,000 mg/kg BW per oral once daily for three days followed by curcuma as positive control, *E. mathaei* extract 400, 800, and 1,200 mg/kg BW once daily at day 4–10). On day 11, the AST/ALT levels were measured from blood specimens, while MDA concentration and histopathology were determined from the liver tissue. This study was reviewed by the Ethical Clearance Committee for Preclinical Research, and obtained approval number code: S.Ket/035/KEPK-FKGUHT/XI/2019.

### Malondialdehyde assays

The rat's MDA levels were obtained from liver tissue samples taken on the 11th day after treatment as follows: all rats were anesthetized with cocktail ketamine, then the liver tissue was taken and euthanized on the rat. The liver tissue sample as much as 0.5 g was crushed together with quartz sand using a mortar until it was smooth, and 200 µL of physiological NaCl was added to the mortar. The homogeneous preparation was put into a centrifuge tube, added 550 µL of distilled water, 100 µL of TCA, then homogenized, added 250 µL of 1N HCl. The mixture was added with 100 µL of Na-Thio1% and then 500 rpm centrifuged for 10 min. Supernatant was taken and then filtered using glass wool. The supernatant obtained was heated using a 100 °C water bath for 20 min. The supernatant that has been heated is then cooled to room temperature. Then determined the absorbance value of the sample using a UV–Vis spectrophotometer at a maximum wavelength of 532 nm. The concentration was expressed as nanomole per gram of tissue.

### Histopathological study

The liver obtained in each group were kept in 10% formalin solution. Then fixed liver was embedded in paraffin, and serial sections were cut for histopathological examination.

### Statistical analysis

Data were expressed as mean ± SD. $p<0.05$ was considered significant.

# Results

The results showed that peroral administration of Paracetamol 2,000 mg/kg BW for three days resulted in a significant increase of AST/ALT, and MDA levels (Table 1), and the number of the necrotic cells of hepatocytes compared to negative control group ($p<0.05$) (Table 2) suggest that the liver damaged has formed and there was shown that the liver damage due to paracetamol-induced showed no improvement of AST level, MDA level, and the number of necrotic cells for seven days after paracetamol

**Table 1:** Effects of *Echinometra mathaei* extract on paracetamol-induced changes in serum hepatic marker enzymes and MDA level.

| Groups | AST, U/L | ALT, U/L | MDA, nmol/g |
|---|---|---|---|
| Normal control | $62.75 \pm 2.06^{b,c}$ | $22.75 \pm 1.71^{b,c}$ | $890.25 \pm 395.93^{b}$ |
| Negative control (only paracetamol 2,000 mg/kg BW) | $90.75 \pm 2.22^{a}$ | $27.00 \pm 0.82^{a}$ | $1{,}219.75 \pm 131.54^{a,c}$ |
| Positive control (curcuma 800 mg/kg BW) | $67.75 \pm 2.06^{a,b}$ | $28.00 \pm 0.82^{a}$ | $718.50 \pm 292.35^{b}$ |
| *E. mathaei* 400 mg/kg BW | $68.00 \pm 0.82^{a,b}$ | $27.25 \pm 0.96^{a}$ | $1{,}096.75 \pm 142.02^{c}$ |
| *E. mathaei* 800 mg/kg BW | $60.00 \pm 4.00^{b,c}$ | $25.33 \pm 0.58^{a,c}$ | $596.67 \pm 245.93^{b}$ |
| *E. mathaei* 1,200 mg/kg BW | $67.40 \pm 1.52^{a,b}$ | $26.00 \pm 1.23^{a,c}$ | $535.20 \pm 87.84^{a,b}$ |

[a]Significant difference with normal control group; [b]Significant difference with negative control group; [c]Significant difference with positive control group; with ($p<0.05$).

**Table 2:** Effects of *Echinometra mathaei* extract on the number of hepatocyte necrotic cells.

| Groups | Number of necrotic cells |
|---|---|
| Normal control | $4.00 \pm 1.00^{b,c}$ |
| Negative control (only paracetamol 2,000 mg/kg BW) | $14.00 \pm 3.00^{a,c}$ |
| Positive control (curcuma 800 mg/kg BW) | $9.00 \pm 2.00^{a,b}$ |
| *E. mathaei* 400 mg/kg BW | $8.00 \pm 2.00^{a,b}$ |
| *E. mathaei* 800 mg/kg BW | $6.00 \pm 1.00^{b,c}$ |
| *E. mathaei* 1,200 mg/kg BW | $10.00 \pm 2.00^{a,b}$ |

[a]Significant difference with normal control group; [b]Significant difference with negative control group; [c]Significant difference with positive control group; with ($p<0.05$).

induction, without drug treatment (irreversible damage during the experimental period).

Paracetamol induction in this study showed different macroscopic observations and histopathological changes. The macroscopic observation showed that the negative control group's liver tissue experiences hepatomegaly from the liver tissues' weight. The histopathological observation showed that paracetamol-induced changes such as loss of the normal structure of hepatic cells, blood congestion, fatty degeneration, increased damaged of the cell and processed to become a necrotic cell. In contrast, animals treated with *E. mathaei* extracts after Paracetamol induction showed an improvement in these changes and the tissue appeared with normal structures (Figure 1).

Treatment with *E. mathaei* extract significantly ($p<0.05$) decreased the elevation of AST/ALT, MDA levels, and the number of necrotic hepatocytes cells in all doses as compared to negative control group, except at the doses of 400 mg/kg BW for SGPT level. *E. mathaei* extract at a dose of 800 mg/kg BW provides the best improvement for AST/ALT, MDA levels, and a decrease in the number of damaged hepatocyte cells compared to treatment with Curcuma tablets.

# Discussion

There have been several studies of natural resources as hepatoprotector, but research on the potency of marine biota *E. mathaei* is still little finding. In a previous study, Soleimani explained that *E. mathaei* has excellent antioxidant properties obtained from its PHNQ pigment [14]. This study is a preliminary test to determine *E. mathaei* extract's effect as a hepatoprotective, which was conducted based on previous research about its excellent antioxidant properties.

In the present study, paracetamol was used as an inductor of liver damage in rats. Administration of Paracetamol at excessive doses (2,000 mg/kg BW) causes hepatotoxicity through complex mechanisms [18]. In the normal intake dose, the enzymes glucoronyl and sulfotransferases metabolize paracetamol [19]. However, excessive dose intake leads the glucuronidation and sulfation pathways to become saturated and activate P450 enzymes to create NAPQI. An appropriate amount of Glutathione (GSH) plays this role in conjugation. NAPQI will be conjugated to form acetaminophen mercapturic at a low level. Still, those continuous processes will lead to depletion of GSH and elevation of NAPQI [20, 21]. As a result, mitochondrial will run into cellular oxidative stress, lead to alteration of cell homeostasis and permeability disorders such as cell swelling, the elevation of liver enzymes, and MDA levels [22].

In liver damage, enzymes such as AST/ALT, release from the cytosol into the bloodstream [23]. Therefore, AST and ALT are quantitative analyzes to determine liver function. There was a significant elevation in AST and ALT after paracetamol induction at toxic doses in this study. Administration of *E. mathaei* extract at all treatment doses showed a significant reduction in AST levels but not with ALT. That result probably the elevation of ALT that lasts longer than AST. This enzyme is more specific as a liver parenchymal enzyme than AST [24].

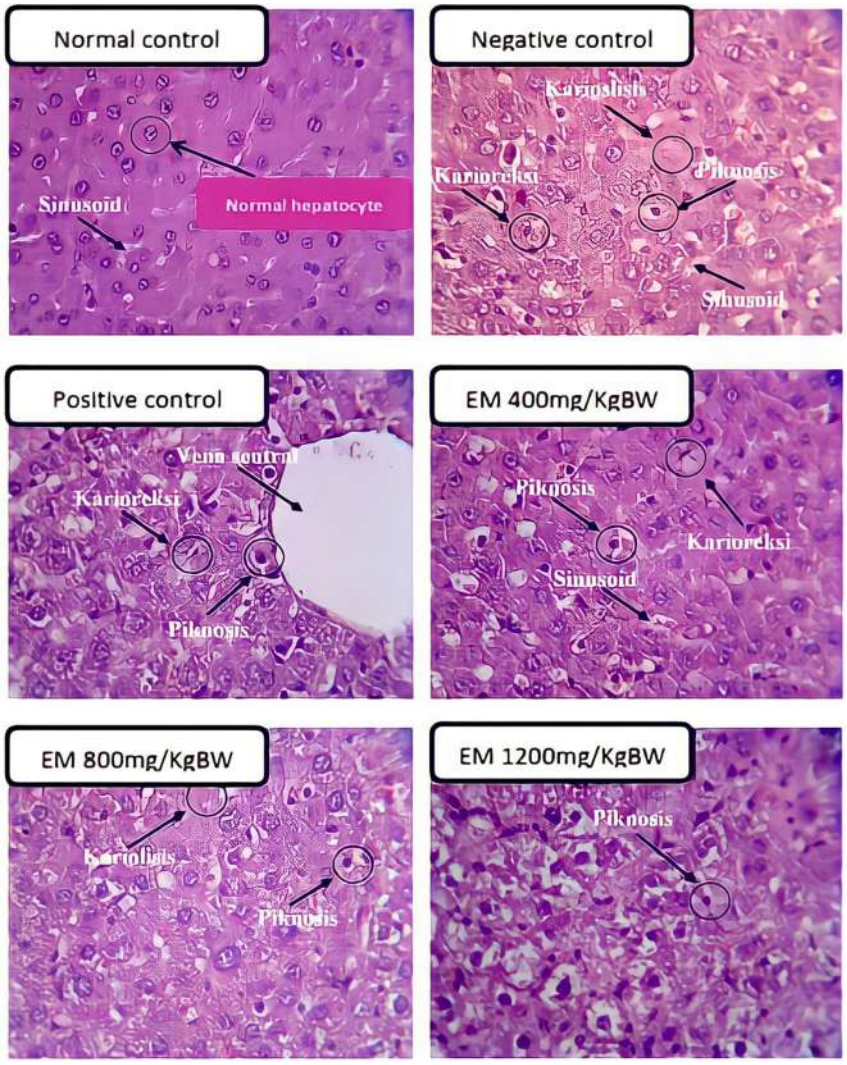


**Figure 1:** Microscopic structure of transverse slices of male white rat liver in each treatment (hematoxylin–eosin staining, 400× magnification).

MDA examination showed an elevation in paracetamol-induced as the negative control group. The treatment groups of *E. mathaei* at 800 mg/kg BW and 1,200 mg/kg BW showed a significant reduction in MDA levels. That result indicates a possible restoration in lipid peroxidation status. Lipid peroxidation is responsible for impaired cell membrane integrity and fluidity, which leads to cell death [25].

Visually, cell damage appears through macroscopic and histopathology examination. In the negative control group, liver enlargement was a hypertrophic condition resulting from liver microsomal metabolic enzyme activity. The activity causes hepatocellular hypertrophy and hepatocellular hyperplasia. Consequently, it possessed a liver enlargement [26, 27]. The histopathological observation showed that paracetamol-induced changes include loss of hepatic cells' normal structure, blood congestion, fatty degeneration, increased damage of the cell, and processed to become a necrotic cell. In contrast, animals treated with curcuma and *E. mathaei* extracts after Paracetamol induction showed an improvement in these changes, and the tissue appeared with normal structures (Figure 1). These visual observation results are proportional to the enzyme and MDA levels examination, confirming *E. mathaei*'s potential as a liver protector.

*E. mathaei* has activity in hepatic repairment as evidenced by the improvement of AST/ALT, and liver tissue MDA levels, which is lower than the negative control group. This result aligns with Soleimani's research where the polyhydroxy naphthoquinone compound derived

from sea urchins *E. mathaei* has antioxidant activity [28]. It suggests that the PHNQ content in *E. mathaei* may effectively repair liver damage, where naphthoquinone captures superoxide ions and breaks the chains between superoxide ions ($O_2^-$), which will inhibit several inflammatory factors and increase Glutathione S-Transferase. Increased glutathione reduces the amount of NAPQI in the liver, utilizing which NAPQI will bind to glutathione to become a non-toxic material [29].

Previous research conducted by Sohair showed that Echinochrome, PHNQ, could repair damage to liver cells [30]. The hepatorepair effect of PHNQ pigment was reported in Sohair study conducted from Sayed et al. [31]. The authors obtained the data that echinochrome pigment reduced the level of MDA against experimentally induced gastric ulcers in rats. Another study by Mohamed found that echinochrome pigment could enhance hepatic protection in septic rats [15]. These findings convinced *E. mathaei* as a potent source of antioxidants. *E. mathaei* extracts could be a potent natural product source and provide a promising hepatoprotective effect against drug-induced hepatotoxicity in rat.

## Conclusions

The *E. mathaei* ethanol extract repaired the hepatic damage induced by paracetamol.

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**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical approval:** The research related to animals used has complied with all the relevant national regulations and institutional policies for the care and use of animals (Ethical Clearance Committee for Preclinical Research, Faculty of Dentistry, Hang Tuah University, Indonesia, and obtained approval number code: S.Ket/035/KEPK-FKGUHT/XI/2019).

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Ni Luh Dewi Aryani, Siswandono Siswodihardjo, Widji Soeratri* and Nadia Fitria Indah Sari

# Development, characterization, molecular docking, and *in vivo* skin penetration of coenzyme Q10 nanostructured lipid carriers using tristearin and stearyl alcohol for dermal delivery

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**Abstract**

**Objectives:** This study aims to develop coenzyme Q10 nanostructured lipid carriers (NLCs) using tristearin and stearyl alcohol as well as isopropyl palmitate (IPP) as solid and liquid lipid respectively for the dermal delivery system.
**Methods:** The coenzyme Q10 NLCs were optimized using tristearin, and stearyl alcohol in different concentrations and further characterized by dynamic light scattering (DLS) for particle size, polydispersity index (PDI), zeta potential, differential scanning calorimetry (DSC) and X-ray diffractometry for crystallinity behavior, Fourier transform infrared spectroscopy (FT-IR) for drug-lipid interaction, scanning electron microscopy (SEM) for particle shape, viscometer for viscosity, and pH meter for pH value. Furthermore, entrapment efficiency (EE), drug loading (DL), and skin penetration *in vivo* were also evaluated while molecular docking was conducted to examine the interaction between coenzyme Q10 and the lipids.
**Results:** The coenzyme Q10 NLCs with tristearin-IPP and stearyl alcohol-IPP as lipid matrix had <1,000 nm particle size, <0.3 PDI, less negative than −30 mV zeta potential, about 41% crystallinity index, and about six as the pH value. Moreover, the EE, DL, viscosity, and *in vivo* skin penetration of the NLCs using tristearin were higher compared to stearyl alcohol, however, the skin penetration depths for both NLCs were not significantly different. Furthermore, the *in silico* binding energy of coenzyme Q10-tristearin was lower compared to coenzyme Q10-stearyl alcohol. Both of them showed hydrophobic and van der Waals interaction.
**Conclusions:** The NLCs of coenzyme Q10 were formulated successfully using tristearin-IPP and stearyl alcohol-IPP for dermal delivery.

**Keywords:** coenzyme Q10; dermal delivery; molecular docking; NLC; skin penetration.

## Introduction

Coenzyme Q10 is a lipid-soluble antioxidant due to the 10 isoprenoid side-chains. Chemically, it is known as 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone [1]. Furthermore, coenzyme Q10 is used for skin anti-aging in cosmetic products. However, the skin penetration is poor due to its lipophilic property and large molecular weight (863.36 g/mol) [2–5]. The nanodelivery system is potentially used to overcome this problem since it enhances dermal penetration due to its active ingredients, lipid nanoparticles constitute part of this system [6, 7].

The two main classes of lipid-based nanoparticles are solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs), the difference between both categories depends on the lipid matrix. For SLNs, only solid lipids are used whereas the lipids matrix for NLCs consisted of solid and liquid lipids. Due to the incorporation of liquid lipids in NLC, the crystal structure arrangement of the solid lipid become disordered hence, the entrapment efficiency (EE), drug loading (DL), and stability of NLC increases [6, 8].

In this study, coenzyme Q10 NLCs were developed using tristearin and stearyl alcohol as solid lipids and isopropyl myristate (IPM) or isopropyl palmitate (IPP) as liquid lipids. Meanwhile, tristearin and stearyl alcohol have different lipophilicity with the former being more lipophilic than the latter [9]. IPM or IPP is considered as a skin penetration enhancer [10].

***Corresponding author: Widji Soeratri**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, E-mail: widjisoeratri@yahoo.com
**Ni Luh Dewi Aryani,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia; and Department of Pharmaceutics, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia
**Siswandono Siswodihardjo,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Nadia Fitria Indah Sari,** Department of Pharmaceutics, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia

Therefore, this study aims to develop the coenzyme Q10 NLCs for dermal delivery using tristearin and stearyl alcohol as solid lipids and IPM or IPP as liquid lipids. The NLC formulas were optimized to obtain optimal coenzyme Q10 NLCs for dermal delivery thereafter, the coenzyme NLCs were evaluated in physicochemical characteristics and *in vivo* skin penetration through rats' skin. Moreover, *in silico* studies via molecular docking were also conducted to elucidate the interactions between coenzyme Q10 and solid lipid.

# Materials and methods

## Materials

The coenzyme Q10 was purchased from Kangcare Bioindustry Co., ltd. Nanjing, China while Tristearin analytical grade was purchased from Sigma Aldrich (St. Louis, MO, USA). Also, Stearyl alcohol, Span 80, phenoxyethanol were purchased from Universal Pharma Chemical (Surabaya, Indonesia) while IPM, IPP, propylene glycol, and Tween 80 were purchased from Bratachem (Surabaya, Indonesia). Furthermore, Ethanol 96%, $NaH_2PO_4$, and $Na_2HPO_4$ (analytical grade) were purchased from E.Merck (Darmstadt, Germany). All materials used in the study fulfilled pharmaceutical-grade unless otherwise stated.

## Preparation of optimized coenzyme Q10 NLCs formulas

Using the high shear homogenization method, the lipids were melted at 80 °C and stirred at 3,400 rpm with ultra turrax for 1 min until a homogeneous mixture was obtained. The 1% Coenzyme Q10 was placed into the mixture and agitated for about 2 min until dissolved. Furthermore, the 2% Span 80 and 18% Tween 80 were heated to 80 °C separately and poured gradually while the 10% propylene glycol and 69% phosphate buffer were also heated to 80 °C and then poured into the lipid phase and stirred until homogeneous for about 1 min, thereafter, the stirring speed was increased to 24,000 rpm for 3 min, at 3,400 rpm, 0.6% phenoxyethanol was added at 40 °C and stirred continuously until room temperature. The coenzyme Q10 NLCs formulations for optimization are shown in Table 1

**Table 1:** The coenzyme Q10 NLCs formulas for optimization (concentration materials in %).

| Materials | Lipid 8% | | | Lipid 10% | | | Lipid 15% |
|---|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 | F7 |
| Coenzyme Q10 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Tristearin | 5.6 | – | – | 7 | – | – | – |
| Stearyl alcohol | – | 5.6 | 5.6 | – | 7 | 7 | 10.5 |
| IPM | – | 2.4 | – | – | 3 | – | – |
| IPP | 2.4 | – | 2.4 | 3 | – | 3 | 4.5 |

NLC, nanostructured lipid carriers; IPP, isopropyl palmitate; IPM, isopropyl myristate.

## Particle size, polydispersity index (PDI), and zeta potential

The nanoparticle analyzer (Nanotrac Wave, Microtrac W3717) was used to measure particle size, PDI, and zeta potential of the coenzyme Q10 NLCs. Meanwhile, before the test, the samples were diluted with appropriate aqua dem.

## Thermal behaviors

Thermal behaviors for coenzyme Q10, the solid lipids, and coenzyme Q10 NLCs were analyzed using differential scanning calorimetry (DSC). Approximately, 4 mg of the samples were heated from 30 to 100 °C in a calorimeter (DSC model 1/500, Mettler Toledo) with a heating rate of 10 °C/min. The percentage of crystallinity index (%CI) was measured using the following equation:

$$\%CI = \frac{\text{enthalpy } (\Delta H) \text{ coenzyme Q10 NLC}}{\Delta H \text{ lipid matrix} \times \text{concentration lipid phase}} \times 100$$

## The X-ray diffraction

Crystallinity behaviors and X-ray diffractions were evaluated using an X-ray diffractometer (Phillip X'pert). The samples were analyzed in $2\theta$ range of 4–40° at 40 kV, 30 mA.

## Fourier transform infrared

Drug-lipid interaction was determined using Fourier transform infrared (FT-IR) spectra. The samples in a KBr were prepared to form a pellet and then scanned at wavelengths of 400–4,000 $cm^{-1}$ using FT-IR Spectrophotometer (Jasco FT-IR 5300).

## Morphology

The coenzyme Q10 NLCs morphology was evaluated using scanning electron microscopy (SEM, ZEISS) with 25,000× magnification.

## Rheology and viscosity

The Cone and Plate viscosimeter (Brookfield AT 17362, spindle CPE-41) were used to determine the rheology and viscosity of the coenzyme NLCs.

## The pH value

The calibrated pH meter (SI analytic LAB 850) was utilized for evaluating the pH value of NLCs.

## The entrapment efficiency (EE) and drug loading (DL)

The indirect method was used to assign the EE and the DL of coenzyme Q10 NLCs. The free coenzyme Q10 was obtained through centrifugation of coenzyme Q10 NLC at 10,000 rpm for 30 min. Prior to this, the

former was diluted with a known amount of aqua dem. The filtrate absorbance was measured using a spectrophotometer UV1800 (Shimadzu) at a wavelength of 275 nm.

### *In silico* studies by molecular docking

The two and three-dimensional (2D and 3D) chemical structures of coenzyme Q10, tristearin, and stearyl alcohol were generated using ChemDraw® Pro 2016 (Cambridgesoft), as well as the energy minimization process using the same program. Also, molecular docking of coenzyme Q10 and solid lipids were performed by Autodock Tools (ADT)1.5.6, and AutoDock Vina (The Scripps research group) while the docking results were visualized with Discovery Studio Visualizer (DSV) (Biovia) and ADT.

### Skin penetration *in vivo*

Skin penetration *in vivo* was performed using male Wistar rats weighing 200–300 g, aged 6–8 weeks without defects nor skin disease, also, there were no wounds after cleaning the rat's hair. Thereafter, the coenzyme Q10 NLC with Nile red as a fluorescent label was spread on the skin of the hairless rats which were split into three groups. The first, second, and third groups were sacrificed via cervical dislocation at 2, 4, 6 h respectively after Q10 NLC application. Furthermore, the rats' skin was made into histological preparations using a frozen cryotome while the depth of penetration was measured using fluorescence microscopy (Olympus FX-1000). The experimental animals were used with permission from the Animal Care and Use Committee (ACUC), Airlangga University (ethical clearance No. 2.KE.174.09.2019).

### Statistical analysis

The Student's t-test was used for evaluating the differences in mean values (n=3) of the physicochemical characteristics with $p<0.05$, whereas in the *in vivo* studies of rats skin penetration, the two-way ANOVA method with $p<0.05$ was used to determine the differences in mean values (n=3) among the formulation groups.

## Results

### Particle size, polydispersity index (PDI), and zeta potential

The Coenzyme Q10 NLCs formulas with different lipid matrix concentrations were evaluated to obtain optimal NLCs. The coenzyme Q10 NLCs had particle size 472–1,063 nm, PDI 0.297–0.293, and zeta potential −11.5 to −20.3 mV. The particle size of (F1) and (F3) were 472.0 ± 47.1 and 684.3 ± 8.0, respectively, hence, the particle size for (F1) was smaller than (F3) whereas, (F2), (F4), (F5), (F6), and (F7) were >600 nm.

### Thermal behaviors

The melting point and enthalpy of coenzyme Q10, tristearin, stearyl alcohol, coenzyme Q10 NLC (F1), and (F3) showed endothermic peaks as presented in Figure 1 and Table 2. The %CI of solid lipids, coenzyme Q10 NLC (F1), and (F3) were 100, 41.47, and 41.44%, respectively.

### The X-ray diffraction

The X-ray diffraction patterns for tristearin had several sharp peaks at 2θ values of 6.113, 21.117, and 23.293°, stearyl alcohol at 2θ values of 20.964 and 24.139° whereas,

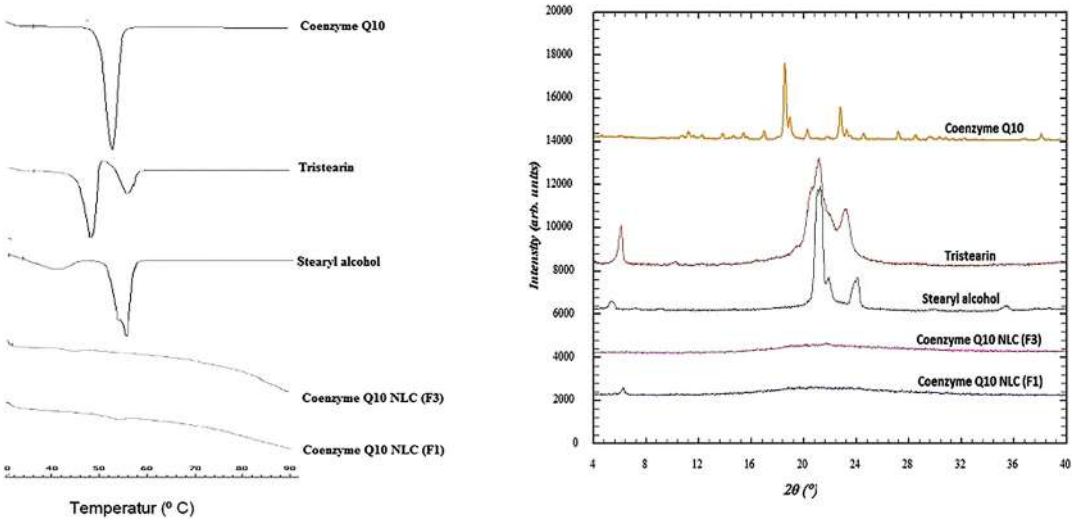


**Figure 1:** (A) DSC thermogram and (B) X-ray diffraction of coenzyme Q10, tristearin, stearyl alcohol, and coenzyme Q10.

Coenzyme Q10 had sharp peaks at 2θ values of 18.544 and 22.746° as presented in Figure 1.

## Fourier transform infrared

FT-IR spectra of coenzyme Q10 compared to coenzyme Q10 NLCs, and the lipids at 4,000–400 $m^{-1}$ are shown in Figure 2. The FT-IR spectra of coenzyme Q10 indicated peaks at 2,962.13, 1,732.73, 1,645.95, and 1,200.47 $cm^{-1}$ for C–H, C=O, C=C, and C–O stretching respectively.

## Morphology

Morphologically, the coenzyme Q10 NLC (F1), and (F3) showed spherical particles as presented in Figure 3.

**Table 2:** Melting point, enthalpy (ΔH), crystallinity index (CI) of coenzyme Q10, tristearin, stearyl alcohol, and coenzyme Q10 NLCs.

| Materials | Melting point, °C | | Enthalpy, J/g | | CI, % |
|---|---|---|---|---|---|
| | Peak 1 | Peak 2 | Peak 1 | Peak 2 | |
| Coenzyme Q10 | 51.63 | – | −153.2 | – | – |
| Tristearin | 48.24 | 56.77 | −125.51 | −43.91 | 100 |
| Coenzyme Q10 NLC (F1) | – | 55.49 | – | −3.8 | 41.47 |
| Stearyl alcohol | 41.17 | 55.58 | 30.57 | −162.29 | 100 |
| Coenzyme Q10 NLC (F3) | – | 44.31 | – | −5.38 | 41.44 |

NLC, nanostructured lipid carriers; CI, crystallinity index.

## Rheology and viscosity

The rheology was determined by examining the viscosity of coenzyme Q10 NLC (F1), and (F3) at various shear rates. The coenzyme Q10 NLCs viscosity decreased by increasing the rate of shear, as shown in Figure 4. In addition, the viscosity of the NLC Coenzymes Q10 (F1), and (F3) were 23,582 ± 1,922 and 17,739 ± 1,126 Cps at 0.5 rpm indicating that (F1) was higher than (F3) ($p<0.05$).

## The pH value

The pH value of coenzyme Q10 (F1), and (F3) NLC were 5.88 ± 0.03 and 5.75 ± 0.10, respectively meanwhile, the pH values of the coenzyme Q10 NLC (F1) and (F3) were not significantly different ($p>0.05$).

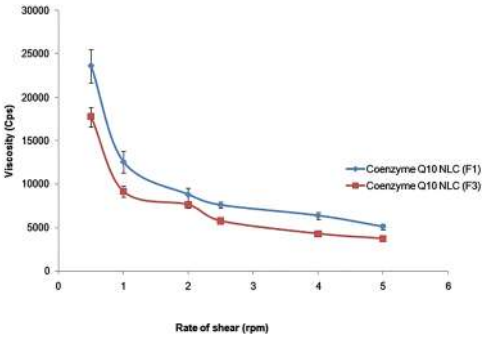


**Figure 4:** Rheology of coenzymeQ10 NLCs.

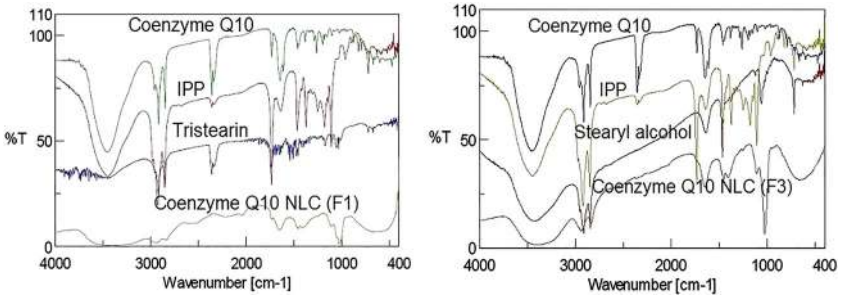


**Figure 2:** FT-IR spectra of coenzyme Q10, IPP, tristearin, stearyl alcohol, and coenzyme Q10 NLCs.

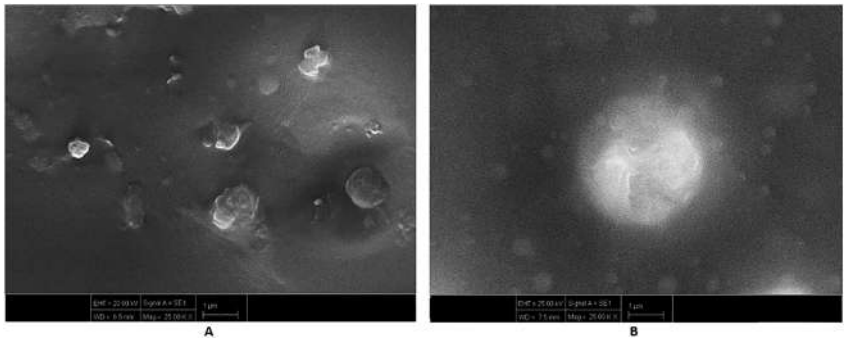


**Figure 3:** SEM image of (A) coenzymeQ10 NLC (F1) and (B) coenzymeQ10 NLC (F3) with a magnification of 25,000×.

### The entrapment efficiency (EE) and drug loading (DL)

The EE of coenzyme Q10 NLC (F1) and (F3) were 90.612 ± 0.908 and 86.138 ± 1.786%, respectively whereas, the DL for both NLCs were 11.327 ± 0.113 and 10.767 ± 0.223%, respectively, indicating higher % EE and % DL values for (F1) compared to (F3) ($p<0.05$).

### *In silico* studies by molecular docking

The binding energy (ΔG) *in silico* of coenzyme Q10-tristearin and coenzyme Q10-stearyl alcohol were −9.2 and −6.6 kcal/mol, respectively. Both of them showed hydrophobic and van der Waals interaction in the 3D visualization using DSV and ADT respectively as shown in Figure 5. Furthermore, this hydrophobic bond was at C18 atoms of tristearin and C48 atoms of coenzyme Q10 with a distance of 4.31 Å. A similar bond was found between coenzyme Q10 and stearyl alcohol at C18 atom of tristearin and C53 atom of coenzyme Q10 with a distance of 4.48 Å.

### *In vivo* skin penetration

The skin penetration depth of coenzyme Q10 NLC (F1) at 2, 4, and 6 h after applications were 298.45 ± 8.70, 332.94 ± 36.27, and 358.34 ± 15.86 μm, respectively whereas, for (F3) with similar intervals, the obtained values were 294.64 ± 38.21, 340.73 ± 10.13, and 349.62 ± 6.59 μm. Hence, the depth of skin penetration for both (F1) and (F3) increased with increasing time ($p<0.05$) and showed no significant difference after 6 h ($p>0.05$). The fluorescence intensity of (F1) was higher compared to (F3), and however, both NLCs could across the stratum corneum and penetrated the skin, as shown in Figure 6.

## Discussion

The Coenzyme Q10 NLC was designed to overcome coenzyme Q10 difficulties in penetrating the skin. Foremost, the coenzyme Q10 NLC formulas were optimized and then prepared using the high shear homogenization method with different concentrations and types of lipid matrix as shown in Table 1. Meanwhile, 1% Coenzyme Q10 in NLC was used due to its ability to induce the fibroblast cells in the mice model [11].

The lipid matrix concentrations include 8, 10, and 15%, while the ratio of solid to liquid lipid was 70:30. hence, the results showed that the lower the matrix concentration, the smaller the particle size. This is because, lower lipid matrix concentration requires less energy to reduce the particle size compared to higher lipid concentrations which increase the viscosity of the system but decrease the shearing capacity of the stirrer, hence, particle size reduction becomes difficult. Moreover, the surfactant concentration was not adequate to match the increasing lipid matrix concentration to cover the particle surface therefore, the particle size increased. The result was in line with other previous studies [12, 13].

The particle sizes for coenzyme Q10 NLC (F1) and F3 were ≤600 nm, whereas, others were >600 nm. Transdermal preparations have particle sizes of about 600 nm [14]. While particle size distribution of the coenzyme Q10 NLC (F1) and (F3) were homogenously dispersed (PDI < 0.3).

The Zeta potentials for (F1) and (F3) were less negative than −30 mV. Even though the zeta potential of NLCs Q10 was less negative than −30 mV, it did not mean physically unstable. Due to the use of nonionic surfactants (Tween 80, and Span 80), these compounds provide steric stability to the system [15]. It failed to ionize into a charged group such as ionic surfactants capable of producing zeta potential due to molecular polarization and constructed electric double-layer [16–18].

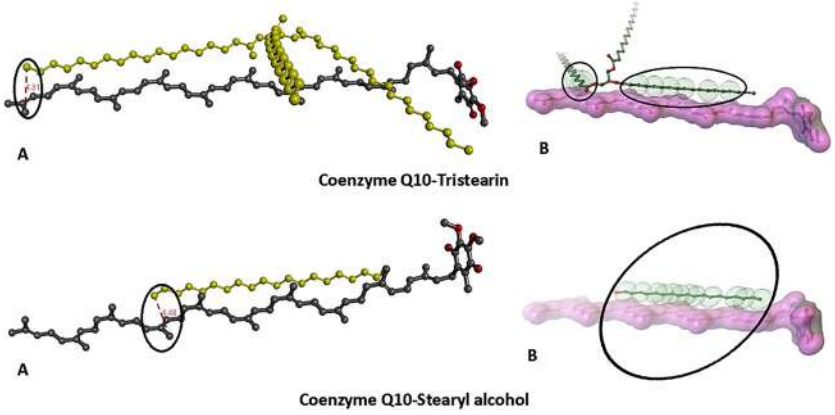


**Figure 5:** Molecular docking of coenzyme Q10 with tristearin and stearyl alcohol (A) using DSV show hydrophobic bonds and (B) using ADT show van der Waals interactions.

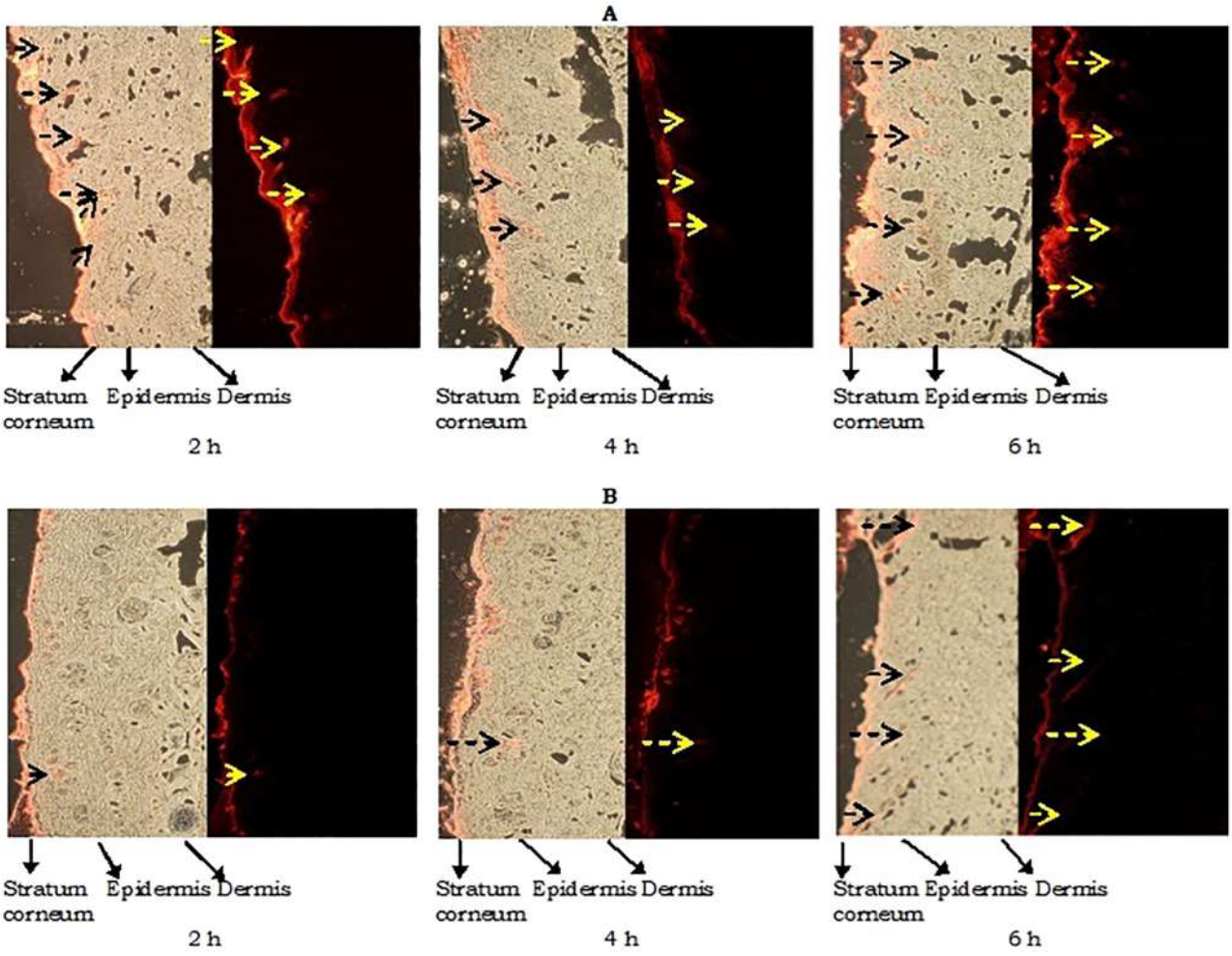


**Figure 6:** *In vivo* skin penetration through rats skin of (A) coenzyme Q10 NLC (F1) and (B) coenzyme Q10 NLC (F3) for 2, 4, and 6 h after applying the coenzyme Q10 NLC using a fluorescence microscope with a magnification of 10×.

Furthermore, the coenzyme Q10 NLC (F1) and (F3) were evaluated for physicochemical characteristics and *in vivo* percutaneous penetration through the rats' skin.

DSC was used to determine the physical characteristics and crystal structure of coenzyme Q10, solid lipids, and coenzyme Q10 NLCs [19]. Meanwhile, the DSC thermogram of tristearin and stearyl alcohol as presented in Figure 1 showed two endothermic peaks, indicating the presence of a crystal structure in tristearin and stearyl alcohol [20, 21]. In general, triglycerides and fatty acids exhibit three different polymorphs [22–24].

The melting point and enthalpy of coenzyme Q10 NLCs were lower compared to that of coenzyme Q10 and solid lipids, as shown in Figure 1 and Table 2. This was caused by the incorporation of IPP as liquid lipid hence, the crystal order of solid lipid became less ordered or amorf state in NLCs. Therefore, coenzyme Q10 was entrapped or dissolved in the lipid matrix [25, 26]. The CI of coenzyme Q10 NLCs were determined by assuming the CIs of solid lipids were 100%, meanwhile, this value decreased compared to the solid lipid. Furthermore, the CI of coenzyme Q10 NLC (F1) and (F3) were less than 50%, as shown in Table 2 indicating that the coenzymes NLCs were in an amorphous state hence, creating enough space for the entrapment of coenzyme Q10 [25–27].

In this study, the DSC analysis was combined with the XRD. Furthermore, the X-ray diffraction pattern was employed to support the crystallinity analysis of the molecules [19]. The X-ray diffraction pattern of coenzyme Q10, tristearin, and stearyl alcohol presented sharp peaks, as shown in Figure 2 whereas, the peak intensity of the coenzyme Q10 NLCs decreased. Meanwhile, sharp peaks point to a highly crystallized particle state [28]. The decreased intensity of the coenzyme Q10 NLCs indicated

that the coenzyme Q10 was entrapped or dissolved in the lipid matrix in an amorphous state [29–31].

The FT-IR spectra of coenzyme Q10 were closely in line with the previous study [32]. There was no significant shift in wavenumber and new peaks for coenzyme Q10 NLCs compared to coenzyme Q10 and lipid using the FT-IR spectra. However, there was a burned peak at 1,732.73 nm, which correlates with functional group C=O stretching in coenzyme Q10. These results indicated that coenzyme Q10 was entrapped in the lipid matrix, and the absence of chemical interaction between coenzyme Q10 and lipid matrix which capable of changing the wavenumber and creating new peaks. Similar results were presented in another study with different drugs and lipid matrix [33–36].

The coenzyme Q10 NLC (F1) had an almost spherical shape while F3 was completely spherical. Meanwhile, an aggregate of molecules appeared prior to SEM analysis with a sticky lipid matrix.

The flow behavior for coenzyme Q10 (F1), and (F3) NLC were non-Newtonian, pseudoplastic due to the decrease in viscosity with increasing shear rate [37] as presented in Figure 4. Therefore, the viscosity of coenzyme Q10 NLC (F1), and (F3) depended on the rate of shear. Both coenzyme NLCs had semisolid consistencies which were suitable for dermal application due to the ease in dispersibility on the skin.

The pH values of the coenzyme NLCs were suitable for dermal preparations as they were similar to the pH of the skin (4–6.5) [38].

The EE and DL of coenzyme Q10 NLC (F1) higher than (F3). This is because coenzyme Q10 is a lipophilic substance with lop p>10 (5), therefore it was more soluble in tristearin compared to stearyl alcohol. Also, the lipophilicity of tristearin, as solid lipid in coenzyme Q10 NLC (F1), was higher compared to stearyl alcohol. Apart from its crystallinity behaviors, the lipophilicity of the lipid matrix also influences EE and NLC drug loading [39, 40].

*In silico* molecular docking between coenzyme Q10 and solid lipids showed that the binding energy (ΔG) coenzyme Q10-tristearin was lower compared to coenzyme Q10-stearyl alcohol. This showed that coenzyme Q10 has a higher affinity for tristearin compared to stearyl alcohol because the former is more lipophilic than the latter [9]. The negative ΔG values indicate that the interactions occur spontaneously [37, 41].

The 3D visualization of molecular docking using DSV showed a hydrophobic bond between coenzyme Q10 with the lipids as shown in Figure 5. Intermolecular interactions include ionic, ion-dipole, and dipole-dipole, hydrogen, hydrophobic, and van der Waals bonds [42]. Furthermore, the van der Waals interactions tend to occur in nonpolar molecules [37]. It is an attractive force, between uncharged molecules or atoms, closely located at a distance of ±4–6 Å. The van der Waals forces are weak interaction which occurs due to the polarity of the induced atoms. However, in large amounts, it produces significant interaction and binding energy between molecules [42]. Coenzyme Q10, tristearin, and stearyl alcohol are nonpolar compounds [9]. The 3D visualization for the van der Waals interactions of coenzyme Q10-lipids was performed using ADT.

The skin penetration depth for (F1) and (F3) was not significantly different after 6 h, however, the fluorescence intensity of (F1) was higher than (F3), as shown in Figure 6. Furthermore, based on the particle size, (F1) was smaller compared to (F3). The EE and drug loading of coenzyme Q10 NLC (F1) were higher compared to (F3). Meanwhile, the skin penetration of NLC was affected by particle size and drug loading, this is supported by a previous study [43].

## Conclusions

The coenzyme Q10 NLCs was developed using tristearin and stearyl alcohol, as well as IPP as solid and liquid lipids respectively. It possessed suitable physicochemical characteristics for dermal delivery and successfully penetrated the skin.

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Abdulloh Machin, Ramidha Syaharani*, Imam Susilo, Muhammad Hamdan, Dyah Fauziah and Djoko Agus Purwanto

# The effect of *Camellia sinensis* (green tea) with its active compound EGCG on neuronal cell necroptosis in *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model

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**Abstract**

**Objectives:** To determine the inhibition effect of *epigallocatechin gallate* (EGCG) and green tea extract on neuronal necroptosis based on necroptosis morphology.
**Methods:** *In vivo* study was performed on male *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model divided into five groups, MCAO-control groups, EGCG 10 mg/kg BW/day, EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract 30 mg/kg BW/day for 7 days treatment. MCAO model was made by modification method using Bulldog clamp. After 7 days of treatment, all *R. norvegicus* were sacrificed. After that, examination using Hematoxylin–Eosin stain was conducted to look at necroptosis morphology in each group.
**Results:** We found that there are significant differences between control group and the other three groups (EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract ($p<0.05$). There is a significant correlation between the number of neuron cell necroptosis and both EGCG and green tea extract ($p<0.05$). The correlation is negative, which means both EGCG and green tea extract will decrease the number of neuron cell necroptosis. EGCG will decrease neuron cell necroptosis starting from the dose of 20 mg/kg BW/day. EGCG 30 mg/kg BW/day produces the best result compared to other doses.
**Conclusions:** *Camellia sinensis* (green tea) with its active compound EGCG decreases neuronal necroptosis morphology in MCAO models.

**Keywords:** *Cammelia sinensis*; EGCG; green tea; necroptosis; neuron.

***Corresponding author: Ramidha Syaharani**, Medicine Undergraduate Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, Phone: +62856 4659 4469, E-mail: ramidha.syaharani-2018@fk.unair.ac.id
**Abdulloh Machin and Muhammad Hamdan,** Department Neurology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Imam Susilo and Dyah Fauziah,** Department Clinical Pathology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Djoko Agus Purwanto,** Department of Pharmaceutical Chemistry Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

## Introduction

Stroke is characterized by abrupt neurological deficit due to focal brain injury in the brain by vascular etiology [1]. Based on WHO Global Health Estimates 2016, stroke is on the second place in the list of noncommunicable diseases that cause death. The national prevalence of stroke in Indonesia in the population above 15 years old is 10.9 per mil. The highest prevalence is found in East Kalimantan with 14.7 per mil. On the other hand, the lowest prevalence is found in Papua with 4.1 per mil. The incidence of stroke increases with age. It mostly occurs in the population above 75, which is 50.2%, followed by 65–74 year old group with 45.3% [2].

Ischemic stroke is a specific type of stroke commonly found in patients rather than hemorrhagic stroke, by percentage of 85% for ischemic stroke and 15% for hemorrhagic stroke [3]. Ischemic stroke happens when there is occlusion in brain vasculature generating obstruction in the brain blood vessel which results in reduced blood flow in the brain [4]. Standardized therapy given to patients with stroke is thrombolysis therapy. The only drug approved by FDA (Food and Drug Administration) to be utilized 3 h after the onset of stroke is intravenous recombinant tissue plasminogen activator (rTPA) [5]. Prior research has showed that the first generation of thrombolytic drugs such as Streptokinase and Urokinase were not effective for treating patients with ischemic stroke [6].

Necroptosis is programmed necrosis and caspase-independent cell death. The main features of necroptosis are organellele swelling and rupture of cell membrane and wall mediated by the death signal pathway [7]. Based on

previous research, necroptosis plays a role in middle cerebral artery occlusion (MCAO) rat *in vivo* process, and its mechanism can be distinguished with apoptosis [8].

Ischemic stroke process initiated by adenosine triphosphate (ATP) derivation in the brain leads to lactic acid accumulation and dysfunction of the intracellular pump [9, 10]. Interferon-gamma (INF-γ), interleukin 1 beta (IL-1β), IL-6, and tumor necrosis factor (TNF-α) activated by the death of astrocyte leads to the increasing number of lactic acid [10, 11]. TNF- α pathway will activate necroptosis process [12]. Dysfunction of intracellular pumps produces ROS (reactive oxygen species), which leads to escalating necroptosis process through some pathways such as autophosphorylation receptor-interacting protein 1 (RIP1) [13].

The second most consumed drinks in the world is green tea [14]. The catechin major component of green tea is epigallocatechin gallate (EGCG) [15]. EGCG can used as an antioxidant, which reduces reactive oxygen species (ROS) and increases antioxidant enzyme. Based on ferric reducing antioxidant power (FRAP), positive correlation is shown by the antioxidant substance of green tea. Green tea has a better antioxidant activity than oolong and black tea [16]. Early therapy of 0.5% green tea extract in 3 weeks will produce inhibition effects towards brain ischemia processes such as peroxidation lipid, level of DNA oxidative damage, neuronal cell death, and infarct in the brain [17].

The information above shows the role of green tea with its active compound EGCG in inhibiting neuronal cell death through necroptosis pathway. Further research on *Camelia sinensis* (green tea) with its active compound EGCG in inhibiting neuronal cell death through necroptosis pathway needs to be done to identify the effect of green tea in decreasing neuronal cell necroptosis in ischemic stroke.

## Materials and methods

This study was designed as randomized posttest only MCAO-control group design true experimental. The study done by using *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model treated with green tea and its active compound EGCG with the approval of Research Ethics Committee of Health Faculty, Faculty of Medicine, Universitas Airlangga. This research was conducted in animal laboratories at the Faculty of Pharmacy, Airlangga University for animal treatment. The morphological examination of neuron cell necroptosis was carried out at the Pathological Anatomy Laboratory of FK UNAIR for 4 months.

The sample of this study included 55 healthy male *R. norvegicus* MCAO models with weight of 200–275 g that met the inclusion and exclusion criteria. The sample was divided into three groups: MCAO-control groups, EGCG 10 mg/kg BW/day, EGCG 20 mg/kg BW/day, EGCG 30 mg/kg BW/day, and green tea extract 30 mg/kg BW/day for 7 days. Treatment was done by simple random sampling with the assumption that all the subjects were treated in a similar method, from taking research subjects to work and laboratory conditions. One group contained 11 rats with a total of 55 rats in all groups.

Before making the MCAO model, male *R. norvegicus* were adjusted to the new environment for 7 days. MCAO model was made by modification method using bulldog clamp to occlude cerebral media artery for 180 min. After the anesthesia stopped, we grabbed the rats by the tail 1 m above the floor and evaluated if there is flexion movement of two front leg toward contralateral of the hemisphere which indicated that the ischemia process was successful, that will cause necroptosis. Next, green tea treatment was done once a day for 7 days. We introduced the green tea treatment by dissolving green tea with aqua bidest with concentration of 1 mg/mL and giving it to the rats by gavage feeding needle. EGCG was obtained and analyzed by Xi'an rongsheng biotechnology and we used pure EGCG 98.7% (HPLC analysis document number 2019070630). Green tea extract used was labeled Meditea (IDM000580138), containing 2.5% of EGCG in 50 g sample analyzed by Angler BioChemlab on HPLC analysis, with certificate number 183689.

After that, all the rats were sacrificed using decapitation method to acquire the brain tissue. After being sacrificed, the brain tissue was taken from the hemisphere that had an infarction of 1.5 cm in front and behind the bregma and was used for histopathological examination. The brain tissue was stained by Hematoxylin eosin. The proportion of necroptosis morphology was examined and classified based on D.C Allred M.D guideline of scoring, proportion classified in score 0–5 [18]. All histopathological examination was carried out directly by pathologist Imam Susilo.

Descriptive analysis and normality test of Kolmogorov–Smirnoff were conducted for each group data. Because data distribution was abnormal, Kruskal–Wallis test was performed, followed by Mann–Whitney test to distinguish EGCG and green tea extract effect toward neuronal cell necroptosis morphology. Kruskall–Wallis test was used to compare necroptosis differences of all group. The analysis was then followed by Mann Whitney test to compare necroptosis differences of each two group after we were sure that there were differences between all groups in Kruskal Wallis test. Lastly, we performed a Spearman correlation test to find out the correlation of EGCG and green tea extract toward necroptosis morphology in MCAO model.

## Results

First, we conducted a descriptive analysis (to evaluate minimum–maximum, mean and standard deviation of our data), followed by a normality test for each group. Our data distribution is abnormal. So, we performed the Kruskal–Wallis test, and the results showed that data of the five groups is significantly different ($p<0.05$). Further, we conducted Mann–Whitney test to differentiate necroptosis morphology between each two groups presented in Table 1. We found that there are significant differences between the normal-control group and MCAO-control group ($p<0.05$), which indicates that the MCAO process happened as shown by the differences of neuronal cell necroptosis.

**Table 1:** Comparison of EGCG and green tea extract effect on neuronal cell necroptosis in MCAO model.

| Group | Mean ± SEM | n | p-Value |
|---|---|---|---|
| Normal control group | 0.44 ± 0.527 | 11 | |
| MCAO control group | 1.80 ± 0.422 | 10 | 0.00[a] |
| EGCG 10 mg/kg BW | 1.5 ± 0.522 | 12 | 0.254[b] |
| EGCG 20 mg/kg BW | 1.31 ± 0.480 | 13 | 0.049[b] |
| EGCG 30 mg/kg BW | 1.08 ± 0.289 | 12 | 0.003[b] |
| Green tea extract 30 mg/kg BW | 1.27 ± 0.467 | 11 | 0.043[b] |

[a]Compared to normal control group, [b]Compared to MCAO-control group. If p-value<0.05 considered statistically significant.

As Table 1 indicates, there are significant differences of neuronal cell necroptosis morphology between the MCAO-control group and the other three groups (EGCG 20 mg/kg BW, EGCG 30 mg/kg BW, and green tea extract) with $p<0.05$. There is a significant correlation between neuron cell necroptosis morphology and both EGCG and green tea extract ($p<0.05$). EGCG 10 mg/kg BW did not decrease neuron cell necroptosis morphology. As found in EGCG 20 mg/kg BW and EGCG 30 mg/kg BW group, EGCG can decrease neuron cell necroptosis. However, based on the p-value, 30 mg/kg BW dose is more effective than 20 mg/kg BW dose.

If we compared EGCG 30 mg/kg BW and green tea extract group, both significantly decreased neuronal cell necroptosis. However, EGCG is more effective as there is an increasing mean value in green tea extract group as presented in Table 1. The correlation is negative, which means an EGCG/green tea extract will decrease neuron cell necroptosis morphology. All of our data indicate that EGCG and green tea extract play a role in decreasing neuronal cell necroptosis during ischemic process. EGCG effect is dose-dependent as increasing EGCG dose increases the decreasing process of neuron cell necroptosis. The differences of each group in histopathological examination can be seen and compared in Figure 1, the necroptotic cell marked as green arrow with pale, fade, nonprominent, pyknotic and karyorrhexis nucleus. EGCG 30 mg/kg BW produced the most significant effect compared to the other groups as can be seen in statistical analysis in Table 1 and Figure 1.

## Discussion

Thrombolysis therapy is the only available therapy for stroke currently and has 4–5 h to its therapy window. While it is too long from the onset, intracranial hemorrhage is commonly found as a major complication of thrombolysis therapy [19]. Necroptosis described as programmed necrosis and apoptosis are regulated cell death mechanisms that happen during neurological damage process in stroke [20]. During its pathway, necroptosis will cause oxidative stress as result of an increasing number of reactive oxygen species. Mitochondrial respiratory chain primarily produces reactive oxygen species, xanthine oxidase and NADPH oxidases which leads to imbalance because of the spontaneous reperfusion or reperfusion caused by administration of pharmacological agents [21].

Green tea is commonly found in 30 countries and it is the most commonly consumed beverage worldwide [22]. Green tea is also used as a herbal plant in India and China [23]. The major catechin component of green tea is EGCG out of four catechin found in green tea [24]. Green tea antioxidant effect is already *in vivo* and *in vitro* approved. Its antioxidant effect shows similarity to antioxidant effect to a-tocopherol. Green tea also contains five times antioxidants effect compared to black tea [22].

Inhibition process of necroptosis was done by decreasing ROS by antioxidant effect produced by EGCG. EGCG will inhibit ROS produced by inhibiting some mechanisms in the necroptosis pathway. First, damaged mitochondria produce ROS, which then stimulate RIP1 and receptor-interacting protein 3 (RIP3) oxidation in three sites of cysteine(c257, c268 and c586) which also promote autophosphorylation of RIP1 and RIP3 at Ser161 so that necroptosis pathway is activated [13]. The second is inhibition toward positive feedback of ROS to increase production of necrosome in necroptosis [25]. Third, EGCG is capable of activating caspase 3 and 8 after 8 h of green tea therapy [26, 27]. Activation of caspase 8 leads to the discontinuation of necroptosis pathway. EGCG also inhibits synthesis of some inflammatory mediators: TNF-α, IL-6 and IL-8 [28, 29]. Fourth, EGCG is able to decrease expression of tumor necrosis factor receptor 1 (TNFR1) and RIP3. TNFR1 and RIP3 start to decrease in administration of 20 mg/kg BW EGCG in rat with MCAO model [30].

Neuron cell undergoing necroptosis is shown as an arrow in Figure 1, neuron cell as pale, faded, nonprominent nucleus. By comparing the intensity of necroptosis cell, our study shows that there is reduction in neuronal cell death necroptosis morphology during histopathological examination. The administration of EGCG 20 mg/kg BW, EGCG 30 mg/kg BW, and green tea extract 30 mg/kg BW shows statistically significant reduction in neuronal cell necroptosis morphology. The most significant dose is EGCG 30 mg/kg BW. The EGCG effect is dose-dependent as there is an increase of EGCG effect as we elevated the dose.

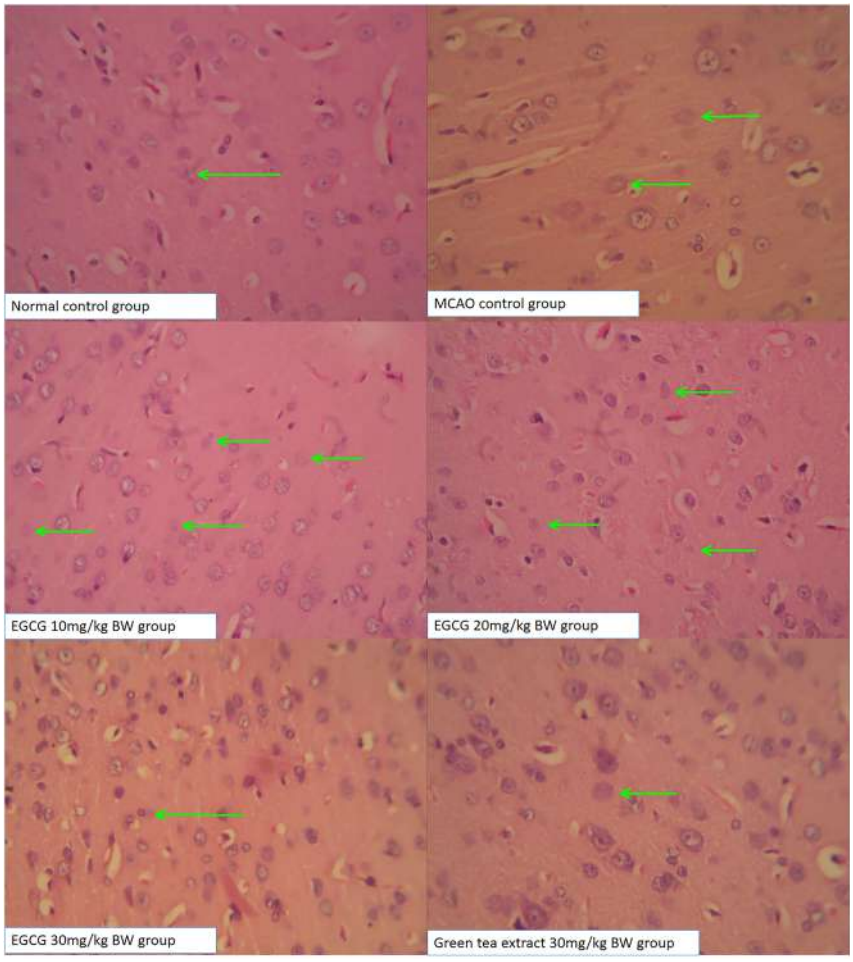


**Figure 1:** Histopathological examination result of hemisphere area in three groups (left to right): MCAO-control group, EGCG group and green tea extract group. Necroptotic neuronal cell showed in green arrow by pale, fade, nonprominent, pyknotic karyorrhexis nucleus. Necroptotic cell count can be seen decreasing in green tea extract group and EGCG group compared to MCAO-control group.

Thirty milligram per kilogram body weight dose EGCG is more effective for decreasing neuronal cell necroptosis rather than green tea extract. This is because one sachet of green tea extract only consists of 2.5% EGCG. It can be concluded that 30 mg/kg BW dose of green extract contains 0.75 mg/kg BW EGCG. As shown by p-value and mean value of proportion score, green tea extract 30 mg/kg BW produces the same effect as EGCG 20 mg/kg BW. This is due to other catechin contents such as (ECG, EC, EGC) also protein, amino acid, fiber, fat, and pigment found in green tea extract. The other catechin contents of green tea mentioned before produce synergic effect that make same antioxidant effect as EGCG of green tea extract achieved in lower dose. So, to produce the same significant effect as EGCG 30 mg/kg BW, we need a dose of green tea extract of 45 mg/kg BW (Using the ratio of 30 mg/kg BW green tea extract (0.75 mg/kg BW EGCG) which produces the same effect as pure EGCG 20 mg/kg BW).

If we applied to humans, the dose of 30 mg/kg BW/day of EGCG in rats is equal to 4.8 mg/kg BW/day EGCG. If we used a standard weight of 70 kg, we need 336 mg of EGCG each day to decrease neuronal cell necroptosis. Using the same method, we need 504 mg of green tea extract each day. Generally, people consume three cups of green tea a day; 240 ml in each cup of green tea contains 187 mg of EGCG. So, each day people consumed 560 mg of EGCG [31]. As mentioned before, we need 336 mg of EGCG or 504 mg of green tea extract each day, and the average daily consumption of three cups a day is enough to achieve the dose of EGCG/green tea extract.

## Conclusions

*Camellia sinensis* (green tea) with its active compound EGCG decreases neuronal cell necroptosis morphology in MCAO models. EGCG effect is dose-dependent starting from 20 mg/kg BW and significantly reduces neuronal cell necroptosis in 30 mg/kg BW dose and 45 mg/kg BW for green tea extract.

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Ika P. Dewi*, Rifdah B. Kwintana, Jihan U. Ulinnuha, Fadhillah Rachman, Fransiska M. Christianty and Diana Holidah

# Hepatoprotective effect of ethanolic extract of sugarcane (*Saccharum officinarum* Linn.) leaves

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**Abstract**

**Objectives:** The sugarcane leaf is rich inphytochemical content. It is rarely used because it is a waste although it has potential activity as antimutation, anti inflammation, and antioxidation. There is no study about its hepatoprotective activity yet. This study was conducted to determine the hepatoprotection of sugarcane leaves in tested animals with liver acute injury induced by carbon tetrachloride ($CCl_4$).

**Methods:** Twenty-four Wistar strain rats were divided into three groups of experimental animals (dose 300, 400, and 500 mg/kg) and three control groups (normal, positive, and negative). The ethanol extract of sugarcane leaves obtained from Panti, Jember, was made using the maceration method. The animals were treated for 14 days by giving the extract to the treatment group. One hour after treatment on the last day, the test animals were given $CCl_4$ intraperitoneally except for the normal group. On the 15th day, the blood of the test animal was taken to be tested for the biochemical value of the liver (aspartate transaminase (AST), alanine aminotransferase (ALT), alanine phosphatase (ALP), and bilirubin) and examined for its liver to be made histological preparations.

**Results:** The results showed that the treatment with a dose of 500 mg/kg was able to decrease AST, ALT, ALP, and bilirubin parameters compared to the negative control. The extract also provided improvements in liver tissue histology compared to the negative control.

**Conclusions:** Sugarcane leaf ethanol extract (SCLE) has a potential hepatoprotective effect.

**Keywords:** $CCl_4$; ethanolic extract; hepatoprotective; rat; sugarcane leaves.

## Introduction

Liver disease causes the death of around two million people every year worldwide. Cirrhosis and liver cancer contribute to 3.5% deaths worldwide. Hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), and cirrhosis related hepatocellular carcinoma are several diseases linked to the liver [1]. Indonesian Ministry of Health states that in 2018 the incidence of hepatitis was 0.39% that spread equally to any age, gender, occupation, and residency [2]. Deaths from hepatocellular carcinoma rank fourth of cancer mortality in Indonesia after lung, breast, and cervix uteri [3].

The liver plays a role in the process of metabolism and detoxification of metabolites or xenobiotics that enter the body [4]. Metabolites or xenobiotics or other agents such as viruses or certain chemicals or free radicals such as reactive oxygen species (ROS) could damage liver cells that result in liver cirrhosis, hepatitis, and liver cancer [5]. Toxic metabolites such as free radicals ROS can trigger an immune response to or have a direct effect on cell biochemistry by damaging the macromolecular structure of liver cells [6]. Carbon tetrachloride ($CCl_4$) is an example of a hepatotoxin that is often used to induce fibrosis and cirrhosis of the liver because its metabolites are free radicals [6, 7]. The metabolic product of $CCl_4$ is trichloromethyl free radicals, which cause oxidative stress by binding to cellular molecules like nucleic acids, protein, and fat. Those will affect DNA synthesis and damage cellular processes causing lipid peroxidation, cell damage, apoptosis, necrosis, inflammation, fibrosis, and malignancy [8, 9].

One of the hepatoprotective ways is to strengthen antioxidant capacity by increasing the number of oxidants in the body, one of which is the intake of antioxidants from outside [4]. Numerous medicinal plants have been

***Corresponding author: Ika P. Dewi**, Preclinical Pharmacology Research Group, Faculty of Pharmacy, Universitas Jember, Jl Kalimantan I/2, Kampus Tegalboto, Jember, East Java 68121, Indonesia, Phone: +62 0331324736, Fax: +62 0331324736, E-mail: ikapdewi@unej.ac.id. https://orcid.org/0000-0002-4841-1533

**Rifdah B. Kwintana, Jihan U. Ulinnuha, Fadhillah Rachman, Fransiska M. Christianty and Diana Holidah,** Preclinical Pharmacology Research Group, Faculty of Pharmacy, Universitas Jember, Jember, East Java, Indonesia

investigated because of their antioxidant activity [6, 10]. These health-promoting properties have gripped attention for their potential as dietary supplements and therapeutic choices for health benefit properties and safer than synthetic agents [4]. Sugarcane has been reported to have antioxidant effects from its raw juice, syrup, molassed, very high polarization (VHP) sugar, and leaves [11, 12].

Sugarcane (*Saccharum officinarum* Linn) is cultivated throughout the world because of its economic value and its benefits as a medicinal plant [13]. Sugarcane is reported to have antioxidant, anticancer, antiproliferative, diuretic, anti inflammatory, antihyper cholesterol, antithrombotic, and antihyperglycemic effects in various studies [12, 14–19]. Sugarcane juice is proven to have a hepatoprotective effect in hepatotoxic test animals induced by the drug isoniazid [14, 18].

The sugarcane leaves are rarely used because they are wastes, although they are rich with phytochemical content [12]. Sugarcane leaves contain polycosanol and phenolic compounds such as flavonoids [15, 20]. Sugarcane leaves methanolic extracts are reported to have various flavones –O– and –C– glycosides [15]. Polyphenolic constituents from water extract of sugarcane leaves such as caffeic acid, ferulic acid, apigenin, and vitexin have the potential antimutation, anti inflammation, as well as antioxidation [12]. Flavonoid is a natural product compound which is famous for its various health benefits, one of which is as an antioxidant in various degenerative diseases such as hepatotoxicity [21]. Research about the hepatoprotective of sugarcane leaves has not been done before especially for liver acute damage. This research was conducted to determine the potential hepatoprotective effect of sugarcane leaves in animal model of acute liver injury by the ($CCl_4$).

# Materials and methods

## Materials

Chemical substances, including methanol and $CCl_4$, were purchased from Merck Company. Milk thistle *Silybum marinum* standardized extract was used as the positive control, containing 80% Silymarin (Puritan's Pride). Commercial kits of aspartate transaminase (AST), alanine phosphatase (ALP), alanine aminotransferase (ALT), and bilirubin were purchased from Analyticon.

## Plant collection and extraction

The samples of sugarcane were collected from Panti, Jember, East Java, Indonesia. The genus and species were confirmed by the herbarium experts using valid identification keys at the Plant Laboratory of State Polytechnic of Jember (No. 21/PL17.13.1.02/LL/2019). The sugarcane leaf samples were dried separately at the temperature of 20–25 °C in shadow and then with mortar the samples were powdered. They were macerated in ethanol 96% with a ratio between powder and ethanol 1:10 (w/v) for two days. The extract was filtered, and the residue was remacerated with a ratio of 1:5 (w/v). The obtained extract was combined and then using the rotary evaporator it condensed at 50 °C. The final product was sugarcane leave ethanolic extracts (SCLEs).

## Grouping and treatment of the rats

In this study, 24 male albino Wistar rats with body weights of 180 ± 20 g were used and divided into six groups. Each group was treated according to Tsai et al. (2010) and Chiu et al. (2018) with some modifications [22, 23]. The protocol of this study was approved by the Ethics Committee of Dentistry Faculty of Universitas Jember (No. 584/UN25.8/KEPK/DL/2019). The liver damage was induced by injecting 1 mL of $CCl_4$ intraperitoneally [24].

Twenty-four rats were randomly assigned to six groups, each consisting of four rats.

Group 1 (Normal): The animals on this group received Carboxymethyl Cellulose Sodium (CMC-Na) 1% for 14 days via gavage and on the 14th day were injected with 1 mL of water.

Group 2 (P): The animals in this group were administered for 14 days with pretreatment 100 mg/kg *S. marinum* standardized extract containing 80% Silymarin and on the 14th day received 1 mL of $CCl_4$ intraperitoneally.

Group 3 ($CCl_4$): The animals on this group received CMC-Na 1% for 14 days via gavage and on the 14th were injected with received 1 mL of $CCl_4$ day intraperitoneally.

Group 4 (D300): The animals on this group were administered for 14 days with 300 mg/kg SCLE and on the 14th day received 1 mL of $CCl_4$ intraperitoneally.

Group 5 (D400): The animals on this group were administered for 14 days with 400 mg/kg SCLE and on the 14th day received 1 mL of $CCl_4$ intraperitoneally.

Group 6 (D500): The animals on this group were administered for 14 days with 500 mg/kg SCLE and on the 14th day received 1 mL of $CCl_4$ intraperitoneally.

Serum levels of AST, ALT, ALP, and bilirubin were measured using standard assay kits according to the manufacturer's instructions. The experiments were conducted according to the instructions provided in each laboratory kit using an Analyticon Biolyzer [25–28] with some modifications as stated below:

AST and ALT Procedure: 100 µL of serum samples were added with 1,000 µL of working solution/Reagent 1 (L-Aspartate for AST procedure and L-Alanine for ALT procedure) followed by 1 min incubation at 37 °C. The solution was added with 200 µL of Reagent 2 ($NaDH_2$ and 2-oxoglutarate), and then its absorbance was measured at 340 nm with Biolyzer 100 photometer.

ALP Procedure: Working solution of Reagent 1 (Magnesium sulfate) and Reagent 2 (p-nitrophenylphosphate) was mixed with ratio 5:1. Five-hundred microliter from this solution added with 10 µL serum samples. The mixture was then incubated for 1 min at 37 °C, and its absorbance was examined at 405 nm with Biolyzer 100 photometer.

Bilirubin Procedure: 50 µL of Reagent 1 (Sodium chloride), 12.5 µL of Reagent 2 (Sulfanilic acid and HCl), 250 µL of Reagent (Sodium nitrite), and 50 µL of serum samples were mixed and then incubated for 10 min at 25 °C. The mixture was then added with 250 µL of Reagent (tartrate/NaOH) followed by incubation for 5 min at 25 °C. The

absorbance of the solution was measured with Biolyzer 100 photometer at 546 nm.

### Morphological and histopathological studies

On the 15th day, the animals were anesthetized and the blood samples were collected from the heart. Then abdominal areas of the rats were opened, and the liver was removed for histopathological analysis. The organs were observed for their presence and color change. The organs were then weighed to find out their absolute weight. Furthermore, the relative weight of the liver was calculated by:

$$\text{Relative liver weight} = \frac{\text{liver weight}}{\text{animal weight}} \times 100\%$$

The bodyweight of test animals used in weighing was the final weight before the animal was sacrificed.

The liver tissues were fixed in 10% formalin for 48 h and then dehydrated in graduated ethanol (70, 80, 90, 96, and 100%, respectively), followed by xylene clearing, paraffin infiltrating, and then embedded in paraffin. Then, the paraffin block was sliced with 4–5 µm thick and stained with hematoxylin and eosin (H–E) dye. The specimens were examined for histopathological changes under the microscope (200×) (Olympus DP21).

## Results

### The effect of sugarcane leave ethanolic extracts on biochemical factors

The effects of SCLEs on various biochemical factors (AST, ALT, ALP, and bilirubin) of the liver in the groups under study are shown in Figures 1–4. The negative control showed a significant increase in AST, ALT, ALP, and bilirubin significantly compared with the control group (normal) ($p<0.05$). Using 300, 400, and 500 mg/kg of SCLE, pretreatment was conducted for 14 days and then given $CCl_4$ on the 14th day leading to the decrease of AST, ALP, and bilirubin; meanwhile, the 400 mg/kg SCLE did not give decrease on the ALT level. The serum ALT level could be affected by some physiological factors and nonhepatic causes like metabolic covariates, celiac disease, and muscle injury [29]. The pretreatment with 500 mg/kg of SCLE had no significance on biochemical factors with the normal groups in all biochemical factors. These results indicate that 500 mg/kg of SCLE has protective functions against $CCl_4$-induced liver damage.

### The effect of sugarcane leave ethanolic extracts on histopathology of liver tissue

The effect of SCLE on liver morphology is given in Figure 5 and Table 1. The macroscopic liver tissue (Figure 5) shows

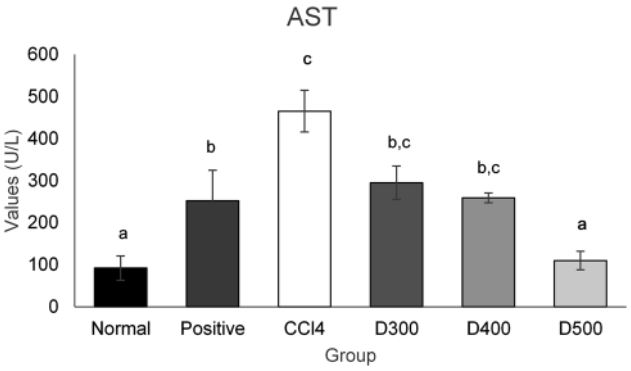


**Figure 1:** Effect of sugarcane leave ethanolic extracts (SCLEs) on aspartate transaminase (AST) level in carbon tetrachloride ($CCl_4$) damaged rats. Each value is represented as the mean ± SE. The same superscript letter indicates that there is no significant difference between treatments based on the Least Significant Difference (LSD) test ($p<0.05$). The letter a, b, and c shows the significant of each group based on the LSD test.

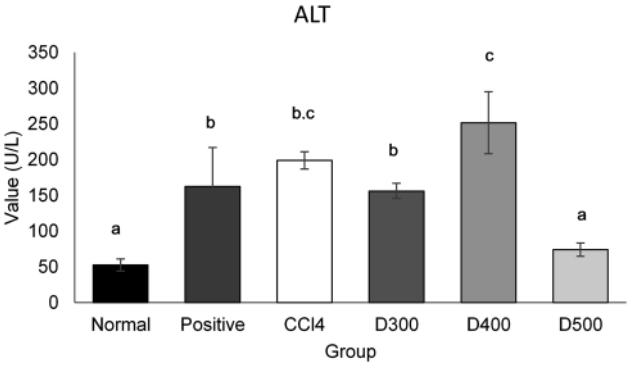


**Figure 2:** Effect of SCLEs on alanine aminotransferase (ALT) level in carbon tetrachloride ($CCl_4$) damaged rats. Each value is represented as the mean ± SE. The same superscript letter indicates that there is no significant difference between treatments based on the Least Significant Difference (LSD) test ($p<0.05$). The letter a, b, and c shows the significant of each group based on the LSD test.

necrosis in the negative group marked by granulomas on some of the liver surfaces. Table 1 shows that the ethanol extract of sugarcane leaves with various doses able to prevent the increase in the relative weight of the rat liver. Pretreatment with SCLE at a dose of 500 mg/kg is known to have the same activity as the positive control because the values are not significantly different. This shows that pretreatment SCLE at a dose of 500 mg/kg could prevent swelling of the rat liver.

The effect of SCLEs on liver histopathology is shown in Figure 6. The negative control in which liver samples are administered with only $CCl_4$ indicates damages such as cell vacuolization and necrosis cells (Figure 6). The groups with pretreatment of SCLE and positive control group show less cell vacuolization and necrosis cells.

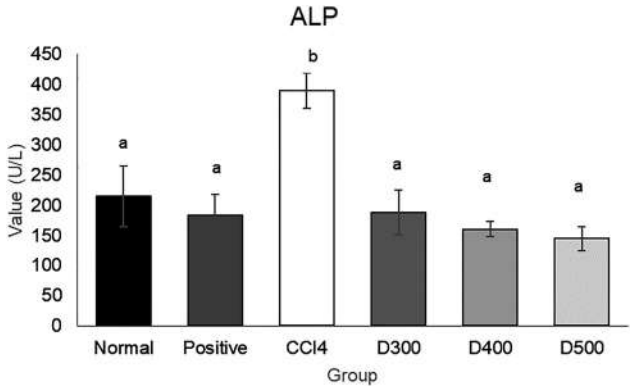


**Figure 3:** Effect of SCLEs on alanine phosphatase (ALP) level in carbon tetrachloride ($CCl_4$) damaged rats. Each value is represented as the mean ± SE. The same superscript letter indicates that there is no significant difference between treatments based on the Least Significant Difference (LSD) test ($p<0.05$). The letter a, b, and c shows the significant of each group based on the LSD test.

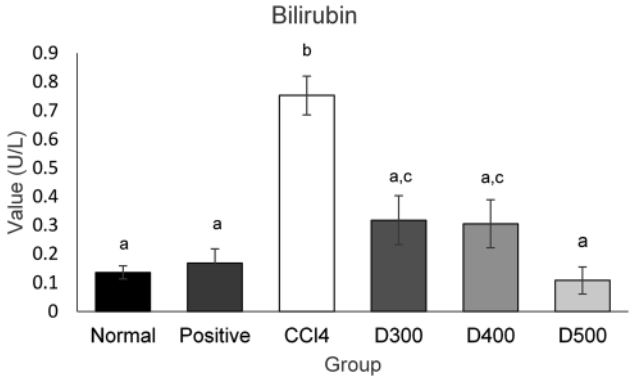


**Figure 4:** Effect of SCLEs on bilirubin level in carbon tetrachloride ($CCl_4$) damaged rats. Each value is represented as the mean ± SE. The same superscript letter indicates that there is no significant difference between treatments based on the Least Significant Difference (LSD) test ($p<0.05$). The letter a, b, c shows the significant of each group based on the LSD test.

## Discussion

This study was conducted to determine the potential of SCLE as a hepatoprotective agent in rats whose liver organ acute damage was induced by $CCl_4$. Ethanol was chosen as a solvent because ethanol is a solvent that can be used to dissolve almost all substances, both polar, semipolar, and nonpolar. In addition, ethanol dissolved flavonoid compounds that are thought to have antioxidant activity in sugarcane [30]. Ethanol is also safer, and its toxicity is not as high as that of other solvents with the same ability to attract flavonoid class compounds [31]. The ethanol and water mixture was the optimal method to dissolve luteolin, one of the flavonoid family which has anti inflammatory, antioxidant, and antitumorigenic properties that sugarcane leaf has [32, 33].

$CCl_4$ acts as an inducer of acute hepatotoxicity because it causes acute and reversible liver injury characterized by centrilobular necrosis [34]. Biotransformation of $CCl_4$ in the liver produces $CCl_3$ and $CCl_3O_2$ free radicals. These free radicals can interact with various intracellular targets in hepatocytes, causing necrosis. $CCl_3$ free radicals inhibit the secretion of lipoproteins in the blood, causing fatty liver. Meanwhile, $CCl_3O_2$ causes lipid peroxidation [9]. This affects the fluidity and permeability of the membrane for ion exchange, which results in leakage of enzymes in the blood and ultimately causes swelling, cytolysis, and cell death [35]. High levels of free radicals in the liver causes oxidative stress stimulated by hepatocytes to give signals for the immune system activation and Kupffer cells. Thus, the process causes liver inflammation by producing various ROS cytokines and proinflammatory cytokines, causing damage characterized by hepatocellular necrosis [34]. Further reactions could lead to cancer initiation or cell death [9].

Oxidative injury due to $CCl_4$ induction disrupts the hepatocellular plasma membrane causing enzymes contained in the cytosol, such as AST, ALT, and ALP to be released into the bloodstream [36, 37]. In a physiologic state, ALP is excreted in bile, but in liver injury due to toxins that damage hepatocytes, ALP releases and increases its serum level [38]. The release of the ALP enzyme mostly occurs from the apical side of the hepatocyte plasma membrane facing the canaliculi [39]. Liver dysfunction, such as that caused by $CCl_4$ induction also disrupts bilirubin metabolism so that bilirubin levels in the body increase [40].

$CCl_4$ induction also causes liver swelling or hepatomegaly [41]. Swelling occurs due to trichloromethyl peroxide free radicals attacking phospholipid molecules, thus increasing the permeability of liver cell membranes [42]. The damage of the plasma membrane causes sodium and water ions to enter the cell while potassium ions leave the cell. When the process happens continuously, the cell will have hydropic degeneration which is characterized by the appearance of clear vacuoles in the cytoplasm [22]. This damage can be seen through liver histopathological conditions [43]. $CCl_4$ induction can trigger inflammation. In this condition, Kupffer cells are stimulated to respond to hepatocellular stress by producing ROS and releasing proinflammatory mediators such as Tumor Necrosis Factor (TNF-α), Prostaglandin (PG) E2, inducible Nitric Oxide Synthase (i-NOS), and Cyclooxygenase (COX)-2 causing cell entry inflammation into the liver cells [44].

The results showed that the ethanol extract of sugarcane leaves in the treatment with a dose of 500 mg/kg was able to provide results for the liver enzymatic parameters close to the values in the normal control group and some parameters like

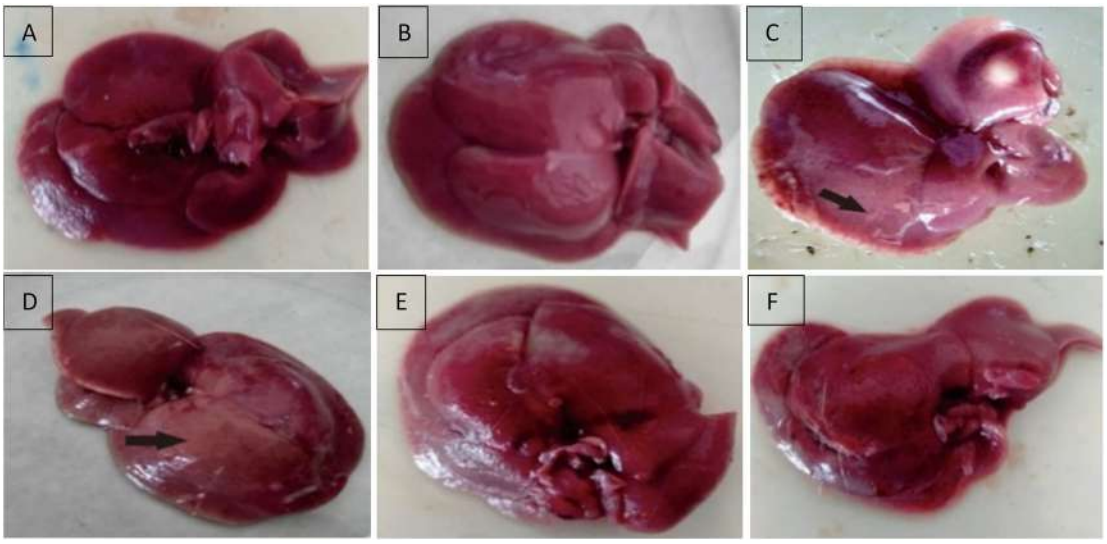


**Figure 5:** Macroscopic liver tissue. A: normal group, B: positive control group, C: negative control (carbon tetrachloride, $CCl_4$), D: SCLE dose 300 mg/kg, E: SCLE dose 400 mg/kg, E: SCLE dose 500 mg/kg. Black arrow shows the necrosis in the liver surface.

**Table 1:** Liver weight and relative liver weight after treatment.

| Group | Liver weight, g | Liver relative weight, % |
|---|---|---|
| Normal | 5.427 ± 0.249 | 2.858% ± 0.039[a] |
| Positive | 5.894 ± 0.131 | 3.175% ± 0.027[c] |
| $CCl_4$ | 7.357 ± 0.318 | 3.819% ± 0.025[b] |
| SCLE dose 300 mg/kg | 6.854 ± 0.169 | 3.662% ± 0.096[d] |
| SCLE dose 400 mg/kg | 6.689 ± 0.065 | 3.389% ± 0.084[e] |
| SCLE dose 500 mg/kg | 6.289 ± 0.072 | 3.227% ± 0.023[c] |

The same superscript letter indicates that there is no significant difference between treatments based on the Least Significant Difference (LSD) test ($p<0.05$). The letter a, b, c, d, and e shows the significant of each group based on the LSD test.

AST and ALP better than the positive control. This result shows that the ethanol extract of sugarcane leaves has potential as a hepatoprotective agent. The hepatoprotective mechanism occurs due to the ability of the extract to stabilize the cell membrane of hepatocytes and prevent the release of enzymes from hepatocyte cells [37]. Another mechanism is due to extract's ability to prevent the formation of free radicals and neutralize them, so the liver is protected from hepatotoxins that trigger oxidative stress [14, 18, 37]. Antioxidants protect the liver from the effects of free radicals by inhibiting and destroying free radical reactions, thereby inhibiting cell damage [45].

The hepatoprotective activity of the ethanol extract of sugarcane leaves is likely due to its phytochemical. Sugarcane leaves contain various kinds of polycosanols and phenolic compounds, such as flavonoids. The High Performance Liquid Chromatography (HPLC) analysis of the methanolic extract of sugarcane leaves showed compounds like flavone glycosides –O– and –C– [15]. Flavone compounds could reduce lipid peroxidation due to the presence of scavenger compounds in them [37]. Coutinho et al. [46] show that the flavone derivatives identified in the hydroalcoholic extract of sugarcane leaves are flavones-O– and –C-glycosides, including apigenin-C-glucoside, luteolin-C-glucoside, diosmetin-C-glucoside, and tricin-O-glucoside. Vila et al. [33] report that sugarcane leaves contain luteolin-8-C-rhamnosylglucoside (flavone glycosides) as the main compound with the activity of capturing radical compounds. Luteolin antioxidant mechanisms are passed by scavenging free radicals, chelating transition metals, inhibiting pro oxidant enzymes, and inducing antioxidant enzymes. Luteolin glucoside has catechol B-ring and a C2–C3 double bond in conjugation with an oxo group at C4, which has the activity of donating hydrogen or electrons to stabilize free radical species and binding activity of transition metal ions such as iron and copper [47].

Luteolin increases antioxidant defense like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity, and glutathione (GSH) levels [48]. Luteolin and flavones like chrysin and apigenin are suggested to inhibit oxidative stress in liver through the extracellular signal-regulated kinase (ERK) 2/nuclear factor-erythroid-2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway upregulation [49]. Luteolin also has been reported to reverse hepatic fibrosis by increased expression of matrix metalloproteinase-9 (MMP-9), removed collagen deposits, and attenuated activation of hepatic stellate cells (HSCs) [50].

The hepatoprotective mechanism of sugarcane leaves, apart from its antioxidant activity, is estimated by its anti

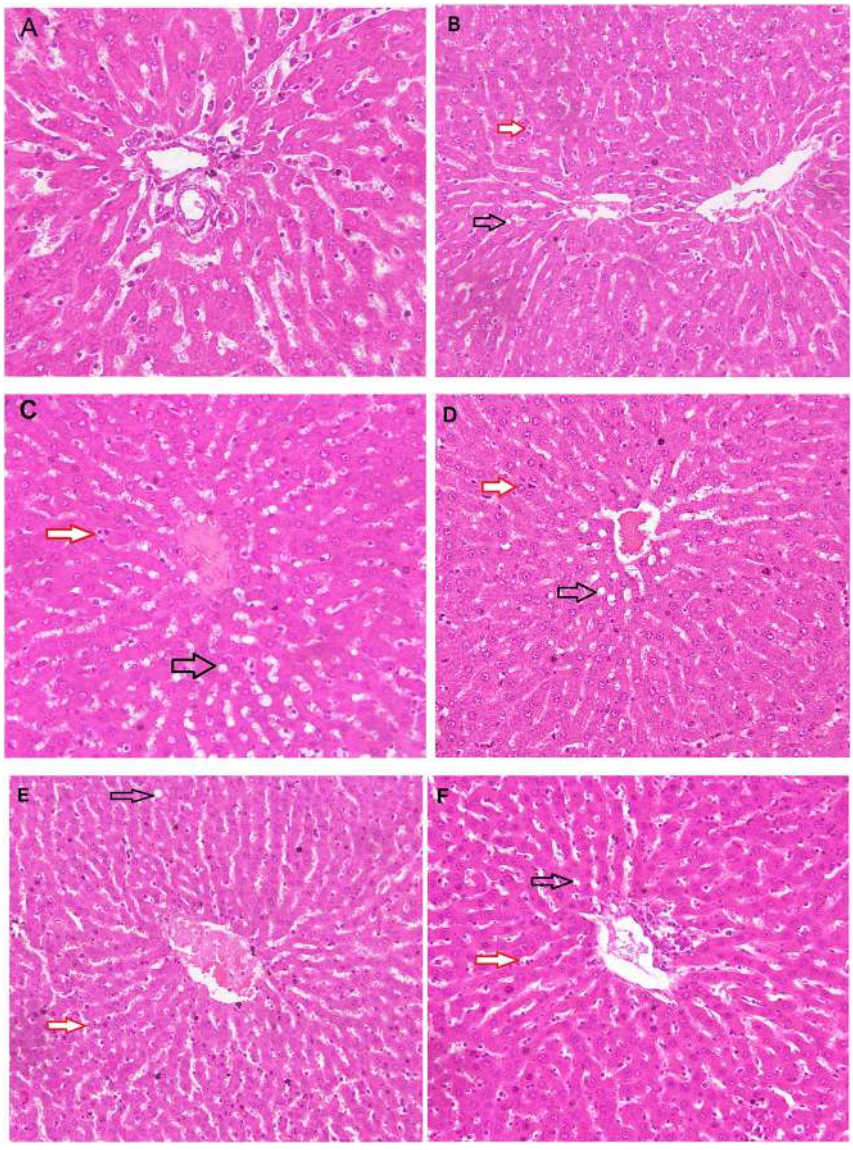


**Figure 6:** Histology of rat liver tissue by hematoxylin–eosin staining, and magnification of 200×. Necrosis cells are shown by red arrows and vacuolization by black arrows. A: normal group, B: positive control group, C: negative control (carbon tetrachloride, $CCl_4$), D: SCLEs dose 300 mg/kg, E: SCLE dose 400 mg/kg, E: SCLE dose 500 mg/kg.

inflammatory effect. It has been proved to have anti inflammatory activity *in vivo* [51]. The Quantitative Structure–Activity Relationship (QSAR) study regarding the potential of flavone-C-glycosides as anti inflammatory shows that glycosylation in ring A increased anti inflammatory activity and flavone-C-glycosides show a stronger anti inflammatory potential compared to flavone-O-glycosides [52]. The luteolin-8-C-glucoside compound (flavone glycoside) has anti inflammatory activity by reducing the expression of proinflammatory enzymes (COX-2 and i-NOS) [52, 53].

## Conclusions

The SCLE shows potential hepatoprotective effect that is observed by biochemical factor and histologic examination. This activity is likely caused by its phytochemistry contents that have antioxidant and anti inflammatory activity.

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**Informed consent**: Informed consent was obtained from all individuals included in this study.
**Ethical approval**: The protocol of this study was approved by the Ethics Committee of the Dentistry Faculty of Universitas Jember (No. 584/UN25.8/KEPK/DL/2019).

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Rozalina Loebis*, Bambang Subakti Zulkarnain and Nadhifa Zahra

# Correlation between the exposure time to mobile devices and the prevalence of evaporative dry eyes as one of the symptoms of computer vision syndrome among Senior High School students in East Java, Indonesia

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**Abstract**

**Objectives:** Computer vision syndrome (CVS) is a group of various eye and vision-related problems from prolonged use of mobile devices. Symptoms include dry eyes, blurred vision, eye strain, headache, and also neck and shoulder pain. This study was carried out to analyze the correlation between the exposure time of High Energy Visible (HEV) from mobile devices' use and the prevalence of evaporative dry eyes in young age.

**Methods:** An observational cross-sectional study was done using quota sampling method for 100 High School students. Data collection was performed using questionnaire to identify daily use of mobile devices (hours) and duration for using mobile devices (years). A classification was determined as mild, moderate, and heavy HEV exposure. Evaporative dry eyes were diagnosed using tear break-up time test (TBUT) of less than 10 s for both eyes.

**Results:** Ninety-four students participated in this study. A total of 82 students (87.2%) experienced evaporative dry eyes. There were 11 students (11.7%) who had dry eyes with mild exposure, 18 students (19.1%) had dry eyes with moderate exposure, and 53 students (56.4%) had dry eyes with heavy exposure. A chi square analysis showed all HEV exposures have similar risk to the prevalence of dry eyes among High School students ($p<0.05$).

**Conclusions:** The risk of developing evaporative dry eyes, as one of the symptoms of CVS in young age with normal tear production, could be induced even with minimal exposure to mobile devices.

**Keywords:** CVS; evaporative dry eyes; High School students; mobile devices; TBUT.

## Introduction

The usage of computers and other digital electronic devices, such as laptops, e-tablets, and smartphones for academic and nonacademic activities, for example, e-mail, social media, internet access, learning media, and entertainment covers almost all Indonesians, namely in children, adolescents, and adults [1]. In children aged 6–18 years old who need a lot of abilities to succeed and do well in school, good eyesight is the key. Reading, writing, working on the blackboard, and using computer are tasks that are carried out every day by students. When the eyes are not functionally well, the learning process will be disturbed [2]. Korean adolescents have reported a twofold increase in eye discomfort and visual symptoms resulting from using a smartphone for more than 2 h a day [3]. High School students aged 15–18 years old in Indonesia are thought to have similar problems due to undeniable excessive mobile devices use nowadays and might experience computer vision syndrome (CVS). CVS is problems related to the eyes and vision that result from prolonged use of mobile devices such as smartphones, laptops, and e-tablets [2]. CVS symptoms include one or more eye complaints from mobile device users such as eye fatigue, burning sensation, irritation, redness, blurred vision, and dry eyes [4]. Dry eyes are the commonest symptom of CVS where a person with dry eyes does not have enough tears to lubricate and nourish the eye surface. Tears are needed to maintain healthy eye surfaces and provide clear vision [2]. Young age has normal tear

***Corresponding author: Rozalina Loebis**, Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, Phone: +628113419344, E-mail: rozalinaloebis2512@gmail.com
**Bambang Subakti Zulkarnain,** Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Nadhifa Zahra,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

production, thus any disturbances which induce dry eyes are potentially due to environmental factors.

Several risk factors associated with the development of CVS such as daily use of mobile devices (hours per day), history of mobile devices (years), gender, age, eye diseases, viewing angles, use of contact lenses, visual display terminal (VDT) filters' use, ergonomically postures, and screen brightness adjustment [5]. A person with a history of computer use within 6 to >8 years may experience higher CVS symptoms than those at <1 year [6]. CVS symptoms are also reported to occur frequently in students who use computers for >2 h a day [7]. Mobile devices produce strong High Energy Visible (HEV) or blue light which accountable for vision-related problems such as cataract or age-related macular degeneration. Prolonged HEV exposures will have a negative impact on the retina. Also, HEV penetration into the macular pigment will reduce eyes protection, making the eyes more susceptible to HEV exposure and cell degeneration. Several *in vitro* models showed HEV is a pathogenic contributor to corneal damage [8, 9]. Therefore, this study was carried out to analyze the influence of exposure of mobile devices, in terms of daily use (hours) and history of their use (years), and the prevalence of evaporative dry eyes in High School students in East Java, Indonesia.

## Materials and methods

This study was a part of the Faculty of Pharmacy Universitas Airlangga community services in rural area in East Java, Indonesia. A sampling quota of total of 100 High School students was allocated for both one public school and one private school. Participants were all second grade High School students and were registered by first come first serve basis a week before community service carried out. All participants were interviewed to recall the average of their daily mobile devices use (hours) and history of using them (years). Next, all students were subjected to the diagnostic examination to test the evaporative dry eyes using the tear break-up time test (TBUT) for dextra and sinistra of their eyes. TBUT was performed to all students using fluorescein staining and slit lamp biomicroscopy examination. A diagnosis of evaporative dry eyes was carried out by an ophthalmologist and was based on the TBUT under 10 s [10]. This study was approved by the Ethical Committee Faculty of Medicine Universitas Airlangga No. 241/EC/KEPK/FKUA/2017 and all participants were provided with informed consent. All confidentiality of the data of the participants were guaranteed.

Based on daily mobile devices use (hours) and history of using them (years), a classification of mild, moderate, and heavy exposure to HEV of the mobile devices was used. These data were collected during the interview session. Amount of HEV exposure on daily basis (hours) was scored to ordinal data of 1, 2, 3, or 4 while duration of mobile devices use (years) was also scored as ordinal data of i, ii, and iii. Multiplication between those ordinal data of amount exposure to HEV (hours) and duration for using mobile devices (years) determines classification of mild, moderate, or heavy exposure. The result of multiplication of 1–2 denotes mild exposure, 3–5 denotes moderate exposure, and 6–12 denotes heavy exposure [11]. Table 1 shows the classification of mild, moderate, or heavy HEV exposure. Furthermore, all data were analyzed using the Statistical Package for the Social Sciences Data version 25. A Chi-squared test was used and the test result is considered statistically significant if p value <0.05.

## Results

A total of 94 students have participated in this study. Demographic data include gender and age. The number of female students was higher than male students but age was almost similar at an average of 16 years old. Table 2 shows the demographic data and also the types of mobile devices that had been used by students. More than half of the students use both laptop/PC and smartphones followed by smartphones alone in the second place. Overall, laptop/PC and smartphones were favorite choice of mobile devices among High School students. About 82 (87.2%) students developed dry eyes based on TBUT examination. Based on gender, more female students had evaporative dry eyes than male (91.3 vs. 76%).

Furthermore, Table 3 shows the prevalence of evaporative dry eyes based on TBUT examination and correlation to HEV exposure either mild, moderate, or heavy.

Table 3 shows that most of the students participated in this study were diagnosed with evaporative dry eyes due to their uses of mobile devices. Overall 82 students (87.2%) developed dry eyes on both eyes. Furthermore, it also shows 11, 18, and 53 students developed evaporative dry eyes respectively on both eyes based on mild, moderate, or heavy HEV exposure. Dry eyes are one of the important CVS symptoms. Also, the statistical analysis showed that all exposures have the similar impact to the prevalence of evaporative dry eyes ($p<0.05$) suggesting that minimal HEV exposure could trigger dry eye, as one of the important symptoms of CVS, even in young age with normal tear production.

**Table 1:** Multiplication of ordinal data between duration (years) and daily exposure (hours) determines HEV exposure [11].[a]

| Duration for using mobile devices, years | Amount of daily exposure to HEV, hours | | | |
|---|---|---|---|---|
| | 1–4 h (1) | 5–8 h (2) | 9–12 h (3) | >12 h (4) |
| 1–4 years (i) | 1 | 2 | 3 | 4 |
| 5–8 years (ii) | 2 | 4 | 6 | 8 |
| >8 years (iii) | 3 | 6 | 9 | 12 |

[a]Mild (1–2), Moderate (3–5), and Heavy (6–12).

**Table 2:** Demographic data of High School students and types of their mobile devices.

| Demographic data | Number of students, % | Number of students diagnosed with dry eyes, % |
|---|---|---|
| Gender | | |
| Male | 25 (26.6) | 19 (76[a]) |
| Female | 69 (73.4) | 63 (91.3[a]) |
| Total | 94 | 82 (87.2) |
| Age | Average of all students were 16 years old | |
| Type of mobile devices | | |
| Laptop/PC | 1 | 1.1 |
| Smartphones | 34 | 36.2 |
| e-tablet | 0 | 0 |
| Laptop/PC and smartphones | 54 | 57.4 |
| Laptop/PC and e-tablet | 1 | 1.1 |
| Smartphones and e-tablet | 2 | 2.1 |
| All of them | 2 | 2.1 |

[a]Percentage for each gender (Male: 19 out 25; Female: 63 out 69).

## Discussion

In the latest century, daily exposure to digital screens has increased dramatically due to human dependency to devices with advance technologies in individual or workplace settings. This also raises the number of complaints of ocular discomfort such as CVS. CVS is a syndrome resulted from prolonged and continuous use of computers, laptops, smartphones, televisions, and tablets or others devices with VDTs. CVS symptoms include ocular and muscular symptoms. Common CVS ocular symptoms include dryness and irritation, burning sensation, blurred vision, hyperemia, diplopia, glare sensitivity, or temporary deception in color perception. While common non-ocular symptoms or muscular symptoms include musculoskeletal pain in the neck, back, and shoulders, carpal tunnel syndrome, and venous thromboembolism, or other dermatological problems. With the fast development of informational technologies, mobile devices, and widespread of internet use, the global burden of screen-induced visual discomfort will rise dramatically [8]. Thus, identifying causes such as time exposure from HEV devices will help to improve health related digital wellbeing and academic performances in students.

**Table 3:** HEV exposure and the prevalence of evaporative dry eyes (dextra/sinistra).

| Exposure | Dextra/sinistra dry eyes n, % | | | | Total n, % | |
|---|---|---|---|---|---|---|
| | No/No | Yes/No | No/Yes | Yes/Yes | | |
| Mild | 1 (1.1) | 0 (0.0) | 0 (0.0) | 11 | 12 (12.8) | p<0.05 |
| Moderate | 4 (4.3) | 0 (0.0) | 0 (0.0) | (11.7) | 22 (23.4) | |
| Heavy | 5 (5.3) | 1 (1.1) | 1 (1.1) | 18 | 60 (63.8) | |
| | | | | (19.1) | | |
| | | | | 53 | | |
| | | | | (56.4) | | |
| Total n, % | 10 | 1 (1.1) | 1 (1.1) | 82 | 94 (100) | |
| | (10.6) | | | (87.2) | | |

In this study, participants were second grade High School students with an average age of 16 years old in Indonesia. This young age group is in the peak performance of health physically and mentally. At this age, the tear production is normal and thus any disturbances to tear production, if no other diseases present, are due to environmental factors. Consequently, most of the students in this study developed evaporative dry eyes as one of the important symptoms of CVS (87.2%) either mild, moderate, or heavy HEV exposure. This figure is almost similar to the study conducted in Elementary School students in Indonesia aged 6–10 years old, which showed that 88% of students developed evaporative dry eyes [11]. A 2017 unpublished small study in undergraduate students in Indonesia also showed that the prevalence of evaporative dry eyes was of 87%. It is estimated that about 50–90% computer users experience symptoms of CVS which is evaporative dry eyes is one of the commonest symptoms [7, 8, 12]. Evaporative dry eyes can be caused by exposure to mobile devices which can increase the evaporation of eye tears. Reduced blink reflexes when starring at a computer screen for a long time can also cause evaporative dry eyes [2]. The small fonts and the insufficient or dark lighting on mobile devices' screens can cause the low-blink rate [11]. An unstable tear film was found in evaporative dry eyes patients with reduced tear production and increased tear evaporation [13]. TBUT is a method for determining the stability of the tear film and is used to diagnose evaporative dry eyes. TBUT is carried out by instilling sodium fluorescein dye to the eyes. The tear film stability is observed under the slit lamp biomicroscopy while the patient is advised to avoid blinking activity until tiny dry spots developed onto the surface of the eye. Generally, TBUT more than 10 s is considered within normal tear film stability [10].

Prolonged use of mobile devices is associated with evaporative dry eyes diagnosis, symptoms, and signs [8]. All of 82 evaporative dry eyes students in this study used mobile devices of more than 5 h a day. A Japanese study involving 102,582 people aged 40–74 years old was carried out to evaluate the correlation between daily hour VDT use

and dry eyes diagnosis. A higher prevalence of dry eyes was found to people with more than 5 h daily use of VDT [14]. In other Japanese study involving 3,549 younger individual aged 22–60 years old, prolonged use of VDT also has been associated with dry eyes [15]. Those studies showed that prolonged VDT use is a risk factor for evaporative dry eyes. Likewise, Table 2 shows that the number of female students with evaporative dry eyes was higher than male (91.3 vs. 76%). This prevalence was similar to other studies conducted in Japan and the United States females were more likely to have dry eyes than men [14–16]. Symptoms such as eye dryness, redness, burning, and blurred vision have been linked to screen use [8]. In a Saudi Arabia study among undergraduate students using self-reported questionnaire for six CVS symptoms, it showed that the most frequent CVS symptoms were burning followed by dryness, blurred vision, and redness. Dryness was the most frequent severe symptom. This Saudi Arabia study also showed that screen time of more than 5 h was correlated with three CVS symptoms [17]. Interestingly, short-term continuous use of 1 h mobile devices has also been linked to dry eyes symptoms [18]. Thus, short- and long-term VDT use is linked to ocular symptoms discomfort such as dry eyes. Furthermore, screen time has also been attributed to dry eyes signs [8]. A Japanese study of 561 VDT users (aged 22–65 years, mean VDT use 7.9 h per day) measured ocular signs included TBUT. This Japanese study found VDT time of more than 8 h was associated with dry eyes and again female had higher prevalence than male [15]. In Turkish study also showed VDT user of more than 6 h daily had significant higher dry eyes symptoms [19]. Also, dry eyes signs have been occurred in children with screen use [11, 20]. Overall, those studies showed prolonged VDT use has been associated with negative effect of tear film stability, volume, and corneal epithelial integrity [8].

In this study among second grade Senior High School students in Indonesia, the least classification (mild HEV exposure) was defined as daily use of 1–4 h for 1–4 or 5–8 years; or daily use of 5–8 h for 1–4 years (Table 1). A chi square analysis among mild vs. moderate vs. heavy HEV exposure showed that all exposures have the same risk of developing evaporative dry eyes ($p<0.05$). Thus, similar to other studies, a minimal HEV exposure of 1–4 h a day was associated as a risk for developing evaporative dry eyes. Several mechanisms have been attributed to the pathophysiology of ocular damage and dry eyes including reduced blink rate, Meibomian Gland Dysfunction, and corneal phototoxicity. Inadequate blinking plays an important role in the loss of tear film homeostasis during VDT use in several studies. Furthermore, HEV or blue light toxicity may be a contributor to dry eyes for VDT users. Blue light can cause reactive oxygen species formation and oxidative damage in corneal cells. The fact that prolonged VDT use has deleterious effect on eyes thus limiting screen time and blink modification are obvious measures to prevent CVS. Resting from computer work for 15 min for every 2 h use and the "20-20-20 Rule" has been recommended by The American Academy of Ophthalmology and American Optometric Association. Also, blinking exercise has been shown to reduce dry eyes symptoms and signs [8].

## Conclusions

This study has shown that a minimally HEV exposure is associated with the risk of developing evaporative dry eyes among individual at young age with normal tear production.

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Mahardian Rahmadi*, Ahmad Dzulfikri Nurhan, Eka Dewi Pratiwi, Devita Ardina Prameswari, Sisca Melani Panggono, Khoirotin Nisak and Junaidi Khotib

# The effect of various high-fat diet on liver histology in the development of NAFLD models in mice

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**Abstract**

**Objectives:** Nonalcoholic fatty liver disease (NAFLD) is exceptionally common around the world. The development of NAFLD is increasing rapidly in the world, along with changes in lifestyle. Excess lipid intake is one of the risk factors for NAFLD. The NAFLD model is induced by a high-fat diet contains SFA, MUFA, and ϕ-6 PUFA. This study aims to assess the effect of high-fat diet variation on liver histology in developing NAFLD models in mice.

**Methods:** Thirty-six male mice (Balb/c) were divided into six groups fed a high-fat diet containing beef tallow 60%, beef tallow 45%, vegetable ghee, animal ghee + corn oil, vegetable ghee + corn oil for 28 days and compared to a control group fed a chow diet. All of the mice were fed with a high-fat diet in the form of pellets *ad libitum* for 28 days. Bodyweight and food intake were measured every day. At the last day of treatment, animals were sacrificed and the Liver were taken for histological analysis.

**Results:** This study showed that NAFLD model development was achieved in all group mice fed a high-fat diet with different degrees of NAFLD. Beef tallow 60% had the worst liver histology.

**Conclusions:** Thus, based on this study, we found that high-fat diet variations influenced the development of NAFLD models in mice, particularly concerning liver histology.

***Corresponding author: Mahardian Rahmadi,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +6281224656516,
E-mail: mahardianr@ff.unair.ac.id
**Ahmad Dzulfikri Nurhan, Eka Dewi Pratiwi, Devita Ardina Prameswari and Khoirotin Nisak,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia
**Sisca Melani Panggono and Junaidi Khotib,** Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

**Keywords:** healthy lifestyle; high-fat diet; liver histology; mice; nonalcoholic fatty liver disease.

## Introduction

Nonalcoholic fatty liver disease (NAFLD) is a condition caused by anomalies in fat accumulation within the liver, without excessive alcohol intake. Some of the characteristics of NAFLD include steatosis, fibrosis, inflammation, and ballooning [1]. Pathologically, NAFLD is classified into the nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL is a relatively mild liver disorder, characterized by steatosis without ballooning and inflammation. Meanwhile, NASH characterized by steatosis, ballooning, with or without inflammation [1, 2]. NASH is also an essential factor in hepatocellular carcinoma development [3].

Several studies have suggested that an increase in NAFLD prevalence is related to increased mortality of liver disease [4, 5]. In Western countries, the prevalence of NAFLD reaches 15–30% in the population. This prevalence increases to more than 50% in overweight individuals and increases to more than 90% in obese non-diabetic individuals [6]. It is currently known that lifestyle changes have implications for the massive development of NAFLD in the world. Improper dietary habits, especially excessive fat intake, are categorized as an essential risk factor related to the pathogenesis of NAFLD [7]. However, animal and human studies of the effects of fat intake on NAFLD development are limited [8].

Furthermore, the high-fat diet (HFD) is one approach that is often used to develop NAFLD models in experimental animals. HFD contains excess fat composition (varies between 45 and 75%) and is given *ad libitum* to experimental animals for a certain period [9]. The variation in the type of fat used is significant because *in vitro* evidence has found that different fatty acids have different effects on hepatocytes [10]. Most NAFLD model studies with experimental animals focus on HFD lard, while other fats are also widely consumed in daily life [8]. Thus, the

study of animal models of NAFLD-induced HFD using fats from other sources is an interesting thing to explore.

Some of the ingredients developed as HFD include beef tallow, animal ghee, vegetable ghee, and corn oil. Beef tallow and animal ghee are dominant in the content of saturated fatty acids (SFA), with a percentage of 55.4 and 65% [11]. Meanwhile, vegetable ghee is dominant in the content of monounsaturated fatty acids (MUFA). Corn oil is rich in linoleic acid (ω-6 polyunsaturated fatty acids; ω-6 PUFA), with a percentage of 50.4% [12].

In principle, HFD adopts a diet low in carbohydrates but high in fat. When glucose reserves in the body have depleted, the body uses fat as an alternative energy source from breaking down fatty acids in adipose tissue [13]. Furthermore, free fatty acids diffuse into the circulation, which has implications for increasing fatty acid levels in the blood. These things trigger the uptake of fatty acids in the blood by the liver, causing fat accumulation in the liver [14]. The body requires a variety of fatty acids with diverse degrees of saturation. In PUFA deficiency, the body tries to compensate for its needs by synthesizing these fatty acids from SFA and MUFA. This condition causes the fatty liver to worsen due to the high SFA took up by the liver [15].

The saturation and size of the carbon chains of fatty acids also affect their metabolism in the body. SFA and MUFA tend to be difficult to use as energy as soon as they are absorbed. Therefore, these two types of fatty acids are mostly stored in adipose tissue. However, if the body lacks energy, there is a massive breakdown of fatty acids in the adipose tissue. Meanwhile, PUFAs tend to be immediately oxidized to be used as energy in the body or diverted to other functions, for example making cell membranes [16].

Thus, the variation of HFD and the success of creating HFD significantly determine the NAFLD model. This research conducts a study related to the effect of various types of HFD on liver histology to determine and develop NAFLD models.

# Materials and methods

## Ethical approval

All experiments on animals were carried out at the Animal Research Laboratory of the Faculty of Pharmacy, University of Airlangga, Indonesia, and refer to the Guidelines for the Care and Use of Laboratory Animals released by the Ethical Committee of the Faculty of Veterinary Medicine, University of Airlangga, Indonesia.

## Animal and experimental design

Balb/c male mice weighing about 20–30 g were used in this study. They were housed in chip-bedded plastic cages at room temperature (22 ± 2 °C) in a 12 h diffuse light/12 h dark cycle at the Animal Research Laboratory of the Faculty of Pharmacy Universitas Airlangga. Mice were divided into six groups (n=6), fed a high-fat diet containing beef tallow 60%, beef tallow 45%, vegetable ghee, animal ghee + corn oil, vegetable ghee + corn oil for 28 days and compared to a group fed a chow diet (control group). Mice were fed according to the treatment group with 8–12 g of pellets. The remaining pellets were weighed daily for a food intake evaluation. Also, the body weight of the mice was weighed every day during treatment. All treatments lasted 28 days. The HFD was developed in our laboratory. The final composition and caloric value of HFD and chow diet are present in Table 1.

## Histological examination

After 28 days of treatment, all mice in each group were sacrificed, and the liver was extracted. Tissue samples were removed directly post-mortem and fixed in 10% formalin, processed, and embedded in paraffin. Then, 3 μm sections were cut and stained with hematoxylin and eosin. The slides were examined under an optical microscope, and the digital images were captured. The liver's histological features

**Table 1:** The final composition and caloric value of HFD and chow diet.

| HFD composition | Composition, % | | | | | | | Caloric value, kcal/100 g |
|---|---|---|---|---|---|---|---|---|
| | Chow diet, 306.20 kcal/100 g | Cornstarch, 360 kcal/100 g | Beef tallow, 902 kcal/100 g | Corn oil, 884 kcal/100 g | Milk, 500 kcal/100 g | Vegetable ghee, 834 kcal/100 g | Animal ghee, 894 kcal/100 g | |
| Chow diet | 100% | N/A | N/A | N/A | N/A | N/A | N/A | 306.20 |
| HFD beef tallow 60% | 15% | 25% | 60% | N/A | N/A | N/A | N/A | 677.13 |
| HFD beef tallow 45% | 35% | 20% | 45% | N/A | N/A | N/A | N/A | 585.07 |
| HFD vegetable ghee | 30% | 20% | N/A | N/A | N/A | 50% | N/A | 580.86 |
| HFD animal ghee + corn oil | 35% | N/A | N/A | 10% | 25% | N/A | 30% | 588.77 |
| HFD vegetable ghee + corn oil | 35% | N/A | N/A | 10% | 25% | 30% | N/A | 570.77 |

(including steatosis, inflammation, ballooning) were analyzed using a scoring system based on the NAFLD activity score.

### Statistical methodology

Bodyweight profile and food intake profile data were statistically analyzed using Two Way ANOVA and followed by post hoc analysis using the Bonferonni test. All calculations were performed using the GraphPad Prism 6 Software (GraphPad, Inc., San Diego, CA, USA). Bodyweight profile and food intake profile data are represented as mean ± SEM.

## Results

### The effect of HFD variation on body weight profile of mice

The results obtained showed that after 28 days of treatment, there was an increase in body weight of about 5% in the group of mice fed with the chow diet. Further, the group of mice given HFD animal ghee + corn oil and HFD vegetable ghee + corn oil showed an increase in body weight over 20%. Meanwhile, the group of mice that were given HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee showed a weight loss of more than 20% (Figure 1).

### The effect of HFD variation on food intake profile of mice

The food intake profile for each mouse was obtained from weighing the remaining feed (pellets) every day for 28 days. Group 1, as a control group, was given 8–12 g chow diet per day containing 306.2 kcal/100 g. Furthermore, the HFD groups were given a variation of HFD 8–12 g per day with almost the same amount of calories for every 100 g of pellets, respectively: group 2 were given HFD beef tallow 60% containing 677.07 kcal/100 g, group 3 were given HFD beef tallow 45% containing 585.07 kcal/100 g, group 4 were given HFD vegetable ghee containing 580.86 kcal/100 g, group 5 were given HFD animal ghee + corn oil containing 588.77 kcal/100 g, and group 6 were given HFD vegetable ghee + corn oil containing 570.77 kcal/100 g. The results obtained showed that the amount of calories intake in the group of mice fed with HFD was higher than the calories intake in the group of mice fed with the chow diet (Figure 2B).

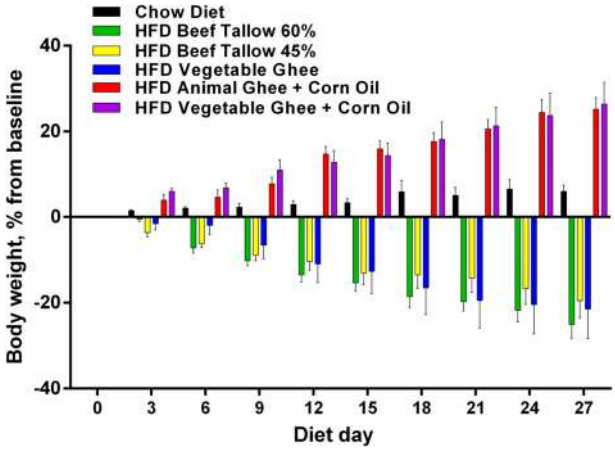


**Figure 1:** Bodyweight profile of each group of tested animals.

### The effect of HFD variation liver histology of mice

Histological examination of the liver was observed under an optical microscope at 400× magnification. Based on microscopic observations, the liver histology of mice fed a chow diet showed nuclei of cells with cytoplasm and sinusoid in a regular pattern around the central vein (Figure 3A). The histology of mice given HFD beef tallow 60% showed macrovesicular steatosis and ballooning accompanied by inflammation that resembled the NASH pattern (Figure 3B). Meanwhile, the histology of mice given HFD beef tallow 45% and HFD vegetable ghee showed an abnormal hepatocyte structure, characterized by microvesicular steatosis, ballooning, and inflammation around the central vein. In both experimental groups, there was also a borderline/probable NASH which is the boundary for NAFL and NASH (Figure 3C, B). Further, in the group of mice given HFD animal ghee + corn oil and HFD vegetable ghee + corn oil showed hepatocytes with mostly normal cell structures, but some cells showed ballooning. Liver histology in these two groups showed a more NAFL-oriented pattern which is the initial stage of NAFLD development (Figure 3E, F).

## Discussion

HFD-induced NAFLD is the preferred method in animal studies for reproducing the conditions observed in humans, including the biochemical and histopathological aspects of developing NAFLD [9]. This study evaluates the effects of various types of HFD, including; HFD beef tallow 60%, HFD beef tallow 45%, HFD vegetable ghee, HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil

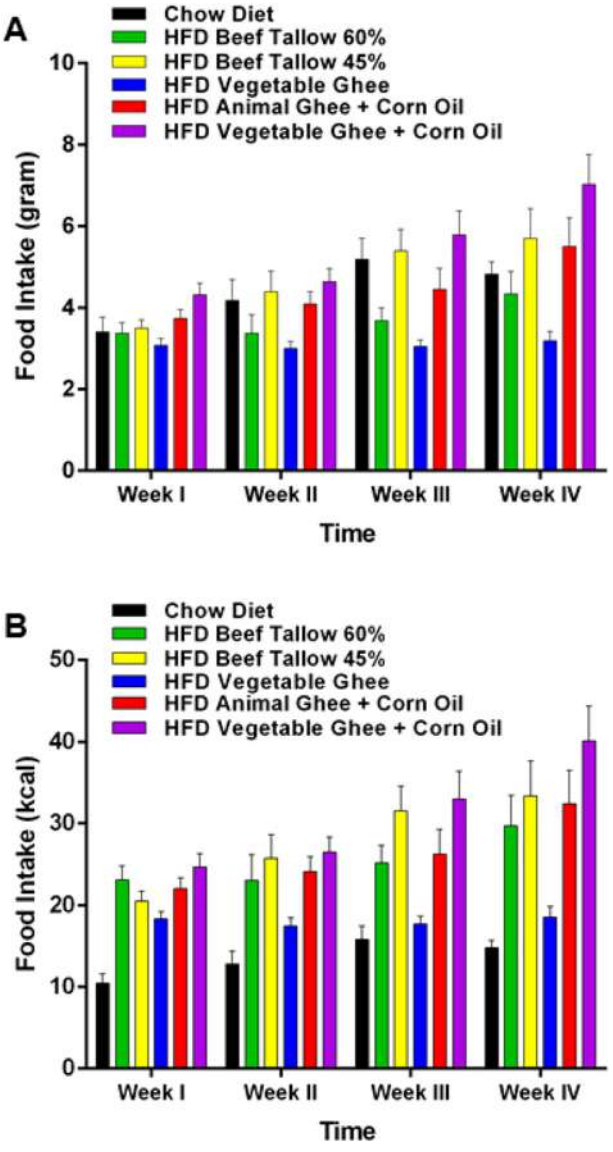


**Figure 2:** Food intake profile of each group of tested animals for 28 days {units of grams (A), units of calories (B)}.

on the development of NAFLD models in mice. The parameters observed in this study include; body weight profile, food intake profile, and liver histology in mice.

The body weight profile of mice related to the lipolysis and esterification processes in adipose tissue. Triglycerides (TG) in adipose tissue are stored statically but may always undergo two-way reactions, namely lipolysis, and esterification. In this study, mice were given HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil showed an increase in body weight. This finding may be due to the esterification process to form TG beyond lipolysis so that TG accumulates in adipose tissue and causes obesity. The presence of PUFA contained in HFD animal ghee + corn oil, and HFD vegetable ghee + corn oil may also inhibit the breakdown of fatty acids in adipose tissue [14].

In contrast, mice fed with HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee showed weight loss (Figure 1). This finding may be due to the breakdown of fatty acids in adipose tissue and lipolysis that is greater than esterification. Fatty acids formed as a result of partial lipolysis impossible to be re-esterified because they were not kept up with the rate of lipolysis. As a result, free fatty acids that accumulate in adipose tissue immediately diffuse into the circulation, leading to weight loss and increased fatty acids in the blood [14, 17]. The high lipolysis caused by HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee may be due to the dominant SFA and MUFA content so that the body compensates for PUFA deficiency by synthesizing these fatty acids from SFA and MUFA. This circumstance results in a greater breakdown of SFA/MUFA in adipose tissue. Besides, HFD, which is low in carbohydrates, causes reduced levels of glucose and insulin in the blood resulting in a decrease in the amount of glucose that enters the adipocytes. This condition results in reduced esterification and increased lipolysis [17].

Furthermore, the results of liver histology in this study showed that all mice fed HFD develop a NAFLD but to varying degrees. The group of mice that were fed with HFD vegetable ghee + corn oil and HFD animal ghee + corn oil, which were rich in SFA/MUFA + PUFA content, experienced NAFLD with a relatively lower degree than the group of mice fed other types of HFD. These findings were in line with the postulates put forward by several previous studies, where PUFA was shown to induce NAFLD because it causes the accumulation of arachidonic acid in the membrane [18]. However, when PUFA is combined with SFA/MUFA, it was known to prevent insulin resistance and avoid SFA/MUFA-induced endoplasmic reticulum stress and prevent inflammation in the liver [19]. These are probably why the development of NAFLD in these two groups had a lower degree than the group that was fed other types of HFD in this study.

Whereas in mice fed HFD beef tallow 60%, HFD beef tallow 45%, and HFD vegetable ghee were found to develop a more severe degree of NAFLD. This condition may be caused by the buildup of excess free fatty acids in the adipose tissue and diffuse into the circulation which causes an increase in fatty acids in the blood and is taken up by the liver in large amounts, exacerbating the condition of NAFLD [14, 17]. Furthermore, several studies suggest that the dominant HFD beef tallow contains SFA injure hepatocyte directly through several mechanisms, including; cell death induced stress of endoplasmic reticulum, causing intrinsic mitochondrial apoptosis, as well as triggering inflammatory

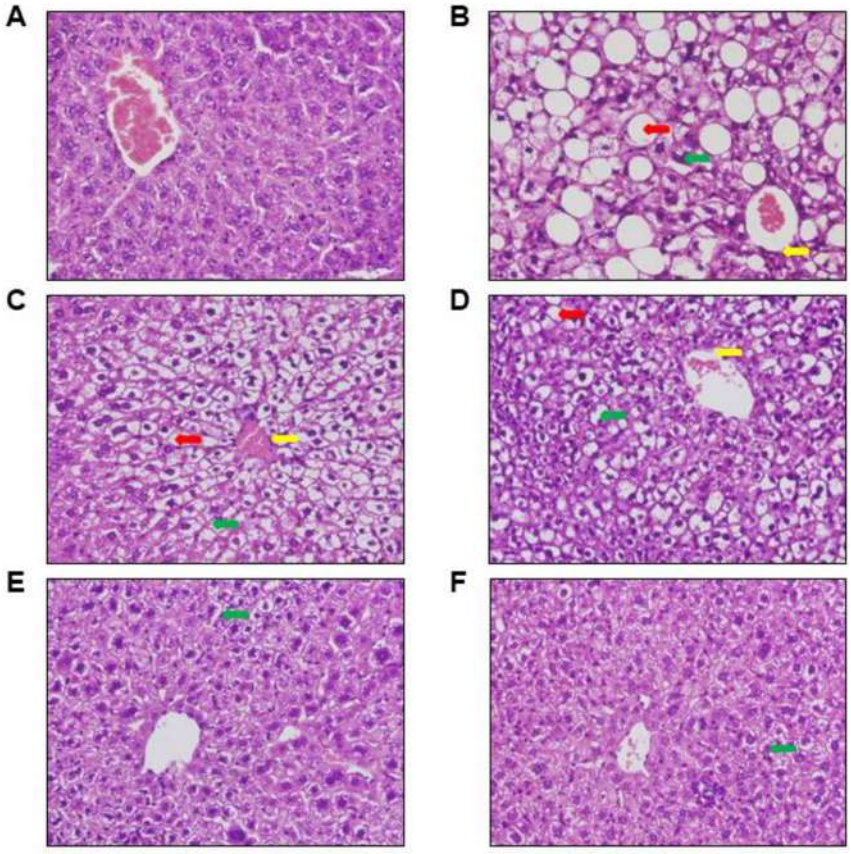


**Figure 3:** Histological analysis with hematoxylin-eosin staining from the liver revealed damage caused by HFD, according to the type of fat used; (A) control group, (B) beef tallow 60% group, (C) beef tallow 45% group, (D) vegetable ghee group, (E) anima ghee + corn oil group, (F) vegetable ghee + corn oil group. Red arrows indicate steatosis, green arrows indicate ballooning, and yellow arrows indicate inflammation.

mediators (e.g., cytokines) [20]. Besides, SFA also causes an increase in adipocyte oxygen consumption and leads to hypoxia of adipose tissue *in vivo* [21]. Meanwhile, MUFA-enriched HFD vegetable ghee was associated with *in vivo* adipocyte hyperplasia. One study has also reviewed that administration of MUFA causes liver steatosis and inflammation of adipose tissue [22].

Studies conducted in humans have shown that SFA tends to induce steatosis, insulin resistance, and proinflammatory cytokines (such as TNF-α) compared to PUFA [23]. These findings also are attributed to this study, where the histological results of mice fed a combination of HFD (HFD vegetable ghee + corn oil, and HFD animal ghee + corn oil) showed less degeneration of ballooning than the mice fed with vegetable ghee or HFD beef tallow only (Figure 3).

A study examining the SFA and MUFA fats found that these two types of fat cause NASH, which is an advanced stage of NAFLD disease. In addition, it was also found that different compositions of the same fat type influence the development of different NAFLD [18]. These findings are in line with the results of this study, where the experimental group fed with HFD beef tallow 45% (predominantly containing SFA) and HFD vegetable ghee (predominantly containing MUFA) showed a more probable NASH pattern (Figure 3C, D). In comparison, the experimental group fed with HFD beef tallow 60% showed a definite NASH pattern (Figure 3B).

In terms of food intake, this study found differences in the food intake profile between the experimental groups (Figure 2). Several studies have tried to examine the factors that may affect the profile of food intake in mice, including mouse strains, genetic background (transgenic and knockout mice), age, gender, stress and habituation, body composition and body fat distribution, diet composition, and appetite [24]. Among these, factors that may have played a significant role in our study were dietary composition and appetite. Our finding is in line with several previous studies, which revealed that diet composition affects appetite, which results in a change in the food intake profile of experimental animals. On the other hand, the variations in the types and characteristics of protein in the diet also produce different metabolic and physiological responses. This condition affects the digestion kinetics and digestibility where it is strongly associated with the resulting satiety [25]. Interestingly, in our study, we also found that each group's food intake profile tended to increase with each week. This condition may be due to

*ad libitum* feeding, which stimulates caloric intake in rodents over time [26].

In particular, several studies reported that MUFA caused more severe NAFLD compared to SFA at the same calorie amount [22]. Interestingly, in our research, we found that the SFA-enriched HFD beef tallow 45% tended to have the same severity as MUFA-enriched HFD vegetable ghee. This finding may be attributed to the total calories intake of the group of mice fed with HFD beef tallow 45%, which tended to be grated than the total calories intake of the group of mice fed with HFD vegetable ghee (Figure 2B), although the HFD of beef tallow 45% and HFD vegetable ghee had the same total calories per 100 g. Thus, apart from HFD composition, the proportion of HFD intake may also influence the severity of NAFLD development in mice.

## Conclusions

The development of the NAFLD model could be achieved in all groups of mice, where the liver histology level was the most severe, respectively; HFD beef tallow 60%, HFD beef tallow 45% and HFD vegetable ghee, and HFD animal ghee + corn oil and HFD vegetable ghee + corn oil. The results of this study may contribute to the development of the NAFLD model as an essential approach to studying the pathological state of disease. Apart from that, it may also be useful to discover and develop relevant therapeutic agents for this disease.

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**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** The authors state no conflict of interest.

**Ethical approval:** All experiments were conducted at the Animal Research Laboratory of the Faculty of Pharmacy University of Airlangga, Surabaya, Indonesia, in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the National Institutes of Health revised in 1985. The Ethics Committee of Faculty of Veterinary Medicine Universitas Airlangga, Surabaya, Indonesia, approved the study protocol.

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Samirah, Aniek Setiya Budiatin, Ferdiansyah Mahyudin and Junaidi Khotib*

# Fabrication and characterization of bovine hydroxyapatite-gelatin-alendronate scaffold cross-linked by glutaraldehyde for bone regeneration

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**Abstract**

**Objectives:** Alendronate are widely used in the treatment of bone disorders characterized by inhibit osteoclast-mediated bone resorption such as Paget's disease, fibrous dysplasia, myeloma, bone metastases and osteoporosis. In recent studies alendronate improves proliferation and differentiation of osteoblasts, thereby facilitating for bone regeneration. The disadvantages of this class are their poor bioavailability and side effects on oral and intravenous application such as stomach irritation and osteonecrosis in jaw. Thus, local treatment of alendronate is needed in order to achieve high concentration of drug. Bovine hydroxyapatite-gelatin scaffold with alendronate was studied. Glutaraldehyde was used as cross-linking agent, increase the characteristics of this scaffold. The objectives of this study were to manufacture and characterize alendronate scaffold using bovine hydroxyapatite-gelatin and crosslinked by glutaraldehyde.

**Methods:** Preparation of cross-linked bovine hydroxyapatite-gelatin and alendronate scaffold with different concentration of glutaraldehyde (0.00, 0.50, 0.75, and 1.00%). The scaffolds were characterized for compressive strength, porosity, density, swelling ratio, *in vitro* degradation, and cytotoxicity (the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay, shorted as MTT assay).

**Results:** Bovine hydroxyapatite-gelatin-alendronate scaffold cross-linked with glutaraldehyde showed lower density than without glutaraldehyde. As glutaraldehyde concentration increased, porosity also increased. Eventually, it reduced compressive strength. Swelling ratio and *in vitro* degradation was negatively dependent on glutaraldehyde concentration. In addition, the scaffold has a good safety by MTT assay.

**Conclusions:** Bovine hydroxyapatite-gelatin-alendronate scaffold was fabricated with various concentrations of glutaraldehyde. The presence of glutaraldehyde on bovine hydroxyapatite-gelatin-alendronate is safe and suitable candidate scaffold for bone regeneration.

**Keywords:** alendronate; bovine hydroxyapatite; gelatin; glutaraldehyde; scaffold; neglected disease.

## Introduction

Bisphosphonates, a bone resorption inhibitor, are drugs currently used for metabolic bone disease, such as osteoporosis, Paget's disease, fibrous dysplasia, myeloma, and bone metastases [1, 2]. Among bisphosphonates, alendronate (Ale) is a drug that is widely used because it effectively inhibits bone resorption by preventing recruitment and differentiation of osteoclasts. Furthermore, in recent study alendronate also improves the proliferation and differentiation of osteoblast, that can accelerates bone regeneration [3, 4]. In oral administration, alendronate has poor bioavailability (1%). Meanwhile, it is associated with side effects including esophageal irritation and osteonecrosis in jaw [2]. Given these drawbacks, the local administration alendronate through scaffold composite is a promising therapy strategy [1, 5].

Hydroxyapatite (HA) is an inorganic component that naturally present in bone tissue and widely used as a main composite for bone tissue regeneration [6]. Bovine hydroxyapatite (BHA) is a natural HA derived from bovine bone. BHA has carbonates substitution that improved activity of osteoblast [7]. Gelatin is a natural polymer which is similar to the organic components of the bone. Gelatin has biodegradable, biocompatible, and osteoinductive properties [8]. Therefore, BHA and gelatin composite is widely used as scaffolds for bone regeneration. Scaffold composed of BHA and gelatin is easily degraded, therefore need a

***Corresponding author: Junaidi Khotib**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 813 318 40710, E-mail: junaidi-k@ff.unair.ac.id
**Samirah and Aniek Setiya Budiatin,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Ferdiansyah Mahyudin,** Department of Orthopaedic and Traumatology, Faculty of Medicines, Airlangga University, Surabaya, Indonesia

crosslink agent such as glutaraldehyde (GA) to improve characteristics of composite [9, 10].

The objectives of this study were to manufacture and characterize of bovine hydroxyapatite-gelatin-alendronate scaffold with various concentrations of GA. The scaffold was characterized for mechanical strength, density, porosity, swelling ratio, *in vitro* degradation and cytotoxicity. The fabrication and characteristic test of the scaffold were carried out to obtain a suitable bone graft candidate for bone regeneration.

# Materials and methods

### Material

Bovine hydroxyapatite powder was obtained from Teaching Industry of Airlangga University, Surabaya, Indonesia. Alendronate sodium was product of Arshine Technology Co., Limited (Wanchai, China). Gelatin 150 bloom was product from Cartino, Thailand. Glutaraldehyde 25%, $KH_2PO_4$, $Na_2HPO_4$, and NaCl were product of Merck Millipore, Germany.

### Scaffold fabrication

Eighteen grams of bovine hydroxyapatite was mixed with 200 mg of Alendronate, and a 20 wt% gelatin 150 bloom solution in a warm mortar. After that, the mixture was granulated with a 1 mm sieve in order to obtain uniform size. The granules then were dried in 40 °C oven for 24 h. Then, the dried granules were cross-linked using glutaraldehyde with concentration of 0.00, 0.50, 0.75, and 1.00% for 24 h until the color change to brownish. After that the granules were washed with distilled water to remove the remaining glutaraldehyde, followed with phosphate buffer saline (PBS) at pH 7.4. After that, the granules were dried again in oven 40 °C. Dried granules (100 mg) were weight and pressed into pellets.

### Mechanical testing

Mechanical behavior of scaffold cross-linked with glutaraldehyde in different concentration was investigated through compression strength measured using an autograph (Shimadzu AG-10 TE, Japan). The scaffold was pressed with a cross head speed of 5 mm $min^{-1}$ in a cylindrical sample with a diameter of 4 mm and a height of 3 mm. Five samples of each group were used for the compressive strength [11–13].

### Density and porosity determination

Density is calculated based on the ratio of dry mass to volume. The porosity of scaffold is determined by weighing the dry mass, then immersing the sample in 5 mL of distilled water for about ±2 min until the sample expands. After that, the filter paper is used to remove the remaining liquid present on the sample. Then the sample is weight again as wet mass. The porosity is the ratio between the difference in wet mass and dry mass divided by the volume of the sample [14].

### Swelling ratio

Swelling ability of scaffold was measured based on the previously described method by [15, 16]. The scaffold was immersed in PBS solution (pH 7.4) at 37 °C for 1, 3, 7, 14, and 28 days. Wet samples were wiped with filter paper to remove excess liquid then weighed as wet weight (Ww). After that, the scaffold was dried at 60 °C for 72 h (Wd). Swelling ratio is measured based on equation = (Ww − Wd/Wd) × 100%.

### *In vitro* degradation

Scaffold degradation was carried out by immersing the sample in PBS in order to mimic the body fluids *in vivo*. The initial weight of scaffold is weighed before the degradation test conducted. The degradation test was carried by immersing the scaffold in PBS pH 7.4 at 37 °C for 1, 3, 7, 14, and 28 days. After immersing the scaffold, the scaffold was dried using oven at 60 °C for 72 h. After that, the scaffold was weight as dry weight. Weight loss is the changes of dry weight after immersion and initial weight before immersion [12, 15].

### Cytotoxicity test

The MTT assay is used to determine the viability of cells, depend on the cell's ability to metabolically reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. The MTT assay was conducted using Baby Hamster Kidney (BHK)-21 fibroblast cell. Each sample was mixed with 2 mL of media and put into well as much as 50 µL/well. After that, the well then added with culture media of 100 µL/well, and incubated for 24 h (37 °C). Then, samples were washed with PBS and added with MTT solution of 10 µL/well. After incubated for 3 h (37 °C), dimethyl sulfoxide (DMSO) as much as 50 µL/well was added and slowly shaked for 5 min to dissolve the formazan crystals which present in purple. The absorbance was measured with ELISA reader with the wavelength of 620 nm. Cell viability was determined by dividing the viability of treated cells with the controls [17].

### Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). The study data were statistically analyzed using software SPSS version 24.0 (SPSS Inc., chicago, IL, USA). The result obtain were submitted to the Shapiro Wilk normality test and One Way analysis of variance (ANOVA). p value less than 0.05 was considered statistically significant. All calculations were performed using GraphPad Prism 6 Software (GraphPad, Inc., San Diego, CA, USA).

# Results

## Mechanical testing

Mechanical strength is the capacity of a material or structure to withstand loads. Mechanical characterization test using autograph is needed to compress the scaffold until it breaks.

Figure 1 is the compressive test results of scaffold with various concentrations of glutaraldehyde. The compressive strength was 12.080 ± 1.156, 10.666 ± 0.808, 10.449 ± 0.946, 9.122 ± 0.670 MPa for scaffold with GA concentration of 0.00, 0.50, 0.75, and 1.00%, respectively. These results indicated that the presence of glutaraldehyde reduced compressive strength. Increasing glutaraldehyde concentration, reduced compressive test value.

## Density and porosity

Density is the ratio between the mass and volume of the substance at a certain temperature and pressure. While porosity is a measure of the empty spaces in a material, that contributes to the cell homing. Based on the porosity and density test, glutaraldehyde concentration affects the scaffold's density and porosity (Figures 2, 3). The density of scaffold with 1.00% GA was significantly different with another scaffold (0.00, 0.50, 0.75% GA) ($p<0.05$). Figure 3 shows the results of the porosity test on the scaffold with different GA concentration. The porosity of the scaffold in GA 0.00, 0.50, 0.75, and 1.00% was 37.837 ± 5.701, 60.914 ± 0.539, 63.306 ± 3.084, and 65.004 ± 4.063%, respectively ($p<0.05$). The result showed that more GA concentration increased the more porosity increased.

## Swelling ratio

Swelling ratio is the fractional increase in the weight of the hydrogel due to water absorption. Figure 4 showed swelling ratio of the scaffold with various concentrations of GA after soaking the scaffold in PBS for 28 days. All groups experienced cracks starting on day one, and there was an increase

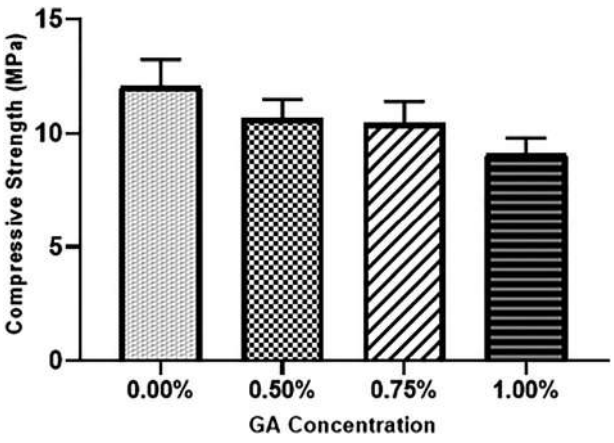


**Figure 1:** Compressive strength of scaffold with 0.00, 0.50, 0.75, and 1.00% GA.

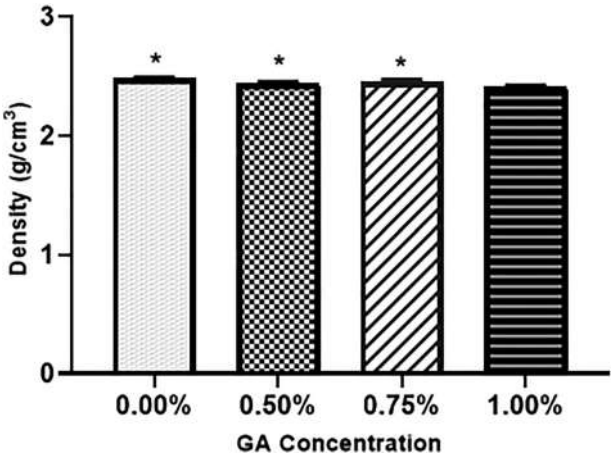


**Figure 2:** Density of four different scaffold as a function of the addition of glutaraldehyde (GA 0.00%, GA 0.50%, GA 0.75%, GA 1%) (*p<0.05 compared with 1.00%).

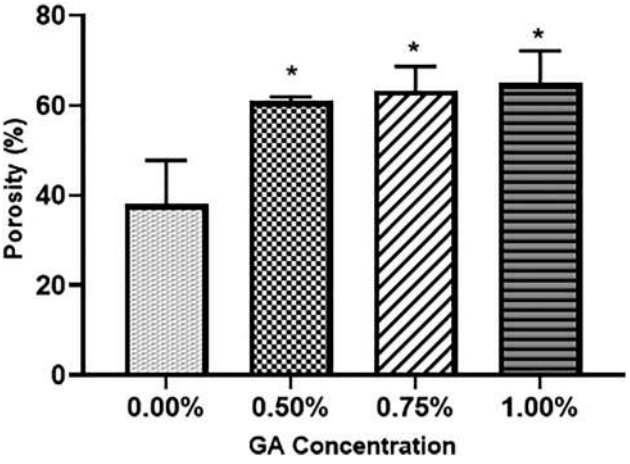


**Figure 3:** Porosity of four different scaffold as a function of the addition of glutaraldehyde (GA 0.00%, GA 0.50%, GA 0.75%, GA 1%) *p<0.05 compared with 0.00%.

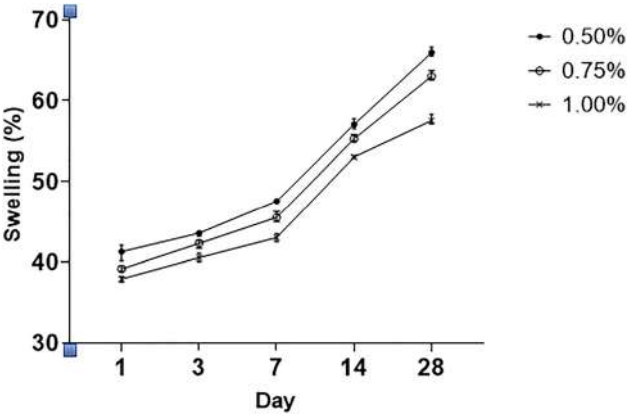


**Figure 4:** Swelling of scaffold crosslinked with various amount of glutaraldehyde concentration after immersion in PBS pH 7.4 at 37 °C for 28 days.

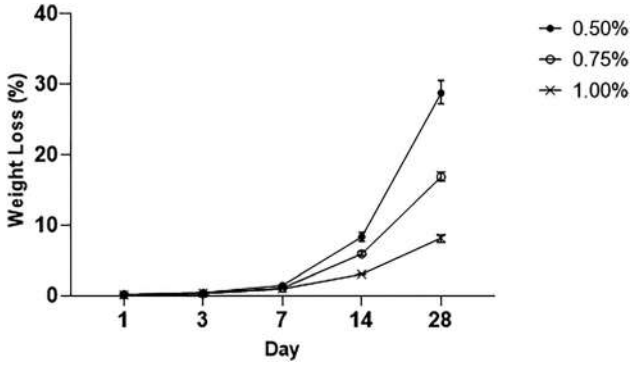


**Figure 5:** Weight loss of scaffold crosslinked with various amount of glutaraldehyde concentration after immersion in PBS pH 7.4 at 37 °C for 28 days.

after day seven and sharply increased from day 14 to day 28. Scaffold with 0.50% GA showed the highest swelling ratio. Cumulative swelling results on day 28 for scaffold 0.50, 0.75, and 1.00% were 65.963 ± 0.318, 63.040 ± 0.365, and 57.543 ± 0.389%, respectively ($p<0.05$).

### *In vitro* degradation

Degradation is gradual decomposition of a material. Figure 5 showed the scaffold's weight-loss after immersion in PBS pH 7.4 for 1, 3, 7, 14, and 28 days. All samples showed additional weight-loss during the time period. The curves were divided into three groups with different concentrations of GA. In the first group, the weight-loss of scaffold with 0.50% GA increased after day seven of immersion and got sharper after the 14th and 28th days. Another group was GA 0.75 and 1.00% show similar profile with first group. The cumulative weight-loss on day 28 for scaffold with GA concentration of 0.50, 0.75, and 1.00% GA scaffold were 28.727 ± 0.954, 16.800 ± 0.369, and 8.150 ± 0.315%, respectively. The minimum weight-loss was in the scaffold with 1.00% GA ($p<0.05$).

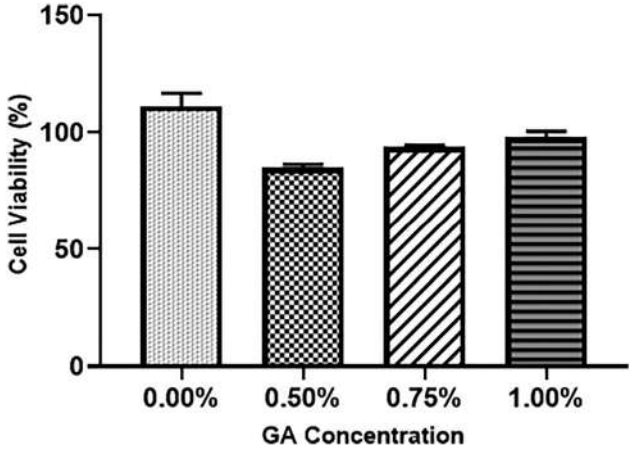


**Figure 6:** Result of cell viability study (MTT assay).

### Cytotoxicity

Cytotoxicity is the property of the chemicals that is harmful to living cells. Figure 6 shows the results of the cytotoxicity test using BHK 21 fibroblasts. The results of cell viability with various GA concentrations showed no toxic effect because the viability was above 50%. The highest cell viability was shown in the sample with GA level of 1.00%. The MTT assay was correlated with cell proliferation and mitochondrial function, the loss of cell viability was indicated by decreased MTT measurement [18].

## Discussion

In this study, scaffold bovine hydroxyapatite-gelatin-alendronate containing alendronate with varying levels of GA as crosslink agent was successfully designed. The characteristic properties of these scaffolds were provided. Furthermore, there was a decrease in the scaffold's compressive test, along with the increase in GA concentration (Figure 1). Higher GA concentration allowed gelatin chain to react with more GA molecules that causes more fragile [19]. The compressive test obtained in this study from 9.122 ± 0.670 to 12.080 ± 1.156 MPa, in line with the compressive strength for femoral bone (9.3 ± 4.5 MPa) [8] and compressive strength for spongy bone (4–12 MPa) [19].

As shown in Figures 2, 3, density was negative dependent to the porosity. The porosity of the scaffold with various concentrations of GA ranges from 60.914 ± 0.539 to 65.004 ± 4.063%, this corresponds to scaffold with high porosity of 50–65%. Scaffold without GA have 37.837 ± 5.701% porosity, appropriate to scaffold with low porosity of 35–45% [20]. The density of the scaffold affects mechanical strength, permeability, and the presence of structural defects. Porosity is an important parameter that defines the properties of biomaterials obtained [6]. Increase of porosity caused a decrease in compressive strength otherwise higher density contributes to higher mechanical strength [8]. High porosity provides a biological environment for proliferation, differentiation, and cell function that benefit the scaffold [21]. Therefore, it is necessary to balance between porosity and density of the scaffold to established specific application [8].

In this study, the swelling ratio showed that all samples with GA experienced cracks. The longer scaffold immersed in PBS, the more water absorbed. This could be because during the immersion process, the scaffold formed a lot of capillary

cavities that could cause PBS to enter and disrupt the bonds between the constituent compositions and then decrease the integrity [1, 15]. Swelling of scaffold facilitates cell adhesion, cell internalization and increases nutrient diffusion, which is the basis for enhancing tissue regeneration [21]. Concentration of glutaraldehyde is inversely proportional with swelling ratio. Porosity is a characteristic related to the swelling ratio. In general, when the scaffold has high porosity, the swelling ratio will also increase. However, different things happened in this study; the porosity test results compared the best with the swelling ratio test results. This may happen because of the effect of alendronate in the formulation. Alendronate formed strong chemical bonds through the phosphonate groups and the calcium ions. These chemical bonds might prevent the excessive swelling of the scaffold [22]. Scaffold with GA 1.00% showed the lowest swelling, is the most suitable one for scaffold application. In line with research conducted by Bigi et al. (2001), that the higher the concentration of glutaraldehyde will reduce the amount of water absorbed [23].

The degradation test results showed that weight-loss inversely related to the concentration of glutaraldehyde [16]. It was found that the scaffold with GA 0.50% had a more reduction in weight (28.727 ± 0.954). Whereas scaffold with GA 0.75 and 1.00% had a degradation for 28 days of 16.800 ± 0.369 and 8.150 ± 0.315%, respectively. The result is consistent with the research of Wang et al. 2009 that indicates that there is good degradation of the scaffold. As the scaffold begins to degrade, it is replaced by a new bone matrix, which can accelerate the bone regeneration process. When the degradation rate of the scaffold equal with the rate of osteogenesis, the scaffold can accelerate the process of regeneration [21].

The MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is based on the conversion of MTT to formazan crystals by living cells, which determines mitochondrial activity. Because for a large part of the cell population, the total mitochondrial activity is related to the number of viable cells [18]. The cytotoxic value was determined based on the IC50 concentration required to achieve 50% growth inhibition compared to the growth of the control [24]. All scaffold synthesized in this study showed an increasing trend of formazan absorbance (Figure 6), thus suggesting an increase in glutaraldehyde concentration by up to 1.00% increased cell proliferation.

Glutaraldehyde is a toxic agent at high concentrations [25]. Several studies that have been conducted by other researchers, including those of Gao et al., stated that the scaffold soaked and washed with the GA cross-link agent concentration of 1 and 2.5% had a toxic effect on chondrocyte cells [26]. The safe and optimal GA concentration is used as a cross-linking agent in the concentration range of 0.5–2% [19]. This study indicated that scaffold with GA 0.50–1.00% does not negatively affect cell proliferation, it can maintain cyto-compatibility properties and achieve good mechanical properties.

## Conclusions

Bovine hydroxyapatite-gelatin-alendronate scaffold was fabricated with various concentrations of glutaraldehyde. An increase in GA will increase the scaffold's porosity, which causes a decrease in compressive strength. The swelling test and *in vitro* degradation test are inversely proportional to the increase in GA. All samples showed non toxicity based on cytotoxic assays. Based on these results, the presence of GA in bovine hydroxyapatite-gelatin-alendronate is safe and suitable candidate scaffold for bone regeneration.

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**Competing interests:** Authors state no conflict of interest.
**Informed consent:** Not applicable.
**Ethical approval:** Not applicable.

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Ria Etikasari*, Tri Murti Andayani, Dwi Endarti and Kartika Widayati Taroeno-Hariadi

# Health related quality of life among postmenopausal woman with hormone responsive HER2– breast cancer in Indonesia

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**Abstract**

**Objectives:** Breast cancer (BC) in women could decrease health-related quality of life (HRQoL). HRQoL becomes important to be assessed to design a relevant treatment that could improve patient outcomes. Furthermore, assessing HRQoL by measuring health state utilities becomes pivotal for health economic evaluation. This study aimed to describe the HRQoL of postmenopausal women with hormone responsive (HR+) HER2– BC using the EQ5D5L instrument in Indonesia.

**Methods:** A cross-sectional study was conducted among 126 patients in Dr. Sardjito Hospital in Indonesia. The HRQoL was assessed by interviewing BC patients using the EQ5D5L questionnaire, and the utility index was calculated using the Indonesian value set. Information regarding clinical characteristic and socio-demographic were gained from patient medical records. One-way ANOVA and post-hoc Scheffe's test was performed to compare the utility score within the health state.

**Results:** Of the 126 patients, a mean ± SD for the age of 59.2 ± 6.1 years. The major problems of patients were pain/discomfort (75.4%) followed by anxiety/depression (54.8%). The mean (SD) of EQ5D VAS was 76.64 (14.91). Mean (SD) of utility score was 0.87 (0.10), 0.77 (0.19) and 0.58 (0.44) for free metastasis (FM), locoregional metastasis (LM) and distant metastasis (DM), respectively. Poor QoL was observed at DM health state ($p<0.05$).

**Conclusions:** HRQoL of postmenopausal women with HR+ HER2– BC was low. The major reported problems were pain/discomfort and anxiety/depression.

**Keywords:** breast cancer; EQ5D5L; Indonesia; postmenopausal woman; quality of life.

## Introduction

Breast cancer (BC) is the third most common type of cancer with an incidence of 2.1 million cases in 2018 and the most common cause of female death in 154 countries. The incidence increased more than two million with 626.679 deaths. In Southeast Asia, there were 38.1 cases per 100,000 women with 14.1 deaths per 100,000 women, and here Indonesia is one of countries with the biggest incident and death in BC by 52.256 new cases in 2018 [1].

The most common cancer type of BC is hormone responsive (HR+) with 75% prevalence of BC patient population [2] and 80% of them are the postmenopausal women aged 51 years old on average [3]. Since the serum hormone level (estrone, estradiol and progesteron) is different between premenopausal and postmenopausal woman, the therapy will be different. Hence, immunohistochemistry laboratory testing is needed to determine the subtype of BC and the choice of therapy [4].

Health technology can be used in prevention and screening, as well as to cure and prolong survival of BC patient. The use of current technologies such as mammography, surgery, chemotherapy, radiotherapy and hormonal therapy [5], not only has a positive impact on the form of tumor shrinkage, cancer cell death, metastasis prevention or increase survival rate, but also has a negative impact on patients such as neutropenia, leukopenia, anemia, neuropathy, fatigue, and dermatological adverse event like photosensitivity and pigmentary change [6, 7]. For BC patients, it requires a long time therapy to achieve a therapeutic outcome. Therefore, BC and the use of health technology would impact on health related quality of life (HRQoL). HRQoL measurement is needed to assess which technology is more considered capable of achieving the desired outcome with a better life quality [8]. HRQoL assessment can be used for patient-reported outcome comprising physical, social

***Corresponding author: Ria Etikasari**, Doctoral Program, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia; and Faculty of Pharmacy, Universitas Muhammadiyah Kudus, Kudus, Indonesia, E-mail: riaetikasari@gmail.com
**Tri Murti Andayani and Dwi Endarti,** Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia
**Kartika Widayati Taroeno-Hariadi,** Department of Internal Medicine, Division of Hematology and Medical Oncology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

and psychological functioning, symptom burden, satisfaction, and wellbeing [9].

HRQoL measurement to the woman with BC could be performed using various specific and generic instruments. Several specific instruments which have been used in many studies include EORTC QLQ-C30 and EORTC QLQ-BR23, FACT-B, FACT-ES, HFRDIS, SLDS-BC LSQ-32 and QLICP-BR instrument. Meanwhile, the generic instruments include SF-36 and EQ-5D instrument. EQ-5D has been recommended and widely used in many countries. The index score (utility), if combined with the quality adjusted life year (QALY) value, can be used for calculating cost utility analysis (CUA) of a health intervention [10].

Several studies evaluating HRQOL on BC patient have been conducted in Indonesia in various stages, settings and instruments. Meanwhile, no studies are found so far assessing HRQOL towards patients with BC on postmenopausal women in Indonesia. This recent study becomes important to provide the utility score that could be the outcome parameter for HTA study. The objective of this study is to measure HRQoL of postmenopausal women with HR+ HER2– BC using EQ-5D-5L instrument.

## Materials and methods

The cross-sectional study was conducted at Dr.Sardjito Hospital, a referral hospital, in Yogyakarta, Indonesia, in the period of January to August 2019. The patient health state previously had been confirmed through the medical record. The inclusion criteria of patient included a postmenopausal woman, diagnosed with HR+ HER2– BC, and receiving therapy for at least four months. Postmenopausal status was determined by age above 55 years old or below but she has received aromatase inhibitor third generation as a hormonal therapy such as anastrozole, letrozole or exemestane. Hormonal status was determined by the patient immunohistochemistry laboratory testing.

Of the 129 eligible participants confirmed, three patients resigned during the interview or did not complete the answers in questionnaire. A total of 126 patients were interviewed face to face and previously should fill out and sign the informed consent. Information regarding clinical characteristic and socio-demographic was gained from the patient medical records.

HRQoL of patient was measured using EQ-5D instrument, which comprised of the descriptive system questionnaire and visual analog scale (EQ-VAS) [11]. Descriptive system questionnaire measured problems in five dimensions: mobility, self-care, usual activity, pain/discomfort and anxiety/depression, while each dimension had five response levels: no problems (level 1), slight problems (level 2), moderate problems (level 3), severe problems (level 4) and extreme problems (level 5). The EQ-5D descriptive system resulted in a utility score, while EQ-VAS represented the overall health of the patient ranging from 0 (worst) to 100 (the best). This study used EQ-5D-5L questionnaire in official Indonesian language and the Indonesian value set to calculate EQ-5D index score (utility) [12]. Patient health state was adopted from the study of Beauchemin et al. [13].

The one-way ANOVA followed by post-hoc Scheffe's test was applied for the comparison of the utility score between groups of the health state. Here, the dependent variable referred to the utility score and the independent one referred to the health state. Statistical analysis was performed using p-values<0.05 indicating the statistical significance. Ethical clearance was gained from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada with the reference number KE/FK1197/EC/2018.

## Results

The patient had age mean of 59.2 years old (ranging from 47 to 78 years old). More than half of patients were the housewives (53.2%) and had high educational level (61.1%). The duration of illness was in the range of 4 months to 15 years (Table 1).

This study showed that the highest proportion of EQ-5D descriptive system of patients was the health state of 11,121 (17.46%), followed by health state of 11,111 (10.32%), 11,112 (9.52%) and 11,122 (7.94%). Five patients (3.97%) had poor health state: 33,333; 44,552; 54,533; 55,542 and 55,533. The health state of 11,121 showed that the patient had no problem in the dimension of mobility, self-care, usual activity and anxiety/depression but had slight problem in the dimension of pain/discomfort. The health state of 11,111 indicated that

**Table 1:** Patient characteristics.

| Characteristic | | n, % |
|---|---|---|
| Age, year | Mean ± SD | 59.2 ± 6.1 |
| Age distribution (n=126) | <55 | 23 (18.3) |
| | 55–65 | 80 (63.5) |
| | >65 | 23 (18.2) |
| Employment status (n=126) | Private employee | 34 (27.0) |
| | Government employee | 25 (19.8) |
| | Housewife | 67 (53.2) |
| Educational status (n=126) | No schooling | 4 (3.2) |
| | Elementary school | 20 (15.9) |
| | Junior high school | 25 (19.8) |
| | Senior high school | 44 (34.9) |
| | University degree | 33 (26.2) |
| Duration of illness (n=126) | <6 bln | 8 (6.3) |
| | 6 bln–5 th | 98 (77.8) |
| | >5 th | 20 (15.9) |
| Cancer stage (n=126) | I | 7 (5.6) |
| | II | 30 (23.8) |
| | III | 46 (36.5) |
| | IV | 43 (34.1) |
| Health state (n=126) | Free metastasis (FM) | 34 (27.0) |
| | Locoregional metastasis (LM) | 49 (38.9) |
| | Distant metastasis (DM) | 43 (34.1) |

patient had no problem in all dimensions. The poor health state of 33,333; 44,552; 54,533; 55,542 and 55,533 indicated that the patient had a problem in all dimensions in which the level was in the range from slight to extreme problems.

The most frequent problem reported was pain/discomfort (75.4%), followed by anxiety/depression (54.8%), mobility (38.9%), usual activity (34.9%) and self-care (16.7%) (Table 2). The patient had an average utility score (SD) of 0.727 (0.315) and an average VAS score (SD) of 76.64 (14.91). The average of the utility score decreased from FM (0.87) to LM (0.77) and to DM (0.58). ANOVA analysis indicated that the utility score was statistically significant ($p<0.05$). Furthermore, in contrast to FM vs. LM ($p=0.285$), post-hoc Scheffe's test showed the significant difference on FM vs. DM ($p=0.000$) and LM vs. DM ($p=0.011$) (Table 3).

## Discussion

Several studies measured utility index of the general population in Indonesia using the EQ-5D instrument. A study reported that the mean of index score (SD) of general Indonesian women was 0.90 (0.12) and the mean (SD) of EQ VAS was 79.08 (14.52) [14]. Other study reported that the mean of index score of an Indonesian woman was 0.8424 and the mean of EQ VAS was 88.99 [15]. Our study showed a decrease in HRQoL of postmenopausal woman with HR+ HER2– BC (0.727). These findings were similar to the International study reporting the mean (SD) utility scores of 0.7 (0.18) [16] and a study in another region of Indonesia found that EQ VAS score female with BC symptom was lower than general population (69.1 vs. 78.6) [17]. Our study indicated that there were some problems among postmenopausal women with BC hormone responsive.

Our study also found the decrease of utility score from health state FM to LM and to DM. The mean (SD) in the utility scores of health state at FM, LM, and DM were 0.87 (0.10), 0.77 (0.19) and 0.58 (0.44), respectively. Also, the decrease was found in VAS score from FM (87.50) to LM (76.20) and to DM (68.56). Utility between the health states was showed a significant difference, but not for FM vs. LM. It meant that the HRQoL of patients between FM and LM was alike although many patients in LM had a lower score in some problems compared to the one in FM due to the symptoms and the therapy. This study had the same result with the previous study using SF-12 [18], EORTC QLQ-C30 and EORTC QLQ-BR23 questioners. DM health state had the lowest score in view of metastatic site emerging more frequent of pain, limiting the patient activities and the health state tending to worsen [19]. The most common problem that occurred in patients was pain/discomfort and anxiety/depression. These findings are similar to the previous several studies related to the quality of life of BC patient [16, 17, 20] and other cancer types: lung, stomach, colorectal, oesophageal [21], cervical [22], HPV-related cancer [23], and colorectal cancer [24].

A review published in 2017 resumed that the pain in BC patient can be caused by the tumor itself and the cancer related therapy. Pain can be resulted from tumor spread to other tissues to produce second malignance. Pain in patient undergoing therapy may occur as an adverse event of therapy, such as post-mastectomy pain, painful neuropathies induced from chemotherapy, plexopathies after radiotherapy and arthralgia while using aromatase inhibitor [25]. The very frequent type of pain occurred to BC patient was bone pain, as a metastatic site for tumor growth [26]. A study using a specific instrument, EORTC QLQ-C30, revealed that 71.75% of respondents experienced pain contributed by metastasis [27]. The management of pain was required to make the disease not worsen. Pharmacological approaches for pain management can be carried out by prescribing analgesic, antidepressant, anticonvulsant even opioid for severe pain [28].

**Table 2:** Pasien's respone to EQ5D5L descriptive system in each dimension.

| Problems | Mobility | Self-care | Usual activity | Pain/ discomfort | Anxiety/ depression |
|---|---|---|---|---|---|
| No | 77 (61.1) | 105 (83.3) | 82 (65.1) | 31 (24.6) | 57 (45.2) |
| Slight | 34 (27.0) | 15 (11.9) | 29 (23.0) | 65 (51.6) | 52 (41.3) |
| Moderate | 10 (7.9) | 2 (1.6) | 8 (6.3) | 24 (19.0) | 17 (13.5) |
| Severe | 1 (0.8) | 2 (1.6) | 1 (0.8) | 4 (3.2) | 0 (0) |
| Extreme | 4 (3.2) | 2 (1.6) | 6 (4.8) | 2 (1.6) | 0 (0) |

**Table 3:** Descriptive of EQ5D5L index score classified by health state.

| Health state | EQ5D index score | | | | | |
|---|---|---|---|---|---|---|
| | Mean | SD | 95% CI of mean | | SE | p-Value |
| | | | Lower | Upper | | |
| Free metastasis | 0.871 | 0.103 | 0.837 | 0.906 | 0.018 | 0.000 |
| Locoregional metastasis | 0.768 | 0.188 | 0.716 | 0.821 | 0.027 | |
| Distant metastasis | 0.584 | 0.444 | 0.451 | 0.717 | 0.068 | |
| All states | 0.727 | 0.315 | 0.672 | 0.782 | 0.028 | |

Our findings were found different from the previous studies stating that the most commonly identified problem of BC woman was anxiety [29, 30]. Anxiety can occur to BC patient because of the diagnosis and the treatment impact on the psychological well-being of the patients. Furthermore, it is the most common psychological states in terms of distress in BC patient [31]. A review published in 2008 concluded that depression can influence human relationship, occupational performance, stress and health perception, and physical symptoms. Thus, depression is associated with the poor life quality of patient [32]. Antidepressants, as a clinical treatment, may have a choice for the depressive symptom while the level anxiety is high [33].

Research that measured HRQoL using EQ-5D instrument have some advantages. Since it only has five questions, it becomes simple to answer and complete the questions. It could assess the utility for many diseases and provides single utility index in which the comparison is simple on the intervention for different health problems. However, it is less sensitive to respond the change of patient health state [23]. As a comparison, a study used QWB-SA, a generic instrument, recognized that respondents found it difficult to understand the content of questions so that they needed more time to complete the questionnaire [34].

Our study has several limitations. First, for using cross-sectional survey, this study could not explain a correlation between variables under study. Second, data were collected from a single public hospital in Yogyakarta, so it has not yet represented the Indonesian population.

## Conclusions

The most frequently reported problem was pain/discomfort and anxiety/depression. The average utility score of patient was 0.727 (95% CI 0.672–0.782). Poor QoL of patients were observed at distant metastasis health state ($p<0.05$).

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Dinda M. N. Ratri*, Arina D. Puspitasari, Cahyo W. Nugroho, Budi Suprapti, Suharjono and Christoper P. Alderman

# Gender differences in the blood glucose type 2 diabetes patients with combination rapid and long acting insulin therapy

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**Abstract**

**Objectives:** Previous research suggests that there may be intergender differences in the profile of glycemic control achievable during the treatment of type 2 diabetes mellitus. This preliminary study was conducted to determine differences in glycemic outcomes in type 2 diabetes mellitus patients amongst men and women in an Indonesian hospital.

**Methods:** The study was conducted at the outpatient internal medicine polyclinic of Universitas Airlangga Teaching Hospital Surabaya. This observational prospective cohort study examining outcomes for 64 patients (32 men and 32 women) treated with insulin therapy. The primary outcome measure was the extent to which subjects achieved concordance with the target blood glucose parameters based on the American Diabetes Association (ADA) guidance.

**Results:** After 3 months of combination basal-bolus insulin treatment, the proportion of subjects who had fasting blood glucose values in the target range did not increase for either gender. For women, there was a significantly higher proportion of subjects who achieved a postprandial glucose value within the target range (p=0.04)

**Conclusions:** In this study, patients achieved postprandial glycemic outcomes for women but not men. More research is required to elucidate the possible intergender difference in results for subjects treated with basal-bolus insulin for type 2 diabetes mellitus.

**Keywords:** blood glucose level; diabet; diabetes; gender; insulin; rapid and long-acting insulin; sex; type 2 diabetes mellitus.

## Introduction

The prevalence of diabetes mellitus (DM) has increased worldwide in recent decades. In 2016, the Ministry of Health of the Republic of Indonesia released data on the costs associated with DM treatment and its complications. It accounted for approximately 33% of the total claims' national coverage expenditure [1]. One significant component of these expenses is attributable to providing insulin treatment, which is relatively expensive, especially in a developing country. For example, standard therapy with a modest insulin dose in Indonesia will cost approximately 450,000 Indonesian Rupiah each month, meaning the price of treatment with insulin (excluding the costs of consumables such as syringes and needles) will amount to at least 10% of the average monthly earnings in this nation [2]. A previous study conducted at Universitas Airlangga Teaching Hospital in Surabaya found that of 240 patients who were treated with insulin, 28% of them combined basal-bolus insulin, which incorporates fast or short-acting insulin with long-acting insulin [3]. Research suggests that targeted blood glucose outcomes for patients older than 60 years are obtained for only 53% of those treated [4]. In another study, targeted blood glucose concentrations were found to be obtained for those treated with insulin therapy in only 20.8–24.4% of cases [3, 5]

***Corresponding author: Dinda M. N. Ratri**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia; and Department of Pharmacy, Universitas Airlangga Hospital, Surabaya, East Java, Indonesia, Phone: +62315933150, E-mail: dindamonika@ff.unair.ac.id. https://orcid.org/0000-0001-6493-3561

**Arina D. Puspitasari and Budi Suprapti,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia; and Department of Pharmacy, Universitas Airlangga Hospital, Surabaya, East Java, Indonesia

**Cahyo W. Nugroho,** Internal Medicine Department, Faculty of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia; and Internal Medicine Department, Universitas Airlangga Hospital, Surabaya, East Java, Indonesia

**Suharjono,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia

**Christoper P. Alderman,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia; and School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia

Differences between genders have been observed in a range of areas relevant to the treatment of diabetes, including insulin sensitivity. Moreover, biological factors such as the differential effects of estrogen secretion's profile upon fatty acid metabolic disposition and the impact of psychosocial factors such as a difference in treatment adherence [6–8]. These gender differences may influence the prevalence of cardiovascular risk factors and mortality rates associated with metabolic syndrome [7–9]. Previous researches have explored the influence of gender upon psychological factors and outcomes of pharmacological therapy of patients with type 2 diabetes mellitus (T2DM) [6, 10, 11]. Some researchers concluded they should consider gender factors in the treatment of T2DM [6, 7, 12]. This study was conducted to determine differences in blood glucose outcomes of type 2 diabetes between male and female patients.

## Materials and methods

This study was a hospital-based, prospective, cohort, observational study conducted at the outpatient internal medicine clinic in the Universitas Airlangga teaching hospital, Surabaya, Indonesia. Subjects were eligible in inclusion criteria if they had a diagnosis of T2DM (with or without complications). Patients were treated with a combination of rapid-acting and long-acting insulin, with or without oral hypoglycemic drugs. Subjects were enrolled in the study using a purposive sampling method during August – September 2017 and completed three visits (at monthly intervals). They had blood glucose data obtained at the first and third visits. Data variables collected included age, the nature of combination oral hypoglycemic drug therapy, and details of comorbid diagnoses obtained from medical record data. Data characteristics such as microvascular complications that were collected are ophthalmic issues, neuropathy and nephropathy. Also, macrovascular complications are coronary heart disease, peripheral arterial disease and cerebrovascular disease. The study examined differences in blood glucose outcomes using post prandial glucose (PPG) and fasting plasma glucose (FPG), measured at the first and third visit. Statistical analysis employed paired t-tests for continuous variables. In contrast, categorical variables, including the use of oral hypoglycemic drugs, comorbid hypertension, and the presence of various complications, were analyzed using the Wilcoxon matched-pairs signed-rank test.

Outcomes of treatment were assigned using FPG and PPG at the first and third visits. Subsequently, it categorized concerning treatment targets according to the parameters of the American Diabetes Association (ADA): FPG concentrations were designated as within the target range if these were within the range of 80–130 mg/dL, and PPG concentrations were designated as within the target range if these were below 180 mg/dL [13]. Next, the blood glucose target compared male patients' outcomes with female patients' outcomes using the Wilcoxon matched-pairs signed-rank test.

## Results

A total of 64 eligible patients were included in the analysis (32 male and 32 female). All patients were treated with rapid-acting insulin (aspart/glulisine) and long-acting insulin (glargine/detemir). Demographic data for the subjects were provided in Table 1. There was no significant difference in the mean age between the male and female groups. The proportions of each gender undergoing concurrent treatment with oral hypoglycemic agents, with comorbid hypertension and the presence of complications of diabetes were not significantly different between men and women ($p>0.05$).

Regarding the targets for treatment outcomes, all subjects (both genders) attaining target values for FPG at baseline was 21.9% at the month first visit and 31.2% at the third-month visit. There is no significant difference between genders in proportions attaining values at each visit. For PPG, in the total population (men and women), all patients achieved target values for 23.4% at the month's first visit and 39% at third visits. The proportion of female subjects attaining the PPG goal was more significant than the male group (25 vs. 50%, $p=0.04$). In contrast, the proportions amongst men for the same parameter were not significantly different (21.9 vs. 28.1%, $p>0.05$) (Table 2).

## Discussion

In this study, after 3 months of basal-bolus insulin treatment, a more significant proportion of women attained

**Table 1:** Patient characteristics.

| Characteristics | Male (n=32) | Female (n=32) | p-Value |
|---|---|---|---|
| **Age, years** | | | |
| Mean ± SD | 57.34 ± 12.33 | 56.72 ± 8.40 | p>0.05 |
| 95% CI of mean | 52.90–61.79 | 53.69–59.75 | |
| **Combination with OAD** | | | |
| No combination | n=19 | n=20 | p>0.05 |
| Combination with 1 OAD | n=10 | n=9 | |
| Combination with 2 OAD | n=3 | n=1 | |
| Combination with 3 OAD | n=0 | n=2 | |
| **Comorbid hypertension** | n=20 | n=23 | p>0.05 |
| **Complication** | | | |
| No complication | n=10 | n=11 | p>0.05 |
| Microvascular complication | n=14 | n=12 | |
| Macrovascular complication | n=5 | n=9 | |
| Microvascular and macrovascular complication | n=3 | n=0 | |

OAD, Oral antihyperglicemic drugs.

**Table 2:** Patients' blood glucose outcome on pre and post therapy.

| Parameter | Patients reaching ADA targets | |
|---|---|---|
| | Male (n=32) | Female (n=32) |
| **FPG, %** | | |
| FPG pre | 25.0% | 18.8% |
| FPG post | 28.1% | 34.3% |
| p value pre-post | >0.999 | 0.23 |
| **PPG, %** | | |
| PPG pre | 21.9% | 25.0% |
| PPG post | 28.1% | 50.0% |
| p value | 0.75 | 0.04 |

FPG, fasting plasma glucose; PPG, post prandial glucose.

target values for post-prandial blood glucose concentrations than men. While, this study did not address body morphology characteristics (e.g., BMI, waist circumference). It is known that the amount of fat tissue in visceral tissue contributes to the incidence of insulin resistance. This can cause gluconeogenesis and dyslipidemia. Furthermore, it may impact gender-based differences in fat seen amongst women and may account for potentially greater sensitivity to insulin [14]. A large proportion of visceral adipose tissue (such as is observed in men) is associated with diminished insulin sensitivity. It can release catecholamine, which induces lipolysis, then producing more angiotensinogen than subcutaneous adipose tissue. For these reasons, it has been postulated that excessive visceral adipose tissue causes a greater predisposition to metabolic disorders than that associated with subcutaneous adipose tissue [15]. The presence of adiponectin hormone, known as hormone-sensitive to insulin in women, is more extensive than in men, and the more significant estrogen presence can influence insulin and the distribution of fat tissue [14]. Adinopectin concentrations tend to be greater amongst women than in men. High adinopectin concentrations are associated with high sex hormone-binding globulin and high concentration of high-density lipoprotein, low glycated hemoglobin value, lower insulin resistance, and lower c-peptide and triglycerides concentrations [16]. It is also possible that psychosocial factors may also contribute to this phenomenology: one study found that women were more convinced and complied with insulin therapy than men [17]. Although not explicitly explored in the design of this small preliminary study. These factors described before might affect women attaining a more excellent proportionate goal amongst the subjects to achieve target post-prandial glucose concentrations after 3 months of basal-bolus insulin treatment.

This study has a range of limitations. The research did not address metabolic variables such as body weight, BMI, and waist circumference, which may have provided additional insights. Different hypoglycemic regimens were used, and a lack of standardization may have contributed to glycemic control variability. As the study involved outpatient treatment, researchers did not assess treatment adherence in detail. Most importantly, the total number of subjects in the study was modest, meaning that if a larger cohort were to have been recruited, this might have enabled greater statistical power and less likelihood of type II error.

The present study is preliminary, but the study appears to be the first research that addresses intergender differences in insulin treatment effects in a Southeast Asian setting. This region accounts for a significant proportion of the world population and has a growing prevalence of diabetes. Besides, body morphology and cultural characteristics in these regions are different from those found in developed nations. This study can provide direction for focusing more detailed research on this topic in this part of the world.

## Conclusions

In this study, women with T2DM treated with basal-bolus insulin for 3 months were more likely to attain PPG targets than men, suggesting that the female gender may be a factor that is predictive for better glycemic control under these clinical circumstances. Further research involving a larger cohort is needed to clarify these results' significance and build an understanding of intergender differences in glycemic control attained with basal-bolus insulin treatment.

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**Ethical approval:** This research was approved by Ethics Committee of the Universitas Airlangga Hospital with serial number 128/KEH/2017 in 29 August 2017.

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Utami Harjantini*, Yulia Lanti Retno Dewi, Diffah Hanim and Ida Nurwati

# Correlation of dietary iron intake and serum iron with thyroid stimulating hormone (TSH) and free thyroxine (FT4) levels in adult hyperthyroid patients

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**Abstract**

**Objectives:** National Baseline Health Research 2013 showed that there were 706,757 (0.4%) hyperthyroid patients in Indonesia. Hyperthyroidism is characterized by abnormal thyroid stimulating immunoglobulin (TSI) which causes low TSH and high FT4 levels. Hyperthyroid patients have a decrease of serum iron levels due to acute phase reactions of hyperthyroidism. This study aimed to analyze the correlation between dietary iron intake and serum iron with TSH and FT4 levels in adult hyperthyroid patients.
**Methods:** This study was conducted in February–July 2020 at the Clinic of Magelang Health Research and Development Center. Sampling of this cross sectional study was based on inclusion criteria in order to obtain 50 adult hyperthyroid patients. Dietary iron intake was collected with $2 \times 24$ h dietary recall, serum iron was measured with colorimetric analysis, the levels of TSH and FT4 were measured by ELISA. The collected data were analyzed using Spearman correlation and multivariate linear regression with 95% confidence level.
**Results:** Deficiencies of dietary iron intake was found in 20 hyperthyroid patients (40%). Low serum iron levels were found in 10 hyperthyroid patients (20%). Spearman correlation analysis showed that dietary iron intake had a negative correlation with TSH ($r=-0.294$; $p<0.05$) but did not correlate with FT4 ($r=-0.142$; $p>0.05$), while serum iron didn't associated with both TSH ($r=0.110$; $p>0.05$) and FT4 ($r=0.142$; $p>0.05$). Furthermore, regression analysis showed that dietary iron intake, serum iron, phytate, and thyrozol intake correlate with TSH levels (R square=0.193; $p<0.05$) and FT4 levels (R square=0.341; $p<0.05$), but there were no independent association between dietary iron intake and serum iron with TSH and FT4 levels ($p>0.05$).
**Conclusions:** Intake and serum of iron didn't correlate with TSH and FT4 levels in adult hyperthyroid patients.

**Keywords:** dietary iron intake; FT4; hyperthyroid; serum iron; TSH.

## Introduction

Hyperthyroidism is an endocrine disorder in which the thyroid gland over synthesizes and secretes hormones, resulting in increased thyroid hormone levels [1]. Hyperthyroidism is characterized by low serum thyroid stimulating hormone (TSH) and high free thyroxine (FT4) level [2]. The hyperthyroidism prevalence of the United States population was 1.2% [3]. Based on National Baseline Health Research 2013 in Indonesia, the prevalence of hyperthyroidism was 0.4% (706,757 hyperthyroid patients) [4]. The prevalence of hyperthyroidism in Central Java Province was higher than the national prevalence, there was 0.5% hyperthyroid population (120.447 hyperthyroid patients) [5].

Thyroid Stimulating Hormone (TSH) is the main thyroid hormone homeostasis regulator produced by the pituitary gland. TSH stimulates thyroid gland to secrete thyroid hormone by feedback mechanism [6]. Increase production of thyroid hormone resulting in TSH production decreases and vice versa [2, 7]. Thyroxine (T4) is the largest hormone produced by the thyroid gland (90%), but when it's released, most of the thyroxine is bound to thyroxine-binding globulin (TBG). Free Thyroxine (FT4) is a free form of thyroxine in the circulatory system. FT4 is valid to diagnose hyperthyroidism because it does not bind to protein [6].

Iron acts as an important cofactor in the body's metabolism [8]. Approximately 10% iron is found in enzymes

***Corresponding author: Utami Harjantini**, Postgraduate Program of Nutrition Science, Sebelas Maret University, Surakarta, Indonesia, Phone: +6285652233866, E-mail: utamihgunawan@gmail.com. https://orcid.org/0000-0002-7844-2090
**Yulia Lanti Retno Dewi and Diffah Hanim,** Department of Nutrition Science, Faculty of Medicine, Sebelas Maret University, Surakarta, Indonesia
**Ida Nurwati,** Medical Faculty, Sebelas Maret University, Surakarta, Indonesia

and cytochromes (350 mg) [9]. The prevalence of iron deficiencies in Indonesian adult population was 36.4% [10]. Deficiency iron intake can cause decreased serum iron and almost all of the iron deficiency was characterized by low serum concentration [9, 11]. Iron deficiency anemia can interfere thyroid hormone metabolism through reduced oxygen transportation [12]. Previous animal studies showed iron deficiency could decrease oxygen transport, impaired the central nervous system of thyroid hormone metabolism, and lowered activity of thyroid peroxidase (TPO) [8, 13]. TSH and FT4 levels will be disrupted if there is an impairment in thyroid hormone metabolism [14]. TPO is an iron-dependent enzyme that catalyzes two opening steps in thyroid hormone synthesis, including thyroglobulin iodination and molecular coupling reaction of iodotyrosine [12, 13].

Hyperthyroid patients have a decrease of serum iron levels due to acute phase reactions of hyperthyroidism [9]. Study on 31 Grave's hyperthyroid patients showed that hyperthyroid phase has lower plasma iron than euthyroid phase [11]. Another study in Pakistan found that hyperthyroid patients have lower serum iron levels compared to healthy subjects [15]. This study aimed to analyze the correlation between dietary iron intake and serum iron with TSH and FT4 levels in adult hyperthyroid patients.

## Materials and methods

This research was conducted at the Clinic of Magelang Health Research and Development Center in February-July 2020. Selection of research sites based on the prevalence of hyperthyroidism and this clinic is specifically for management and treatment of thyroid disorders. This research protocol was approved by the Ethics Committee of the Medical Faculty, Sebelas Maret University, Surakarta, Indonesia (No. 014/UN27.06/KEPK/EC/2020).

This is a cross-sectional observational study, which collected data from hyperthyroid population at one specific point in time. The sample size was calculated using this following formula for finite population [16].

$$n = \frac{t_\alpha^2 * p * q * N}{(N-1) * e^2 + t_\alpha^2 * p * q}$$

Annotation: n=calculated sample size; N= population of hyperthyroid patients in the Clinic of Magelang Health Research and Development Center (137 hyperthyroid patients based on data in 2019); $p$=proportion of hyperthyroid population (5%); $q$=1–$p$ (opposite of proportion); $e$=margin of error (5%); $t_\alpha$=the value of confidence level (1.96 for 95% confidence level).

$$\begin{aligned} \mathrm{n} &= \frac{1.96^2 \times 0.05 \times (1 - 0,05) \times 137}{(137-1) \times 0.05^2 + 1.96^2 \times 0.05 \times (1 - 0.05)} \\ &= 47,8 \\ &= 48 \end{aligned}$$

The minimum sample size was 48 subjects and added by 10% to anticipate subjects who were lost to follow-up or drop out, so that 53 subjects were obtained. Three subjects were dropped out at the time of the research because one subject had lysed blood samples, one subject had too high serum iron levels, and one subject had too low serum iron levels. Therefore, the total sample in this study becomes 50 subjects. Inclusion criteria of this study are hyperthyroid patients who are 19–59 years old, TSH levels <0.3 mIU/mL, and patients with or without stroma. Exclusion criteria included subjects who were taking iron supplementation, pregnant, menstruation, and patients with chronic disease or malignancy.

The independent variables were dietary iron intake and serum iron concentrations. The dependent variables were TSH and FT4 levels. Phytate and thyrozol intake as confounding variables. Dietary iron and phytate intake were measured by the 24 h dietary recall which interviewed two times on different days. Food or dietary intake analysis used Indonesian Nutrisurvey application and 2019 Indonesian Food Composition Table. Serum iron were obtained using colorimetric analysis, while TSH and FT4 levels were measured by the ELISA method. Data of thyrozol intake were obtained from medical record patients and matched with pharmacist data.

Subjects were collected in stages based on hyperthyroid patients who underwent laboratory tests of TSH ±2 weeks before the study day. Subjects who had TSH levels <0.3 mIU/mL and agreed to participate in this study, filled out the informed consent, then took a blood sample of $5 \times 10^{-6}$ $m^3$ from a vein in the subject's arm for analysis of serum iron TSH and FT4 levels. After that, researchers interviewed food intake using 24-h dietary recall and performed anthropometric measurements on the subjects.

Collected data was analyzed by SPSS version 21 program. Normality of data was verified by Kolmogorov-Smirnov test. Independent t-test was used to compare the means of male and female subject groups. The correlation of dietary iron intake and serum iron with TSH and FT4 levels was analyzed using Rank Spearman's correlation test and multivariate linear regression with 95% confidence level.

## Results

A total of 50 subjects consist of nine males and 41 females who have characteristics in age, nutritional status, dietary iron and phytate intake, serum iron, TSH and FT4 levels according to gender (Table 1). The mean age of hyperthyroid subjects was 40.4 years old. Body mass index's subjects in this study had an average about 22.97 kg/$m^2$. The mean intake of dietary iron, dietary phytate, and thyrozol were 15.08, 808.9, and 7.1 mg/day, respectively. The average of serum iron, TSH, and FT4 levels of hyperthyroid patients in this study were 83.74 µg/dL, 0.14 mIU/mL and 2.49 ng/dL, respectively. The average molar ratio of phytate:iron was 4.67 mmol/day. Differences were found in height between male and female subject groups ($p<0.05$). In other characteristics, there were no significant differences between male and female subject groups ($p>0.05$).

Table 2 shows the distribution of subjects characteristic. Most of the subjects in this study had age range 30–49

**Table 1:** Subjects characteristic according to gender (mean ± SD).

| Characteristic of subject | Total (n=50) | Males (n=9) | Females (n=41) | p-Value |
|---|---|---|---|---|
| 1. Age, years | 40.4 ± 10.07 | 45.11 ± 6.75 | 39.37 ± 10.43 | 0.12[a] |
| 2. Weight, kg | 55.77 ± 9.85 | 57.36 ± 7.52 | 55.42 ± 10.34 | 0.60[a] |
| 3. Height, cm | 155.63 ± 7.76 | 165.30 ± 5.84 | 153.5 ± 6.43 | 0.00[b] |
| 4. Body Mass index, kg/m$^2$ | 22.97 ± 3.57 | 20.99 ± 2.51 | 23.4 ± 3.64 | 0.06[a] |
| 5. Dietary iron intake, mg/day | 15.08 ± 6.32 | 16.27 ± 4.46 | 14.82 ± 6.68 | 0.54[a] |
| 6. Phytate intake, mg/day | 808.09 ± 500.6 | 976.16 ± 328.4 | 771.19 ± 527.0 | 0.27[a] |
| 7. Phytate:iron molar ratio, mmol/day | 4.67 ± 2.13 | 5.63 ± 3.0 | 4.45 ± 1.87 | 0.14[a] |
| 8. Serum iron, µg/dL | 83.74 ± 24.93 | 95.00 ± 24.24 | 81.27 ± 24.68 | 0.14[a] |
| 9. TSH level, mIU/mL | 0.14 ± 0.24 | 0.16 ± 0.34 | 0.14 ± 0.22 | 0.78[a] |
| 10. FT4 level, ng/dL | 2.49 ± 2.05 | 2.29 ± 1.90 | 2.54 ± 2.11 | 0.75[a] |
| 11. Thyrozol intake, mg/day | 7.10 ± 3.74 | 7.22 ± 3.41 | 7.08 ± 3.85 | 0.92[a] |

[a]Mean difference is significant. [b]Mean difference is not significant; Statistic analysis by independent t test.

years old (64%). Half of the subjects had normal nutritional status (52%) and all obese subjects were female hyperthyroid patients. Deficient dietary iron intake was found in 20 hyperthyroid patients (40%), while low serum intake was found in 10 subjects (20%) in this study.

The correlation of dietary iron intake and serum iron with TSH and FT4 levels showed in Table 3. Dietary iron intake had negative correlation with TSH (r=−0.294; p=0.038) but did not correlate with FT4 (r=−0.142; p=0.326), while serum iron did not correlate with both TSH (r=0.101; p=0.485) and FT4 (r=0.142; p=0.327). Confounding variable, including phytate intake did not correlate with both TSH (r=−0.261; p=0.067) and FT4 (r=−0.137; p=0.341) while thyrozol intake had negative association with TSH (r=−0.431; p=0.002) and positive association with FT4 (r=−0.470; p=0.000).

Correlation between iron and thyroid hormone has been still controversial, so that further analysis was carried out in this study. Result of multivariate linear regression can be seen in Table 4 and Table 5. Dietary iron intake, serum iron, phytate, and thyrozol intake were associated with TSH levels (R square=0.193; p=0.042) and FT4 levels (R square=0.341; p=0.001).

Thyrozol as a medicine of hyperthyroidism had correlation with TSH (p=0.007) and FT4 (p=0.000). There was no independent association between dietary iron intake with both TSH (p=0.311) and FT4 (p=0.447) as well as serum iron didn't have correlation with TSH (p=0.502) and FT4 levels (p=0.395), independently.

**Table 2:** Distribution of subjects characteristic according to gender.

| Characteristic of subject | Total (n=50) | Males (n=9) | Females (n=41) |
|---|---|---|---|
| 1. Age, years | | | |
| 18–29 | 7 (14%) | 0 | 7 (17%) |
| 30–49 | 32 (64%) | 7 (77.8%) | 25 (61%) |
| 50–59 | 11 (22%) | 2 (22.2%) | 9 (22%) |
| 2. Nutritional status[a] | | | |
| Underweight (<18.5 kg/m$^2$) | 3 (6%) | 1 (11.1%) | 2 (4.9%) |
| Normal (18.5–22.9 kg/m$^2$ | 26 (52%) | 6 (66.7%) | 20 (48.8%) |
| Overweight (23–24.9 kg/m$^2$) | 6 (12%) | 2 (22.2%) | 4 (9.8%) |
| Obese (>25 kg/m$^2$) | 15 (30%) | 0 | 15 (36.5%) |
| 3. Dietary iron intake | | | |
| Adequate (≥80% RDA)[b] | 30 (60%) | 9 (100%) | 21 (51.2%) |
| Deficient (<80% RDA)[b] | 20 (40%) | 0 | 20 (48.8%) |
| 4. Serum iron | | | |
| Normal[c] | 40 (80%) | 8 (88.9%) | 32 (78%) |
| Low[d] | 10 (20%) | 1 (11.1%) | 9 (22%) |

[a]Nutritional status category based on body mass index of individual. [b]RDA (Recommended Dietary Allowances) of dietary iron: male 9 mg/day, female 18 mg/day. [c]Normal serum iron: male 70–200 µg/dL, female 62–173 µg/dL. [d]Low serum iron: male <70 µg/dL, female <62 µg/dL.

**Table 3:** Spearman's correlation test results.

| Variable | TSH, mIU/mL | | FT4, ng/dL | |
|---|---|---|---|---|
| | r | p-Value | r | p-Value |
| Dietary iron intake, mg/day | −0.294* | 0.038 | −0.142 | 0.326 |
| Serum iron, µg/dL | 0.101 | 0.485 | 0.142 | 0.327 |
| Phytate intake, mg/day | −0.261 | 0.067 | −0.137 | 0.341 |
| Thyrozol intake, mg/day | −0.431* | 0.002 | 0.470[a] | 0.000 |

*Correlation is significant.

## Discussion

Subjects in this study were hyperthyroid patients which had 0.14 mIU/mL of TSH concentration and 2.49 ng/dL of FT4 level. Hyperthyroidism found in individuals with TSH concentration less than 0.3 mIU/mL and FT4 level more than 2.0 ng/dL [17]. Overt hyperthyroidism is defined as a low serum TSH with elevated serum levels of FT4.

**Table 4:** Multivariate analysis between serum iron, intake of iron, phytate, and thyrozol with TSH.

| Variable | B | Std. Error | β standardized | t | p-Value |
|---|---|---|---|---|---|
| Constant | 0.348 | 0.147 | | 2.360 | 0.023 |
| Dietary iron intake | −0.007 | 0.007 | −0.196 | −1.025 | 0.311 |
| Serum iron | 0.001 | 0.001 | 0.091 | 0.067 | 0.502 |
| Phytate intake | $4.19 \times 10^{-6}$ | 0.000 | 0.009 | 0.046 | 0.964 |
| Thyrozol intake | −0.024 | 0.009 | −0.379 | −2.821 | 0.007 |
| **R Square** | 0.193 | | | | |
| **F** | 2.697 | | | | |
| **Sig, F** | 0.042* | | | | |

*Correlation is significant.

**Table 5:** Multivariate analysis between serum iron, intake of iron, phytate, and thyrozol with FT4.

| Variable | B | Std. Error | β standardized | t | p-Value |
|---|---|---|---|---|---|
| Constant | −0.194 | 1.142 | | −0.170 | 0.866 |
| Dietary iron intake | −0.043 | 0.056 | −0.132 | −0.767 | 0.447 |
| Serum iron | 0.009 | 0.010 | 0.105 | 0.859 | 0.395 |
| Phytate intake | 0.000 | 0.001 | 0.120 | 0.695 | 0.491 |
| Thyrozol intake | 0.312 | 0.067 | 0.569 | 4.684 | 0.000 |
| **R Square** | 0.341 | | | | |
| **F** | 5.811 | | | | |
| **Sig, F** | 0.001* | | | | |

Subclinical hyperthyroidism is defined as a low serum concentration of TSH and normal reference level for FT4 [3, 17]. In terms of gender, the amount of female subjects was more than male subjects. Gender proportion of this study in accordance with the proportion of hyperthyroidism in Indonesia. The prevalence of hyperthyroidism in Indonesia according to gender was 0.6% in women and 0.2% in men [4]. The average age of subjects was more than 40 years old and 42% subjects had overweight and obese. Study on subjects with autoimmune thyroid disease showed associations among age at diagnosis and disease severity. Autoimmune thyroid disease patients had elevated thyroid hormone and low serum of TSH [18]. Study in Chinese population showed substantial influence of gender and age on thyroid function [19]. Hyperthyroidism increases with age and is more frequent in women [1, 4].

Approximately 40% of subjects had deficiencies of dietary iron intake and all of them were women. Female subjects had lower dietary iron intake than male subjects (14.82 vs. 16.27 mg/day). Almost half of female subjects had iron intake deficiencies. Previous study showed that the adult population in Indonesia have 14.5 mg/day of dietary iron intake. The prevalence of iron deficiencies on hyperthyroid patients was higher than the adult population in Indonesia (40 vs. 36.4%) [10]. Low serum iron levels were found in 10 hyperthyroid patients (20%). The average of serum iron in male and female subjects were 95 μg/dL and 81.27 μg/dL, respectively. Study on 49 hyperthyroid patients found that the average of serum iron was 13.32 μg/g and serum iron in females was lower than males, it is the same with present study [15].

The average serum iron on hyperthyroid subjects in this study was 83.74 μg/dL, not much different from previous study. Previous study in Turkey showed hyperthyroid subjects have higher serum iron than hypothyroid subjects ($p<0.05$) i.e. 89.03 and 49.22 μg/dL, respectively. High serum iron of hyperthyroid patients is due to an elevation of ferritin levels. Ferritin plays a dual role in the storage and transportation of iron, one ferritin has a transport capacity of 4,500 iron atoms. Increases in serum iron and ferritin during hyperthyroidism have been associated with the stimulatory effect of thyroid gland. Disorders in the synthesis and secretion of thyroid hormones alter iron metabolism [14]. Study on Grave's Hyperthyroidism had results that thyroid hormones have lineal impact on mRNA expression of hepcidin in the cell culture model, which is consistent with the clinical findings of increased hepcidin and ferritin levels in their hyperthyroid subjects [11]. Furthermore, iron deficiency causes a decrease in the activity of heme-dependent thyroid peroxidase enzyme, which results in the impairment of thyroid hormone synthesis [20].

The average intake of phytate in this study was 808.09 mg/day. Study in Chinese population showed phytate had an counteractive effect on the uptake of iron from diets and plays an important role in the deficiencies of iron. Intake of phytate on Chinese people was between 648 and 1,433 mg/day [21]. There are two forms of dietary iron, i.e. heme and non-heme. Hemoglobin and myoglobin are proteins containing heme iron, which are available in animal foods, whereas non-heme iron is found in both plant foods and animal flesh. Digestion of heme and non-heme iron have separate pathways [22]. Heme iron digestion is hardly affected by the food compositions, and it depends particularly on the individual's body iron status. Non-heme iron gets into a general pool of non-heme iron on the gastric juices. The quantity of non-heme iron absorption depends largely on the presence of enhancers and inhibitors substance in the diet and the body iron status of each individual [21].

In this study, the average molar ratio of phytate: iron was 4.67 mmol/day. The phytate: iron molar ratio has been used to predict the inhibitory effect of phytate on iron availability in the diet and foodstuffs. The molar ratio of phytate: iron more than 1 mmol/day is an indication of poor iron bioavailability [21, 23], therefore hyperthyroid subjects in this study had poor absorption of iron intake and it affected serum iron levels and their relationship with TSH and FT4 levels. Phytate provides an inhibitory effect on iron by forming an insoluble and indigestible iron-phytate complex [24]. Phytic acid from beans, seeds, legumes, and cereal grains such as tempeh, tofu, and peanut are the main inhibitory substances of iron absorption [23].

This study showed that intake and serum of iron didn't significantly correlate with TSH and FT4 levels ($p>0.05$). Subjects in this study have higher intake of plant foods compared to animal foods which causes the intake of non-heme iron higher than heme iron. During digestion in the intestinal tract, most of the non-heme iron is bound to phytate, which can sequester some forms of non-heme iron and greatly decrease iron absorption [20]. Case control study was found an association of body iron status with hypothyroidism ($p<0.05$) not hyperthyroidism [25]. Another study in Nepal showed that transferrin saturation and hemoglobin had negative correlation with TSH ($p<0.05$), while serum iron did not correlate with TSH [26].

Previous study by El Masry et al. (2018) in children with iron deficiency anemia showed no significant relationship between serum iron levels and FT4 ($r=0.06$; $p=0.647$) [27]. The thyroxine 5-deiodinase enzyme is disrupted during iron deficiency conditions, this enzyme is responsible for the peripheral conversion of the hormone T4 to T3. Enzymatic conversion from T4 to T3 by the thyroxine 5-deiodinase enzyme depends on the body's iron status so that iron deficiency causes levels of thyroid hormone T3 or FT3 to decrease [28]. Previous studies showed a decrease in FT3 levels was in line with a decrease in serum iron levels and an increase in the severity of iron deficiency anemia, but there was no significant decrease in FT4 or TSH levels [27].

Thyrozol as a confounding variable affects TSH and FT4 levels but it doesn't interfere with the body's iron status. Thyrozol is a drug to prevent excessive hormone production by the thyroid gland. Thyrozol contains thiamazole is an antithyroid drug that's used to reduce thyroid hormone production in people with hyperthyroidism or excess thyroid hormone in the blood [29]. There were several limitations of this study, such as the sample size was small and high differences of the sample size in male and female subjects.

## Conclusions

Intake and serum of iron did not correlate with TSH and FT4 levels in adult hyperthyroid patients. Hyperthyroid patients with deficient iron intake and low serum iron levels must improve their iron status in order to not add new health problems. Iron supplementation should be recommended for hyperthyroid patients with inadequate iron intake or low serum iron levels. Hyperthyroid patients should consume animal protein sources according to the guidelines for balanced nutrition, i.e. 2–3 servings/day so that iron absorption increases and inhibition of iron absorption by phytate can be reduced.

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Mahacita Andanalusia, Yunita Nita* and Umi Athiyah

# The effect of pillbox use and education by pharmacist toward medication adherence in diabetes mellitus patients in a Primary Health Care Center in Mataram

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**Abstract**

**Objectives:** Nonadherence to a long-term therapy, including diabetes mellitus, is one of the global problems that need to be overcome. This study aims to determine the effect of pillbox use and education by pharmacists toward medication adherence in patients with diabetes mellitus in a Primary Health Care Center in Mataram.
**Methods:** This research was an experimental research design with pretest-posttest with control group design. The study was conducted from October to December 2019 at Tanjung Karang Primary Health Care Center, Mataram. Measurement of adherence was done using the Adherence to Refill and Medication Scale questionnaire. The higher the score, the more nonadherence the patients. Patients were divided into three groups, which were the control group, educational intervention group, and pillbox and educational intervention group. Each group consisted of 11 patients.
**Results:** Patients' medication adherence increased from 19.54 (SD 4.37) to 15.18 (SD 2.64) in the education and pillbox intervention group (p=0.004). Whereas, in the education and control group, the adherence did not provide a significant change (p>0.05). Based on the difference in adherence scores, it was known that what contributed to changes in compliance was refilling medicine and intentional nonadherence in taking medicine subscale (p=0.024).
**Conclusions:** Providing education and pillbox done by pharmacists at the Primary Health Care Center can increase adherence to the therapy of diabetes mellitus patients. Pharmacists at the Primary Health Care Center can use the intervention model to improve the level of adherence of patients with chronic illness.

**Keywords:** adherence; diabetes mellitus; education; pillbox.

## Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia. Etiopathology in diabetes mellitus consists of impaired insulin secretion, insulin action, and both, as well as disorders of carbohydrate, fat, and protein metabolism. Type 2 diabetes mellitus contributes as many as 90–95% of all diabetes mellitus with the highest proportion in low and middle-income countries [1]. IDF survey in 2019 shows that the number of people with diabetes mellitus in Indonesia is ranked the seventh in the world [2]. The prevalence of diabetes mellitus in West Nusa Tenggara based on the results of the Indonesian Ministry of Health's survey increased from 2013 to 2018 [3].

Nonadherence to therapy is one of the drug therapy problems associated with therapeutic outcomes in diabetes mellitus patients. Studies in large populations showed that the value of glycemic control will worsen when patients didn't comply with treatment [4]. Nonadherence can also lead to a risk of complications [5] and high costs [6, 7]. However, the level of adherence to long-term therapy in developing countries is still below 50% [7]. Previous studies showed that medication adherence in patients with diabetes mellitus in Indonesia was still low [8–10].

Nonadherence to therapy can occur due to intentional or unintentional reasons. Unintentional nonadherence is a passive process where the patient has limited capacity to comply. Whereas, intentional nonadherence is an active process in which the patient decides to not comply [11]. According to WHO, the reasons for nonadherence can be divided into several factors, which are socioeconomic factors, factors related to health systems, disease factors, factors related to therapy, and patient factors [7].

***Corresponding author: Yunita Nita**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +628123014911, E-mail: yunita-n@ff.unair.ac.id
**Mahacita Andanalusia,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Umi Athiyah,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

Various interventions to improve adherence have been carried out, including interventions related to treatment, clinicians, patients, families, and group discussions. The interventions given need to be adjusted according to any obstacles that can cause patients to not comply [12]. In this study, education and pillbox were given as interventions. Education was given to overcome intentional nonadherence problems, meanwhile, pillbox was given to resolve the unintentional nonadherence problem [12]. The objective of the present study was to analyze the effect of pillbox use and education by the pharmacist to improve patients' adherence level to their treatment regimen.

## Materials and methods

This research was a true experimental study with a pretest-posttest with a control group design. The level of adherence was measured before and after the intervention was given. Respondents were divided into three groups, that were the control group, the education group, and the education and pillbox group. The study was conducted in patients with type 2 diabetes mellitus at Tanjung Karang Primary Health Care Center, Mataram. Group distribution was done randomly. Randomization was done by using code for each group in the patients who met the requirement during baseline data collection.

The inclusion criteria in this study consisted of outpatients who had been diagnosed with diabetes mellitus for at least one month, patients were not performing insulin therapy, patients with diabetes mellitus aged ≥18 years, patients who did not have high adherence scores (Adherence to Refill and Medication Scale [ARMS] score>12 [13]), patients who had never received educational interventions and pillbox, as well as patients who were willing to become research respondents by filling out consent sheets. A number of respondents who were successfully recruited were 45 respondents, with each group consisted of 15 respondents. There were 12 respondents who dropped out. Therefore, the respondents who managed to get a follow-up were 33, with each group consisted of 11 respondents.

The research variables included independent, dependent, and moderator variables. Independent variables were the interventions in the form of education and pillboxes. Dependent variables were the adherence to therapy, both overall and on each subscale (refilling medicine and intentional nonadherence in taking medicine, unintentional nonadherence in taking medicine, and persistence in refilling medicine). The moderator variables assessed consisted of data on characteristics of age, occupation, education, income, duration of therapy, number of drugs taken per day, frequency of drug use per day, side effects of drugs, and belief in medication prescribed.

The study was done from October to December 2019 and consisted of two phases. The first phase was the collection of baseline data (pretest), which was conducted during October 2019. In this phase, interventions were carried out. The second phase was the collection of data on follow-up (posttest), which was conducted from November to early December 2019.

Data were obtained through filling out questionnaires, consisting of a demographic questionnaire, ARMS questionnaire, and beliefs about medicines questionnaire (BMQ)-Specific questionnaire. The ARMS questionnaire was used to assess adherence to therapy [13], while the BMQ-Specific questionnaire was used to assess the patient's belief in medication prescribed [14]. The ARMS questionnaire had been tested for its validity and reliability in Indonesia. The validity test had been done with a correlation value of 0.368–0.794 and a reliability test with a score of 0.865 [9]. In addition, an assessment with the Naranjo scale was also used to ascertain the incidence of side effects.

The education intervention obtained material related to the goals of diabetes therapy, how to use the drug, side effects, and how to prevent it, as well as discussions about the ability, challenges of patients in conducting diabetes therapy, and providing patient motivation. Education was given verbally and face-to-face for 15–20 min. A pictogram as educational aid that explained the procedures about the drug consumption was given. On the other hand, pillbox intervention was given as an addition to the education and pillbox group. Pillbox given was pillbox that had been customized by giving day labels in Indonesian and drug labels that referred to their pictograms. In the control group, patients were given general information about how to keep and dispose of medication. The control groups were also given the education and pillboxes after data collection was done.

The results for each characteristic distribution were shown in frequency and percentage. The level of adherence and belief were shown on average and standard deviation. To determine differences in patient characteristics and belief variables, the fisher's exact and one-way analysis of variance (ANOVA) tests were used. A comparison of adherence before and after the intervention was given in each group was assessed by paired t-test. The difference in adherence (Δ) in each group was determined by the one-way ANOVA test. This research had received ethical approval from the Research Ethics Committee in the Faculty of Nursing, Universitas Airlangga (No. 1763-KEPK/2019).

## Results

Table 1 shows the demographic and clinical characteristics of the data in each group. Based on these characteristic data, it was known that the majority of respondents were housewives, aged 55–64 years, didn't graduate from elementary school, had an income of IDR 50,000–100,000 per day, had received diabetes therapy for 1–5 years, got two types of drugs from the Primary Health Care Center with a frequency of use three times a day, and did not have side effects. Based on the Fisher's exact test, it was found that there were no significant differences between each characteristic ($p>0.005$).

Table 2 shows the distribution of beliefs about medication prescribed in each group. Based on the distribution results, it was known that the overall average and each group had a positive belief. Based on the one-way ANOVA test, it was found that there were no significant differences in the beliefs of each group ($p>0.765$).

Table 3 shows the results of the adherence scores before and after the intervention was given to each group. In the control group and the education group, there was no significant effect on scores before and after the administration of the intervention ($p>0.005$). However, in the education

**Table 1:** Patients' characteristics and clinical data.

| Variables | | Control, n | Education, n | Education + pillbox, n | Total n, % | p-Value[a] |
|---|---|---|---|---|---|---|
| Age | 35–44 years old | 2 | 2 | 1 | 5 (15.2%) | 0.721 |
| | 44–54 years old | 3 | 4 | 4 | 11 (33.3%) | |
| | 55–64 years old | 3 | 5 | 4 | 12 (36.4%) | |
| | 65–74 years old | 3 | 0 | 2 | 5 (15.2%) | |
| Occupation | Housewives | 3 | 4 | 4 | 11 (33.3%) | 0.882 |
| | Private employee | 1 | 0 | 0 | 1 (3.0%) | |
| | Entrepreneur | 3 | 4 | 3 | 10 (30.3%) | |
| | Retired | 3 | 1 | 1 | 5 (15.2%) | |
| | Others | 1 | 2 | 3 | 6 (18.2%) | |
| Formal education | Do not graduate from school | 3 | 5 | 6 | 14 (42.4%) | 0.393 |
| | Elementary school | 3 | 1 | 0 | 4 (12.1%) | |
| | Junior high school | 1 | 0 | 3 | 4 (12.1%) | |
| | Senior high school | 2 | 3 | 1 | 6 (18.2%) | |
| | Diploma | 2 | 2 | 1 | 5 (15.2%) | |
| Average income/day (IDR) | <50,000 | 1 | 4 | 5 | 10 (30.3%) | 0.502 |
| | 50,000–100,000 | 5 | 2 | 4 | 11 (33.3%) | |
| | 100,000–150,000 | 2 | 1 | 1 | 4 (12.1%) | |
| | 150,000–200,000 | 2 | 1 | 1 | 4 (12.1%) | |
| | 200,000–250,000 | 0 | 2 | 0 | 2 (6.1%) | |
| | >250,000 | 1 | 1 | 0 | 2 (6.1%) | |
| Therapy duration | <One year | 1 | 3 | 1 | 5 (15.2%) | 0.848 |
| | 1–5 years | 8 | 6 | 7 | 21 (63.6%) | |
| | >Five years | 2 | 2 | 3 | 7 (21.2%) | |
| Number of medication obtained | One medication | 0 | 1 | 1 | 2 (6.1%) | 0.696 |
| | Two medications | 6 | 5 | 8 | 19 (57.6%) | |
| | Three medications | 4 | 3 | 2 | 9 (27.3%) | |
| | Four medications | 1 | 2 | 0 | 3 (9.1%) | |
| Frequency of medication consumption per day | Once | 0 | 1 | 1 | 2 (6.1%) | 0.367 |
| | Twice | 2 | 0 | 0 | 2 (6.1%) | |
| | Three times | 4 | 6 | 8 | 18 (54.5%) | |
| | Four times | 4 | 2 | 2 | 8 (24.2%) | |
| | >Four times | 1 | 2 | 0 | 3 (9.1%) | |
| Adverse reaction effect | None | 11 | 11 | 9 | 31 (94.0%) | 0.312 |
| | *Doubtful* | 0 | 0 | 1 | 1 (3.0%) | |
| | *Probable* | 0 | 0 | 1 | 1 (3.0%) | |

[a]Fisher's exact test.

**Table 2:** Belief toward medication prescribed (BMQ-Specific).

| Variables | Control, $\bar{x} \pm$ SD | Education, $\bar{x} \pm$ SD | Education + pillbox, $\bar{x} \pm$ SD | p-Value[a] |
|---|---|---|---|---|
| Belief score | 0.79 ± 0.67 | 0.60 ± 0.54 | 0.64 ± 0.67 | 0.765 |
| Total | | | 0.68 ± 0.62 | |

[a]*One-way* ANOVA test.

and pillbox groups, there was a significant increase for each adherence subscale and total score between before and after the intervention was given ($p<0.005$).

Table 4 shows the comparison of differences in adherence scores (posttest scores minus pretest scores) before and after the interventions were given to each group. A minus value indicated a decrease in the score, meaning that there was an increase in adherence at the subscale. Meanwhile, a positive value indicated an increase in the score, signifying that there was a decrease in adherence at the subscale. The results showed that there were differences in adherence changes in each group in the refilling medicine and intentional nonadherence in taking medication subscale ($p=0.027$) and overall adherence ($p=0.049$). This showed

**Table 3:** Medication adherence (ARMS score).

| Groups/subscales | ARMS score before intervention | | ARMS score after intervention | | p-Value[e] |
|---|---|---|---|---|---|
| | Min–Max | $\bar{x} \pm$ SD | Min–Max | $\bar{x} \pm$ SD | |
| Control group | | | | | |
| Refilling medicine and intentional nonadherence in taking medicine[a] | 5–10 | 6.73 ± 1.74 | 5–12 | 7.36 ± 2.66 | 0.240 |
| Unintentional nonadherence in taking medicine[b] | 5–16 | 8.64 ± 3.20 | 5–19 | 6.54 ± 1.63 | 0.616 |
| Persistence in refilling medicine[c] | 2–6 | 4.00 ± 1.67 | 2–7 | 4.27 ± 1.62 | 0.602 |
| Total[d] | 13–30 | 19.36 ± 5.24 | 14–28 | 19.82 ± 4.73 | 0.745 |
| Education group | | | | | |
| Refilling medicine and intentional nonadherence in taking medicine[a] | 5–11 | 6.27 ± 1.85 | 5–7 | 5.45 ± 0.82 | 0.095 |
| Unintentional nonadherence in taking medicine[b] | 5–10 | 7.18 ± 1.47 | 5–9 | 6.54 ± 1.63 | 0.396 |
| Persistence in refilling medicine[c] | 2–8 | 4.36 ± 2.34 | 2–6 | 3.00 ± 1.61 | 0.077 |
| Total[d] | 14–26 | 17.54 ± 4.46 | 12–22 | 15.00 ± 3.71 | 0.108 |
| Education + pillbox group | | | | | |
| Refilling medicine and intentional nonadherence in taking medicine[a] | 5–11 | 7.18 ± 2.36 | 5–7 | 5.73 ± 0.90 | 0.042* |
| Unintentional nonadherence in taking medicine[b] | 5–11 | 7.64 ± 1.69 | 5–9 | 6.18 ± 1.33 | 0.038* |
| Persistence in refilling medicine[c] | 2–7 | 4.73 ± 1.74 | 2–5 | 3.27 ± 1.19 | 0.034* |
| Total[d] | 15–29 | 19.54 ± 4.37 | 12–20 | 15.18 ± 2.64 | 0.004* |

[a]Total question items=5; range score=5–20, [b]Total question items=5; range skor=5–20, [c]Total question items=2; range score=2–8, [d]Total question items=12; range score 12–48, [e]*Paired t-test.*

that the refilling medicine and intentional nonadherence in taking medication subscale contributed to changes in adherence.

Table 5 shows a comparison of the difference in adherence score (Δ) in each group for the refilling medicine and intentional nonadherence in taking medication subscale and overall adherence score. It was known that there was a difference between the education and pillbox group and the control group. This indicated that interventions that could provide changes in adherence were educational and pillbox interventions.

## Discussion

Education and pillbox interventions were chosen based on the conditions observed. For example, some patients had specific conditions, such as older age, low-level education, and low income. These conditions showed that education is more suitable to be done face-to-face with pictogram aids. A pillbox can also be used to help improving adherence for patients with these conditions [12]. Pillbox given to respondents was customized to the patient's needs. There were day labels in Indonesian and drug labels with color on each drug to help vulnerable patients to identify their medicine easily.

In this study, the intervention of education and pillbox gave improvement to patient's medication adherence. The main determinant that contributes to the improvement was refilling medicine and intentional nonadherence in taking medicine subscale. This can be caused by internal factors of the patient, where the patients perform actions that they think are rational causing the patient to comply. Internal factors that can affect motivation can be mediated by

**Table 4:** Comparison of the difference in adherence score (Δ).

| Subscales | Control, $\bar{x} \pm$ SD | Education, $\bar{x} \pm$ SD | Education + pillbox, $\bar{x} \pm$ SD | p-Value[a] |
|---|---|---|---|---|
| Refilling medicine and intentional nonadherence in taking medicine | 0.64 ± 1.69 | −0.82 ± 1.47[b] | −1.45 ± 2.07[b] | 0.027* |
| Unintentional nonadherence in taking medicine | −0.45 ± 2.91 | −0.64 ± 2.38[b] | −1.45 ± 2.02[b] | 0.603 |
| Persistence in refilling medicine | 0.27 ± 1.68 | −1.09 ± 2.26[b] | −1.54 ± 1.86[b] | 0.091 |
| Total | 0.45 ± 4.50 | −2.54 ± 4.78[b] | −4.36 ± 3.93[b] | 0.049* |

[a]One-way ANOVA test, [b]Negative (–) value showed decreasing score, positive value showed increasing score.

**Table 5:** Comparison of the difference in adherence score (Δ) (Post Hoc).

| Subscales | Groups | p-Value[a] |
|---|---|---|
| Refilling medicine and intentional nonadherence in taking medicine | Control vs. education | 0.146 |
| | Control vs. education + pillbox | 0.024* |
| | Education vs. education + pillbox | 0.677 |
| Total | Control vs. education | 0.265 |
| | Control vs. education + pillbox | 0.041* |
| | Education vs. education + pillbox | 0.604 |

[a]Post Hoc test in *One-way* ANOVA.

external variables, such as the quality of communication and information from other sources. Besides, patient satisfaction can also be a reason for the improvement in adherence, where satisfaction with attributes given can motivate them in dealing with therapy [11]. Previous studies have shown that patients who were satisfied with the treatment given had better adherence than those who are less satisfied [15, 16]. In this study, the education and pillbox group received the most complete attributes.

Various studies have shown inconsistent results from the relationship between giving education to adherence improvement. In the previous study, it was known that education could improve adherence to therapy in patients with diabetes mellitus [17]. Another study showed that education cannot improve medication adherence in glaucoma patients [18]. In this study, the result indicated that providing education alone cannot significantly improve medication adherence. This condition can be caused by several matters. First, most patients have low education or low literacy. The condition can trouble them to understand additional information beyond the information in the pictogram. Pictogram is intended to facilitate individuals in understanding something, but some things cannot be expressed through pictograms, such as theory [19]. Besides, patients tend to have a passive role, so they cannot describe their existing trust [20, 21]. Good education requires active participation between practitioners and patients. This can be a critical point for determining how patients will be able to decide to follow their treatment management. The pictogram used in this study was also just an explanation of how to consume the medicine. Other educational contents, especially those involving patients' participation in communication could not be described with symbols.

Different results between the two groups can be explained by the concept of Cone of Experience by Dale. This concept explains that learning media is divided into several groups with the most abstract and most concrete categories. Education as the verbal symbols is the most abstract media, where individual abilities in the learning process are at the stages of defining, describing, making lists, and explaining. The pictogram aid is the form of visual symbols where individuals can demonstrate what is done. Meanwhile, the pillbox is a tool used by patients to gain direct experience. Direct experience is the most concrete media. Individuals are not only capable of practicing behavior, but also analyze, plan, shape, and even evaluate their behavior. This concept has been updated to Multimedia Cone of Abstraction (McoA). According to McoA, the combination of media will reinforce the process of learning experience [22]. This explained the more prominent learning process in this group, so that patient's medication adherence improved.

Another study with the MCoA concept in educational research has been done before. A review about media augmented reality (AR) and virtual reality (VR) use was known to give more advantages in the learning experience [23]. Although in McoA, virtual reality technology is assumed to be the most realistic and concrete media, this concept is still relevant in explaining the result in media combination. Combination use between verbal education, pictogram, and pillbox showed a more optimum learning process, so that comprised the improvement in patients' adherence to therapy

There is a limited time in this study that can make a different result in adherence improvement. Therefore, there is a possibility that the respondent will experience a relapse behavior after six months [24]. However, improvement in a short time indicated a potential for the health care professional to provide interventions repeatedly to prevent the relapse. Another limitation is some respondents dropped out without a clear reason, and it could cause bias in the interpretation of results. Nevertheless, the number of respondents has met the minimum sample based on the sample size calculation. Using one instrument to measure adherence also appears to be one of the limitations. This study also did not measure the knowledge and satisfaction, so the process of changing behavior cannot be proven.

## Conclusions

Providing education and pillbox in Primary Health Care Center can improve medication adherence in patients with diabetes mellitus. This intervention model can be used by pharmacists as a part of therapeutic management in patients with chronic diseases.

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**Ethical approval:** The research related to human subjects has complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee (Research Ethics Committee in the Faculty of Nursing, Universitas Airlangga, No. 1763-KEPK/2019).

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Mikhania Christiningtyas Eryani, Esti Hendradi* and Siswandono

# Variation concentration effect of propyleneglycol, glycerin, and polyethyleneglycol 400 to physical properties and dissolution rate of loratadine liquisolid tablet

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**Abstract**

**Objectives:** This study aimed to evaluate the variation concentration effect of propyleneglycol, glycerin, and polyethyleneglycol 400 as a nonvolatile solvent on the physical properties and dissolution rate of the loratadine liquisolid tablet.
**Methods:** The tablet was formulated into 10 formulas, where nine were liquisolid and one was conventional (CT). The concentration of propyleneglycol, glycerin, and polyethyleneglycol used in liquisolid tablets were 14, 15, and 16%. Furthermore, the mixture was evaluated based on flow properties and compressibility index. The tablet was evaluated based on hardness, friability, disintegration time, and dissolution, and the data obtained was evaluated with ANOVA or Kruskal–Wallis statistic program.
**Results:** The result showed that flow properties, disintegration time, and dissolution have a significant value less than 0.05. The tablet friability for all concentration solvents, hardness at 14 and 15% solvent concentration, and compressibility index at 15 and 16% have significant value more than 0.05. The 16% propyleneglycol type solvent concentration tablet has the physical properties and contains the best solution
**Conclusions:** From the result, it is reasonable to conclude that F7 is the tablet with all the physical properties and the best dissolution.

**Keywords:** liquisolid; loratadine dissolution; nonvolatile solvent.

## Introduction

Drug synthesis is developing rapidly these days, and some synthesized types have poor water solubility. This affects the dissolution bioavailability in the blood and the therapeutic effect of the drug. In addition to solubility, the therapeutic effect of a drug is also influenced by the rate of dissolution [1].

Loratadine is a nonsedative antihistamine drug used to reduce allergy symptoms, and it belongs to Class II Biopharmaceutical Classification System (BCS), that have poor solubility but good permeability [2]. Furthermore, the key factor bioavailability of BCS class II drugs is determined by their solubility and dissolution. Therefore, the formulation factor plays an important role in the bioavailability of these drugs [3].

Liquisolid technique was developed to increase the water solubility of active ingredients. It involves dissolving the active ingredient which is lipophilic in a nonvolatile solvent into a suspension or liquid form. This is further converted into a powder that is easy to flow and is ready to be compressed after adding a carrier and a coating [4]. The increase in the solubility of drugs in the liquid-solid technique occurs through various mechanisms, namely an increase in the specific surface area and in the ability to absorb water and get wet [1]. This is because increasing the drug's surface area makes it easier for it to dissolve in water.

The constituent components of liquisolid tablets include nonvolatile solvents, carriers, and coating materials [5]. The use of nonvolatile solvents plays an important role in liquid–solid tablets, for example in wetting particles by reducing the surface pressure between the dissolving medium and the tablet surface to increase dissolution [6]. The nonvolatile solvents used in the liquisolid technique include propyleneglycol, glycerin, and polyethyleneglycol (PEG). They comprise a different number of hydroxy (OH)

***Corresponding author: Esti Hendradi**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, E-mail: esti-h@ff.unair.ac.id
**Mikhania Christiningtyas Eryani,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Siswandono,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

groups which affect their hydrophobicity [7, 8] due to different dielectric constants. Therefore, two compounds with a similar soluble principle such as polarity can dissolve together, and the solubility indicator can be determined from their dielectric constant value.

This study aimed to evaluate the variation concentration effect of propyleneglycol, glycerin, and polyethyleneglycol 400 as nonvolatile solvents to physical properties and dissolution rate of loratadine liquisolid tablet.

# Materials and methods

Tablet was formulated into 10 formulas as shown in Table 1.

The tablet was made by direct compression method by mixing loratadine with a nonvolatile solvent (propyleneglycol, PEG 400, or glycerin). Furthermore, Avicel PH 102 and sodium starch glycolate were added to the mixture with aerosil. After the mixing, the bulk was checked for flowability and compressibility to form a tablet with a hardness of 4–8 kg. The bulk was evaluated in the following subsections:

### Flowability

About 100 g of powder were put into the funnel for the flowability test. The funnel cover is opened to allow the powder to come out on a flat surface. Furthermore, the granule flow times were recorded [7].

### Compressibility

The powder was weighed and put in a measuring cup while recording the volume. Furthermore, the tapping procedure occurred 500 times and the test volume before ($V0$) and after ($V$) was recorded. The compressibility index (I) was measured as follows [7]:

$$I = \frac{V0 - V}{V0} \times 100$$

### Hardness

The tablet hardness test was conducted using the testing tool by sliding the measuring rod on the tester to zero. The tablet was placed on the end of the tool and then the screw was turned on the end of the tool until it breaks. The measurements were repeated for 10 tablets and the average was calculated [7]

### Friability

The friability test procedure was conducted by cleaning and weighing a tablet equivalent to 6.5 g with adhered powder. It was inserted into the friability tester, and the tool was rotated for 100 turns. Thereafter, it was cleaned off the sticking powder and then weighed. Therefore, the percent of friability is obtained by dividing the tablet's weight loss by the initial weight [9].

### Disintegration

The tablet disintegration was conducted on six tablets which were put into a basket containing the medium of 900 mL of distilled water at 37 °C. The time it took for the disintegration was then recorded [9].

### Dissolution

The dissolution test was determined using the II USP method (paddle type), 900 mL of 0.1 N HCl dissolution medium. This was conducted with a stirring speed of 50 rpm and a temperature of 37 ± 0.5 °C for 60 min [9].

### Statistical analysis

Statistical analysis was performed using the ANOVA or Kruskal–Wallis statistic program to obtain significance values for each solvent concentration. A significance value of less than 0.05 is said to be different [10].

# Results

The results of tablet evaluation described in Table 2 below.

Table 2 showed that the best powder flow rate is at F9 and the best compressibility was at F3. From Figure 1, it was shown that F7 had the higher dissolution among all

**Table 1:** Formulation of loratadine liquisolid tablets.

| | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | CT |
|---|---|---|---|---|---|---|---|---|---|---|
| Loratadine, mg | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Propyleneglycol, % | 14 | – | – | 15 | – | – | 16 | – | – | – |
| PEG 400, % | – | 14 | – | – | 15 | – | – | 16 | – | – |
| Glycerin, % | – | – | 14 | – | – | 15 | – | – | 16 | – |
| Avicel PH 102, mg | 160 | 160 | 160 | 200 | 200 | 200 | 240 | 240 | 240 | 200 |
| Aerosil, mg | 8 | 8 | 8 | 10 | 10 | 10 | 12 | 12 | 12 | 10 |
| Sodium starch glycolate, mg | 5 | 5 | 5 | 6 | 6 | 6 | 7 | 7 | 7 | 6 |
| Unit dose weight, mg | 213 | – | – | 266 | – | – | 319 | – | – | 226 |

**Table 2:** Evaluation of loratadine liquisolid tablets.

| | Flowability, g/s | Compressibility index, % | Hardness, kg | Friability, % |
|---|---|---|---|---|
| F1 | 8.53 ± 0.10 | 20.97 ± 0.85 | 4.09 ± 0.30 | 0.74 ± 0.00 |
| F2 | 6.83 ± 0.1 | 18.11 ± 0.81 | 4.09 ± 0.30 | 0.74 ± 0.00 |
| F3 | 5.29 ± 0.00 | 17.65 ± 0.00 | 4.09 ± 0.30 | 0.67 ± 0.01 |
| F4 | 7.07 ± 0.08 | 17.87 ± 0.00 | 4.63 ± 0.51 | 0.81 ± 0.01 |
| F5 | 4.81 ± 0.03 | 19.53 ± 0.83 | 4.18 ± 0.41 | 0.47 ± 0.00 |
| F6 | 7.67 ± 0.12 | 18.58 ± 0.81 | 4.18 ± 0.41 | 0.44 ± 0.00 |
| F7 | 6.52 ± 0.08 | 19.53 ± 0.83 | 4.73 ± 0.47 | 0.51 ± 0.02 |
| F8 | 7.54 ± 0.04 | 20.00 ± 0.83 | 4.18 ± 0.41 | 0.50 ± 0.01 |
| F9 | 10.87 ± 0.14 | 18.11 ± 0.81 | 4.09 ± 0.30 | 0.43 ± 0.00 |
| CT | 7.66 ± 0.66 | 17.88 ± 0.58 | 4.09 ± 0.30 | 0.24 ± 0.02 |

Data are the average of three time replications ± SD.

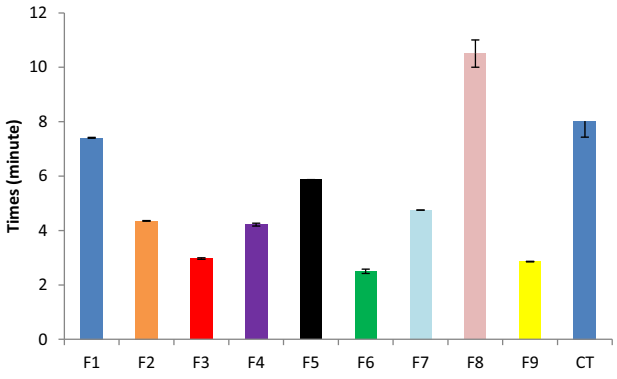


**Figure 2:** Loratadine liquisolid tablet disintegration time in distilled water at 37 °C. Data are the average of the three time replications ± SD.

formulas. Figure 2 showed the disintegration test result while Table 3 described the statistical analysis tests.

Table 3 showed that friability, hardness at 14 and 15% solvent concentration, and compressibility at 15 and 16% solvent concentration have significant values above 0.05.

## Discussion

Liquisolid Loratadine tablets are made by a direct compression process by dissolving the lipophilic active ingredient in a nonvolatile solvent as a suspension or as a liquid. Furthermore, it is converted into a powder that is easy to flow and ready to compress after adding a carrier and coating. The nonvolatile solvents usually used are propyleneglycol, PEG 400, and glycerin.

Propylene glycol is commonly used in the pharmaceutical industry as a humectant and cosolvent in ointments for medical applications. The main function is to dissolve and produce a homogeneous mixture of active ingredients in the formulation. Liquid polyethylene glycols (PEG 400) are widely used as solvents and solubilizing agents for active substances and excipients in liquid and semisolid preparations [8]. Meanwhile, glycerin is widely used in the pharmaceutical industry as solvent and cosolvent [11]. The evaluation conducted are flowability, compressibility, hardness, friability, disintegration time, and tablet dissolution.

Powder flowability increases the filling reproducibility within the compression chamber during the manufacturing process to give a better uniformity of dosage weight. It also has significant pharmacological effects since a good flow time is at least 4 g/s. The results showed that all formulas have a good flow rate because they are more than 4 g/s. The statistical results showed that the powder flow rate for all variations in the concentration of solvents has a significant difference with outstanding rates.

Compressibility is the ability of a granule to remain compact in the presence of pressure. The percentage is calculated based on data obtained from measurements of real and compressive density, and should not exceed 20%. However, two out of the total number of formulas failed to meet the percent compressibility requirements, namely F1 and F8. Since compressibility can indicate the flowability of the tablet mass, therefore, the smaller the value, the better the flow capability. Statistical analyzes show that the formula with a solvent concentration of 30 mg has a significant value less than 0.05, therefore there is a significant compressibility difference in the group.

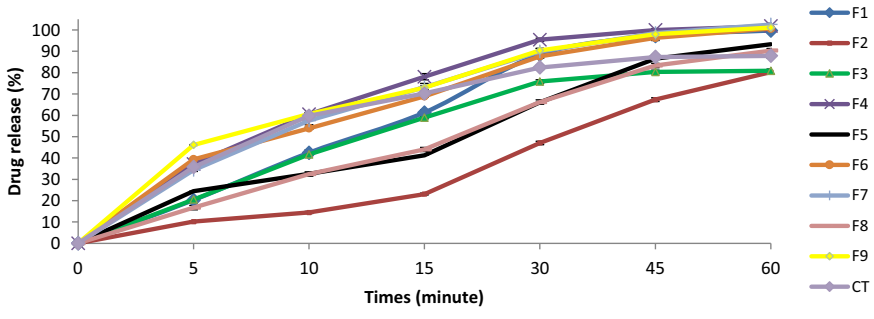


**Figure 1:** Loratadine liquisolid tablet dissolution profile in 0.1 N HCl dissolution medium at 37 ± 0.5 °C. Data are the average of the three time replications ± SD.

**Table 3:** Significance analysis of loratadine liquisolid tablets.

| Solvent concentration | Significance | | | | | |
|---|---|---|---|---|---|---|
| | Flowability | Compressibility index | Hardness | Friability | Disintegration time | Dissolution |
| 14% | 0.000 | 0.034 | 0.368 | 0.18 | 0.018 | 0.018 |
| 15% | 0.027 | 0.061 | 0.517 | 0.18 | 0.024 | 0.018 |
| 16% | 0.027 | 0.07 | 0.001 | 0.18 | 0.021 | 0.018 |

Tablet hardness describes compressive strength during production, packaging, and distribution. This process was carried out by applying pressure to the tablet until it breaks, and all formulas meet good hardness requirements, which is 4–8 kg. Based on the results of statistical analysis, the formula with 16% solvent concentration has a significance value less than 0.05 therefore there is a significant hardness difference in the group.

The friability test ensures the resistance of tablets to mechanical forces in the packaging and distribution process. The measurement was carried out by calculating the percentage of tablet weight lost during rotation on the friabilator at 25 rpm for 4 min. Furthermore, all formulas meet the friability requirements, which do not need to exceed 1%. Based on the statistical test for all variations in the concentration of the solvent, it has a significant value of more than 0.05. Therefore, the tablet friability does not have a significant difference.

Disintegration is the first physical change of a drug when it enters the body. Therefore, this test is performed to simulate the disintegration with the time calculated based on the most recently crushed tablet. The time requirement for uncoated tablets is less than 15 min, for sugar-coated and nonenteric-coated, it is less than 30 min. Enteric tablets should not be crushed within 60 min in an acidic medium and should be destroyed immediately in an alkaline medium [12]. All formulas meet the disintegration time requirement since it was less than 15 min. Furthermore, the statistical tests for all variations in the concentration of the solvent have a significance value less than 0.05. Therefore, there is a significant difference in the disintegration time of the tablets.

In the dissolution process, the solid drug is dissolved in a solvent at a certain temperature. Changes in solubility and dissolution rate will cause changes in drug absorption. Therefore, dissolution tests can be used to predict bioavailability and also differentiate their formulation factors [13]. Several factors that influence dissolution include stirring speed, medium temperature, medium viscosity, substance solubility, and particle size [14]. Furthermore, tablet wettability by dissolution media is one of the mechanisms for explaining the enhanced rate. The nonvolatile solvent present in the liquisolid system facilitates the wetting of the particle by decreasing interfacial tension between the dissolution medium and tablet surface [15].

The dissolution requirement of loratadine is not less than 80% dissolved below 60 min. The results showed that all formulas met the requirements, and F7 has the greatest dissolution. This is because it contains the highest concentration of propyleneglycol as a nonvolatile solvent. Propyleneglycol is widely used as a solvent for the liquisolid system and facilitates the wetting of drug particles to increase dissolution. Statistical tests for all variations in the concentration of the solvent showed a significance value less than 0.05.

## Conclusions

From the results, it is reasonable to conclude that F7 fulfilled the physical properties and has the best dissolution. This is because of its highest concentration of propyleneglycol as a nonvolatile solvent. The use of propyleneglycol can facilitate the wetting of drug particles to increase dissolution.

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Sylvia Anggraeni*, Menul Ayu Umborowati, Damayanti Damayanti, Anang Endaryanto and Cita Rosita Sigit Prakoeswa*

# Role of *Centella asiatica* and ceramide in skin barrier improvement: a double blind clinical trial of Indonesian batik workers

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**Abstract**

**Objectives:** Batik dyes contain irritant chemicals that increase the risk of skin barrier disruption. This study aims to determine the effect of *Centella asiatica* and ceramide in transepidermal water loss (TEWL), hydration of the stratum corneum and skin acidity (pH).

**Methods:** This was a double blind clinical trial of 30 Indonesian batik workers who suffered from skin dryness, but had no clinical manifestation of contact dermatitis. Subjects were given cream containing *C. asiatica* or ceramide that formulated and randomly labeled by manufacturer (PT Paragon Technology and Innovation). Both subjects and researchers were blinded to the type of the cream. Cream was applied to the hands and arms twice a day. Biological function of the skin (TEWL, stratum corneum hydration level, and skin acidity) was examined by Cutometer dual MP-580. Baseline was recorded in the first examination, followed by second and third examinations at two and four weeks after treatment.

**Results:** After four weeks treatment, there were significant improvement of *C. asiatica* application in evaluation of corneometer palmar (p=0.007; CI 95%), corneometer dorsum (p=0.001; CI 95%), and skin acidity dorsum (p=0.017; CI 95%). Ceramide application also gave significant improvement of corneometer palmar (0.038; CI 95%), skin acidity palmar (p=0.001; CI 95%), TEWL dorsum (p=0.023; CI 95%), corneometer dorsum (p=0.002; CI 95%) and skin acidity dorsum (p=0.011; CI 95%). There were no significant differences of *C. asiatica* effectiveness compared to ceramide in skin barrier improvement.

**Conclusions:** *C. asiatica* and ceramide can improve skin barrier hydration in order to prevent the risk of contact dermatitis in batik workers.

**Keywords:** *Centella asiatica*; ceramide; Indonesian batik workers; skin barrier.

## Introduction

Batik is a world cultural heritage of Indonesia, declared by UNESCO on October 2nd, 2009. Batik has a historical precious value. Almost every region in Indonesia has its own color and pattern. Madura's Batik has a bright and bold color for their characteristic. Paseseh Village, Tanjung Bumi Subdistrict, in Bangkalan Madura Regency, is a well-known center of batik craft in the region [1, 2]. Batik making is not a simple process, it is done through at least three steps that can be repeated depending on the batik pattern: painting using canting, giving color (dipping), then drying [3, 4].

Batik workers are directly or indirectly exposed to chemicals. Natural and synthetic dyes are used, especially in the dipping process, which can induce the risk of skin dryness and irritant-contact dermatitis. Those synthetic dyes are among the main culprits. The substances that can induce reaction are reactive chemicals. A study from Jogjakarta, Indonesia showed that contact dermatitis was the most often occupational disease of batik workers. Batik dyes contain sodium hydroxide, heavy metals, suspended solids, or organic substances [3, 5, 6]. Repeated exposure to the skin can disrupt skin barrier function especially in hands area. Skin barrier function can be evaluated by increasing of trans-epidermal water loss (TEWL) and decreasing of stratum corneum hydration and acidity of the skin [7].

***Corresponding authors: Cita Rosita Sigit Prakoeswa**, Department of Dermatology and Venereology, Faculty of Medicine Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, Phone: +62811328199, E-mail: cita-rosita@fk.unair.ac.id; and **Sylvia Anggraeni**, Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; and Department of Dermatology and Venereology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, E-mail: sylvia.anggraeni@fk.unair.ac.id
**Menul Ayu Umborowati and Damayanti Damayanti,** Department of Dermatology and Venereology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya, Indonesia
**Anang Endaryanto,** Department of Pediatrics, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Academic Hospital, Surabaya, Indonesia

*Centella asiatica* is one of the traditional plants proven to provide moisturizing effect. It contains oleanane-type pentacycloc triterpenoid saponin, called as centelloids. The substances of this plant can stimulate fibroblast proliferation and activate the SMAD pathway, which causes an increase in type 1 collagen production, thereby increasing skin stretching and inflammatory reactions. Ceramide is also known to have a moisturizing effect which can improve skin barrier function. They are sphingolipids consisting of shingoidbases, which are amide-linked to fatty acids. Moisturizer application in batik workers is expected to improve physiological the condition of the skin and prevent contact dermatitis [8]. This study aims to evaluate skin barrier improvement and compare the efficacy between *C. asiatica* and ceramide, with respect to the skin barrier function in batik workers.

## Materials and methods

This study design was a double blind clinical trial involving 30 female batik workers in Paseseh Village, Tanjung Bumi Subdistrict, Bangkalan Madura Regency, Indonesia. All participants were included in this study since the sampling method was total sampling. The inclusion criteria of this study are batik workers aged 15–50 years old, work specialty in giving color (dipping), suffered from skin dryness and willing to join the study. Subjects who suffered from contact dermatitis and systemic disease were excluded. Diagnosis of skin dryness and contact dermatitis were established by dermatologists through anamnesis and clinical examination. All subjects were divided in two groups with random allocation method. Each group consisting of 15 subjects was given *C. asiatica* or ceramide cream formulated and randomly labeled as cream A (*C. asiatica* 2% in ceramide based cream) and cream B (ceramide 5% cream) by the manufacturer (PT Paragon Technology and Innovation). Both subjects and researchers were blinded to the type of cream, applied on hands and arms twice a day. Biological function of the skin (TEWL, stratum corneum hydration level, and skin acidity) was examined using Cutometer dual MP-580 (Figures 1–3). Baseline was recorded in the first examination in both dorsum and palmar region of the hand, followed by second and third examinations at two and four weeks after treatment, then analyzed by repeated measure of ANOVA, statistically significant at $p<0.05$. This study was approved by ethical committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (1678/KEPK/XI/2019).

## Results

The study included 30 participants of batik workers with work specialty in giving color (dipping). All subjects were female, aged 15–50 years old, suffered from skin dryness but had no clinical manifestation of contact dermatitis. Baseline of biological condition was recorded on the first week, in both dorsum and palmar region of the hand. The means of TEWL (palmar) were 60.91 and 58.20; TEWL (dorsum) were 28.80 and 29.75; corneometer (palmar) were 51.79 and 48.18; corneometer (dorsum) were 47.12 and 54.29; skin acidity (palmar) were 5.46 and 5.45; skin acidity (dorsum) were 5.35 and 6.72 in group A and group B, respectively.

This study showed that application of *C. asiatica* gave significant improvement in the evaluation of corneometer palmar (p=0.007; CI 95%), corneometer dorsum (p=0.001; CI 95%), and skin acidity dorsum (p=0.017; CI 95%) after four weeks treatment. There was no significant improvement of TEWL palmar, TEWL dorsum and skin acidity palmar (Table 1) and (Figure 4). Ceramide cream application also gave significant improvement in evaluation of corneometer palmar (p=0.038; CI 95%), skin acidity palmar (p= 0.001; CI 95%), TEWL dorsum (p= 0.023; CI 95%), corneometer dorsum (p=0,002; CI 95%) and skin acidity dorsum (p=0,011; CI 95%) after four weeks treatment. TEWL palmar did not reach statistical significance (Table 2) and (Figure 5). There were no significant differences of *C. asiatica* effectiveness compared to ceramide in skin barrier improvement (Table 3).

## Discussion

Batik workers are directly or indirectly exposed to irritant materials of dyes. This condition leads to skin dryness and irritant contact dermatitis. Skin dryness is a condition of decreased quantity and quality of lipids and hydrophilic

**Figure 1:** TEWL, skin hydration, and skin acidity measurement of batik workers.


**Figure 2:** Palmar and dorsal manus inspection of batik worker in the 1st week before *Centella asiatica* application.


**Figure 3:** Palmar and dorsal manus inspection of batik worker in the 1st week before ceramide application.

**Table 1:** The comparison of skin biological function after two or 4 weeks treatment of *Centella asiatica*.

| | Baseline | | Week 2 | | Week 4 | | P |
|---|---|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | |
| TEWL palmar | 60.91 | 10.82 | 55.75 | 10.00 | 56.42 | 8.48 | 0.260 |
| Corneometer palmar | 51.79 | 19.52 | 66.51 | 24.13 | 62.88 | 29.70 | 0.007 |
| pH palmar | 5.46 | 0.18 | 5.39 | 0.15 | 5.47 | 0.21 | 0.385 |
| TEWL dorsum | 28.80 | 14.00 | 35.40 | 11.63 | 29.73 | 9.80 | 0.056 |
| Corneometer dorsum | 47.12 | 21.18 | 76.63 | 32.77 | 58.90 | 25.61 | 0.001 |
| pH dorsum | 5.35 | 0.21 | 5.21 | 0.19 | 5.45 | 0.22 | 0.017 |

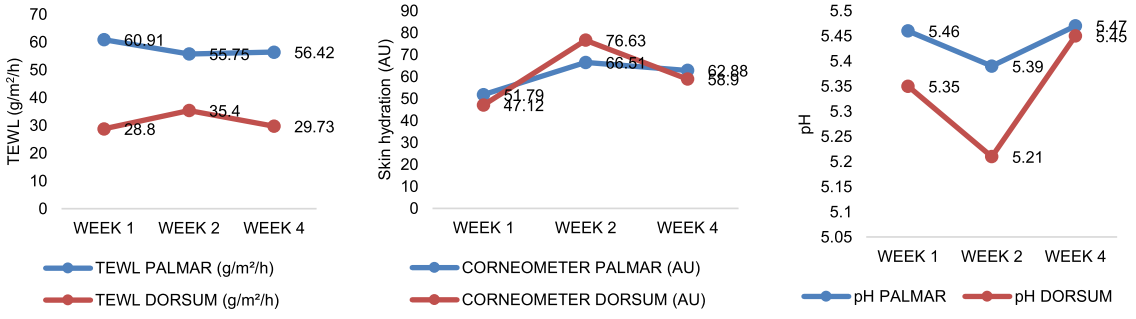


**Figure 4:** The comparison of skin biological function after 1, 2 and 4 weeks treatment of *Centella asiatica*.

**Table 2:** The comparison of skin biological function after two or four weeks treatment of ceramide.

| | Baseline | | Week 2 | | Week 4 | | P |
|---|---|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | |
| TEWL palmar | 58.20 | 13.25 | 51.48 | 11.90 | 52.84 | 9.33 | 0.080 |
| Corneometer palmar | 48.18 | 20.15 | 59.88 | 25.56 | 59.72 | 19.39 | 0.038 |
| pH palmar | 5.45 | 0.15 | 5.39 | 0.11 | 5.67 | 0.22 | 0.001 |
| TEWL dorsum | 29.75 | 13.21 | 38.26 | 15.16 | 28.55 | 10.72 | 0.023 |
| Corneometer dorsum | 54.29 | 19.76 | 84.94 | 24.86 | 60.75 | 19.90 | 0.002 |
| pH dorsum | 6.72 | 5.67 | 5.24 | 0.16 | 5.46 | 0.24 | 0.011 |

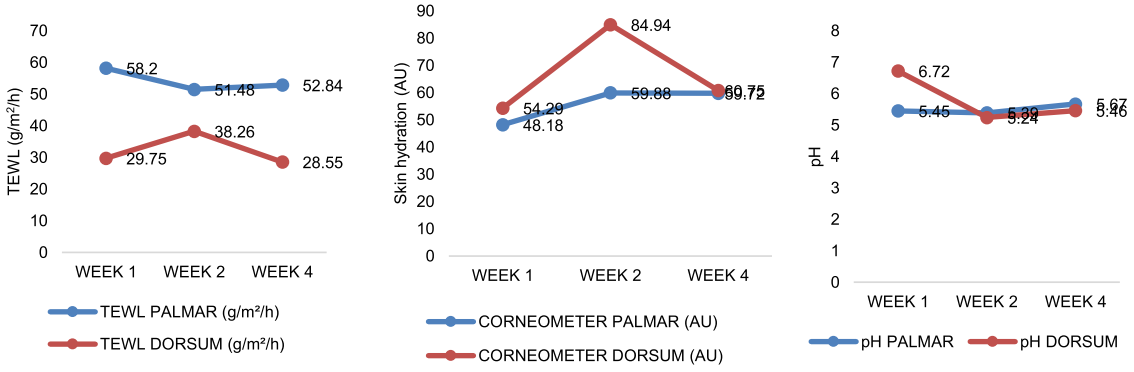


**Figure 5:** The comparison of skin biological function after 1, 2 and 4 weeks treatment of ceramide.

substances. Clinical manifestations are pruritus, roughness, wrinkles, tightness, burning sensation and pain, followed by lack of elasticity, texture coarsening, and wrinkling [9]. This study aims to evaluate the improvement of skin barrier in Indonesian batik workers after moisturizer application.

There was significant skin barrier improvement after *C. asiatica* application in this study. *C. asiatica* is known to have a moisturizer effect. Moisturizer helps to improve barrier function and increase stratum corneum hydration. *C. asiatica* has an important ingredient called centelloids that consist of asiaticoside, madecassoside, Asiatic acid and madecassic acid. Those ingredients are related to the function of this plant as antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic. In the dermatology field, *C. asiatica* has been reported good as adjuvant treatment for burns, psoriasis and scleroderma [10, 11]. Several studies have shown its role in hydrating the surface of the skin, improving the function of the stratum corneum barrier and as an anti-inflammatory agent [8]. At four weeks evaluation, *C. asiatica* gave significant improvements in the palmar and dorsal stratum corneum hydration, while skin acidity had significant improvements in the dorsum region. Triterpene saponin of this plant is a glycone chain that can bind water in the occlusive layer [10]. Improvement of TEWL and skin acidity palmar were also recorded in second week's evaluation, although not statistically significant. Decreasing of TEWL was also found in other studies. It is said that *C. asiatica* can bind water to the stratum corneum layer and increase the epidermal barrier layer [10, 12]. Its active ingredients, called saponins, flavonoids and phenolic acids, also have capability to reduce erythema and indirectly decrease level of TEWL and skin acidity [13].

**Table 3:** The comparison of skin biological function after four weeks treatment of *Centella asiatica* and ceramide.

| | *Centella asiatica* | | Ceramide | | P |
|---|---|---|---|---|---|
| | Mean | ±SD | Mean | ±SD | |
| TEWL palmar | −4.48 | 13.14 | −5.35 | 11.41 | 0.848 |
| Corneometer palmar | 11.09 | 17.03 | 11.54 | 18.04 | 0.112 |
| pH palmar | 0.01 | 0.27 | 0.22 | 0.22 | 0.027 |
| TEWL dorsum | 0.92 | 12.56 | −1.20 | 13.09 | 0.653 |
| Corneometer dorsum | 11.78 | 18.75 | 6.46 | 23.70 | 0.756 |
| pH dorsum | 0.01 | 0.27 | −1.26 | 5.74 | 0.406 |

Result findings in this study also showed significant skin barrier improvement after four weeks application of ceramide. Ceramide is known as good moisturizer that is able to improve the skin barrier function and increase protection from chemical exposure. Lipid products are

important components of skin barrier, especially ceramides, which are major constituents of the free extractable stratum corneum lipids. Ceramide has a significant role in maintaining and structuring water permeability barrier of the skin [14]. Its ingredients, such as salt of pyroglutamic, help to restore the hydration of the stratum corneum. It also acts as a humectant, which simulates sebum and natural lipids, and as an emollient, which occurs as intracellular bilayers of the stratum corneum [15]. All measurements of skin biological function in this group refer to significant improvement ($p<0.05$; CI 95%), except the TEWL (palmar). TEWL level depends on endogenous, exogenous and environmental factors. Endogenous factors consist of age, gender, anatomical position, skin temperature, sweating, circadian rhythm and skin health. TEWL values tend to be highest on the palm and the dominant forearm, the level is higher when sweating because of thermal, emotional, and physical mechanisms. Exogenous factors able to influence TEWL values are skin washing and wet-work, frequency of exposure to chemicals, occlusion and skin damage. Skin and probe temperature differences and also calibration of measurement instruments should be considered [9].

The application of both *C. asiatica* and ceramide moisturizing cream has proven to decrease TEWL, increase stratum corneum hydration and skin acidity, which is expected to provide protection of batik workers from chemical exposure to dyes and prevent contact dermatitis. Although there were no significant differences of *C. asiatica* effectiveness compared to ceramide in skin barrier improvement, we believe that study with larger sample size, and novel formulation will give statistical significance.

## Conclusions

*C. asiatica* and ceramide can improve skin barrier hydration in order to prevent the risk of contact dermatitis in batik workers.

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Teodhora*, Munawarohthus Sholikha, Asniatul Ania and Ika Maruya Kusuma

# Secondary metabolite and antipyretic effects of Maja (*Crescentia cujete* L.) in fever-induced mice

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**Abstract**

**Objectives:** Fever is a condition when the body experiences an increase in average body temperature above normal level. Maja fruit (*Crescentia cujete* L.) contains chemical compounds including alkaloid, flavonoid, saponin, and terpenoid, suspected as potential antipyretics.

**Methods:** The study aimed to determine the antipyretic activity of ethanol extract of Maja fruit. A total of 25 male white mice of the DDY strain (20–30 g). These treatments divided into three groups with a dose extract of 125, 250, 500 mg/kg BW, standard groups of ibuprofen 400 mg/kg BW, and control groups of CMC-Na 1%. Mice were injected intraperitoneally with 0.1 cc of DPT vaccine-induced. Observations were made by measuring the rectal temperatures of mice using a digital thermometer before DPT vaccine injected or average temperatures, at 0 min (after DPT vaccine injected), 60, 120, 180, and 240 min after administering the test material. The differences between the positive control group, test group, and the negative control group were compared using statistical analysis using one-way variance analysis (ANOVA). The results were considered statistically when the value is ($p<0.05$).

**Results:** The above phytochemical screening results showed that alkaloids, flavonoids, and saponins were present in the Maja fruit powder and extract (*C. cujete* L.). Based on the results of the statistical analysis obtained, i.e., Group II was not significantly different from Group III and Group IV ($p\leq0.05$) and was significantly different from Group I and Group V. Group I was significantly different from Group II, Group III and Group IV and was not significantly different from Group V ($p\geq0.05$).

**Conclusions:** The study showed that Maja fruit mice's antipyretic behavior at doses of 125, 250, and 500 mg/kg BW was confirmed as a result in reducing the body temperature of male mice. The 500 mg/kg BW dosage of Maja fruit extract (*C. cujete* L) effectively reduced fever.

**Keywords:** antipyretics; *Crescentia cujete* (L.); *Diphtheria-pertussis-tetanus* vaccine; fever.

## Introduction

The globe is facing the Covid-19 pandemic, which significantly affects people's mobility to carry out activities. As one of the afflicted nations, Indonesia imposes large-scale social sanctions to break the chain of transmission. The transmission is caused by microorganisms, including viruses that invade the body, impair organ functions, and cause a risk of death if not treated properly. Medical effects that are developed due to microorganism infection can send signs, including fever, to the body. A stimulation of cells that are responsible for the immune system will increase the body temperature [1], namely the production of cytokinesis (IL-1, IL-6, and IFN-a). By growing the PGE-2 synthesis pathway, leading to a rise in body temperature, this activation stimulates the hypothalamus to improve cytokines. If the state of the body decreases, microorganisms will defeat the immune system within the body [2]. When body temperature increases above normal levels, people take paracetamol and ibuprofen as antipyretic drugs which function by inhibiting prostaglandin production. Drugs, however, have many side effects and might potentially damage tissues, such as tissues in the liver and kidneys, when taken for a long term [3, 4].

Several researchers in Indonesia are searching for medicines during the pandemic to maintain patients' endurance and avoid consequences of microorganisms within patients that have been infected or exposed to the virus. Many Indonesian researchers are devising a treatment therapy to strengthen the immune system to avoid the exposure to microorganisms. Starting from evolving medicines that have been circulating to looking for new drugs from different varieties of plants that are predicted to produce compounds used as drug action targets that can pharmacologically improve the immune system [5]. Many plants have pharmacological activities from various studies that had been conducted, including Maja (*Crescentia cujete* L), which

***Corresponding author: Teodhora**, Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia, E-mail: c.teodhora@istn.ac.id
**Munawarohthus Sholikha, Asniatul Ania and Ika Maruya Kusuma,** Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia

reportedly provides pharmacological activity in overcoming bronchitis, influenza, asthma, pain, diarrhea [6], anti inflammation [7], stimulating insulin production [8], lowering blood pressure [8], and increasing antioxidants [9] and antimicrobials [10]. There are fewer side effects from the use of alternative medicine [11].

Maja fruit has a lot of identified pharmacological activities. Fortunately, the efficacy of Maja fruit compounds as an antipyretic has not been documented. Therefore, if Maja's fruit ability to relieve fever is demonstrated, various previous studies on its pharmacological action linked to the immune system, such as antioxidants, analgesics, anti inflammatory, and antimicrobials, will be sponsored. This study aims to include knowledge about its pharmacological ability to dramatically improve the body's immune system by creating new medicines.

# Materials and methods

## Materials

Ethanol 70% ($C_2H_5OH$), Sodium Hydroxide (NaOH) 1 N (Merck), *Diphtheria, Pertussis, Tetanus* (DPT) Vaccine (Pentabio), Alumunum Chloride ($AlCl_3$) 1% (Merck), Hydrochloric Acid (HCl) 2 N (Merck), Mayer reagent, Dragendorf reagent, Ferrous (III) Chloride ($FeCl_3$) 1% (Merck), Sodium Carboxymethylcellulose (CMC-Na) 1% (Merck), Acetate Anhydrase ($C_4H_6O_3$) (Merck), Sulfuric Acid ($H_2SO_4$) (Merck).

## Preparation, extraction of plant materials

The Maja fruit was collected from the South Jakarta National Institute of Science and Technology, Jakarta. The determination of the desired species of *C. cujete* L. was confirmed by the Indonesian Institute of Science, Bogor, Biology Research Center, West Java. The confirmed desired species are *Crescentia cute* L. and the Bignoniaceae tribe (Certificate Number of 2108/IPH.1.01/1f.07/XI/2019). Five kilograms of the fruit was washed under running water. Then, the clean fruit was peeled and separated from the peel, sliced thinly, and dried at 60 °C for 48 h Six hundred and fifty grams of fine Maja fruit powder was filtered with a mesh with a size of 40, resulting in 2 kg of dried fruit powder, which was then pollinated. The dried Maja fruit powder was macerated in ethanol 70% at a room temperature for 3 days and shielded from the sunlight. The extract was filtered and concentrated under vacuum using a rotary evaporator until the ethanol in the extract evaporated.

## Preliminary phytochemical test

The preliminary phytochemical screening went through the identification of alkaloid, flavonoid, saponin, and tannin using the extract. The screening aimed to establish pharmacognosy standards.

**Alkaloids**, 1 g of Maja fruit powder and extract were moistened in a beaker glass with 5 mL of 25% ammonia ($NH_3$). Twenty millilitres of chloroform were applied until the substance immersed, then stirred, heated, and filtered over a water bath. The filtrate was then halfway evaporated. The residue was poured into a test tube and added with 1 mL of 2 N hydrochloric acids (HCl), then shaken and left to form two layers; the formed transparent layer was taken and equally placed in three test tubes. The Mayer reagent was attached to the first tube, the second tube with the Bouchard reagent, and the third tube with the Dragendorff reagent. The deposition of white deposits in the Mayer reagent, brown deposits in the Bouchard at reagent, and red sediments in the Dragenddorff reaction suggest alkaloids. Alkaloid screening can be declared as positive if at least two experiments with the reagents form deposits [12].

**Tannin**, 1 g of Maja fruit powder and extract were separated and purified in 100 mL of hot water. Five millilitres of filtrate was inserted into a test tube and a few drops of 1% ferric chloride ($FeCl_3$) solution were applied. If a green–purple or black color is produced, the test substance involves tannins [12].

**Flavonoids**, 1 g of Maja fruit powder and extract were filtered in 100 mL of hot water. In the test tube, 5 mL of filtrate was applied, then 1 mL of 5% sodium nitrite solution ($NaNO_3$) and 1 mL of 10% ammonium chloride solution ($AlCl_3$) were added and shaken, and 2 mL of 1 N hydroxide solution was applied. If the test substance contains flavonoids, the color will turn red or orange [12].

**Saponin**, 1 g of Maja fruit powder and extract were extracted and purified in 100 mL of hot water. In the test tube, 10 mL of filtrate was mounted and shaken vertically for 10 s. The production of steady foam with the height of 1–10 cm suggests saponin. If the test substance includes saponin with the addition of one drop of 2 N HCl, a stable ±1 cm foam will be formed [12].

Male DDY mice weighing 20–30 g, between 2 and 3 months, were collected from the IPB University Faculty of Animal Science, Indonesia. The UPNVJ Health Research Ethics Committee (Letter Number B/2378/1/2020/KEPK) has accepted all experimental protocols for pharmacological trials to support researchers in the preservation of research ethics and attempts to protect human rights and the welfare of animals as research objects. The mice were previously acclimatized for one week, which helped the animals adapt with the laboratory environment. Before checking the antipyretic activity of the ethanol extract of the Maja fruit, an experimental test was done in advance.

This experimental test helped decide what needs to be done in the final test to achieve more reliable and precise data. The preliminary test included the administration of a dosage of DPT and a dosage of Maja fruit extract. In this test, there were four groups consisted of two mice for each group; a wireless thermometer was used to measure the original rectal temperature before the DPT vaccine was triggered. The DPT vaccine, a fever inducer, was administered in the intraperitoneal (IP) and intramuscular (IM) routes with separate volumes of 0.1 and 0.2 cc to each mouse in each group; the temperature was again determined after 30 min of the induction of the DPT vaccine. A fever was identified by the initial increase of body temperature. The second preliminary test was carried out to evaluate the initial dosage of the test substance to be used on the mice for the antipyretic behavior test. The initial dosage of 250 and 500 mg/kg BW of Maja fruit extract (*C. cujete* L.) were used along with the oral administration route in this preliminary research. BW, 300, and 600 mg/kg BW had an antipyretic effect with the highest antipyretic effect obtained at a dosage of 600 mg/kg BW [13] and Maja fruit (*Aegle marmelos* L. Corr.) anti inflammatory test with doses of 100, 200, and 400 mg/kg BW [14]. The initial dose of 250 and 500 mg/kg BW, based on the preliminary

examination, indicated that antipyretic activity occurred in the form of a decrease in the mice's body temperature following a fever with the DPT vaccine induction. The additional dose of 125 mg/kg BW is the lowest dose among the original doses intended to perform antipyretic action.

The mice consisted of randomly chosen healthy five male mice. Fever using DPT vaccine 0.1 cc was induced intraperitoneally for 1 h to achieve a fever.

### Evaluation of antipyretic potential

Based on the preliminary test, the amount and route of administering the DPT vaccine used in the antipyretic activity test were 0.1 cc (IP) as a fever induction dose, and 250 and 500 mg/kg BW as the initial dose of Maja fruit extract (*C. cujete* L.). The five research groups in this test are as follows:

| No. | Groups | Treatment |
|---|---|---|
| 1 | I | 0.5% CMC-Na (Control Groups), orally |
| 2 | II | Ibuprofen 400 mg/kg BW (Standard Drug), Orally |
| 3 | III | Extract-125 mg/kg BW suspended in 0.5% CMC-Na, Orally |
| 4 | IV | Extract-250 mg/kg BW suspended in 0.5% CMC-Na, Orally |
| 5 | V | Extract-500 mg/kg BW suspended in 0.5% CMC-Na, Orally |

The rectal temperature was measured by a digital thermometer for 240 min for every 60 min after the mice was treated. Both mice with various temperature changes, varying from 1.3 to 1.8 °C, were infected by the DPT vaccine fever.

### Data analysis

The data obtained from this research is quantitative. A decline in fever temperature is a quantitative data. The results were then examined statistically. The differences between the positive control group, test group, and the negative control group were compared using statistical analysis using ANOVA if the data were normally distributed and homogeneous, followed by a Tukey's test. The results were considered statistically when the value is ($p<0.05$).

## Results

This study's test material was the volleyball-shaped fruit of Maja (*C. cujete* L.) with a diameter of 25 cm; with smooth and shiny green skin that was hard and woody, and soft white and fragrant flesh. The dried Maja fruit was collected and sorted to be separated from impurities that were left in the dried fruit and unwanted plant components. The above phytochemical screening results showed that alkaloids, flavonoids, and saponins were present in the Maja fruit powder and extract (*C. cujete* L.). This shows that the Maja fruit extract (*C. cujete* L.) contains secondary metabolites thought to have pharmacological effects as antipyretics. As shown in Table 1.

The IP route had a higher absorption rate than IM as the route of administration directly penetrated the abdomen and had a large absorption surface. The medication could quickly enter the systemic bloodstream, providing a faster response. The DPT vaccine, which functioned as a pyrogen, induced the release of endogenous pyrogens (IL-1 and TNF-α) released by polymorphonuclear cells by increasing the temperature in mice. These pyrogens released arachidonic acid in the anterior hypothalamus's chemoreceptive region, which activated prostaglandin production and affected the increase of body temperature/fever [15]. Compared with positive and negative control therapies, the mice treated with oral administration of Maja fruit extract (*C. cujete* L.) showed numerous temperature changes as shown in Table 2.

Based on the effects of the average rectal temperature calculation data on mice, the average rectal temperature decrease of mice was determined to assess the potential of treatments I, III, IV, and V to decrease the mice's rectal temperature. The average decrease in temperature indicates the test substance's antipyretic role in reducing the body temperature of mice, obtained by measuring the temperature 60 min after the induction of the DPT vaccine minus the temperature at a certain point after treatment. Table 2 shows the effects of the average decrease in the mice's rectal temperature. The findings of the average decrease in mice's rectal temperature, based on Table 2, demonstrate a difference in the temperature drop in 240 min per 60 min of each group. The drop in temperature

**Table 1:** Result for phytochemical constituents of Maja fruit.

| Constituents | Theory [12] | Test | Result | |
|---|---|---|---|---|
| | | | Extract | Powder |
| Alkaloid | Mayer: Formed white deposits | White deposits | (+) | (+) |
| | Bouchardat: formed brown deposits | Brown deposits | (+) | (+) |
| | Dragendorff: formed red brick deposits | Redbrick deposits | (+) | (+) |
| Flavonoid | Reddish orange | Reddish orange color | (+) | (+) |
| Saponin | Formed froth ±1 cm stable | Stable ±1 cm froth | (+) | (+) |
| Tannin | Formed greenish black color | Greenish black color | (+) | (+) |

**Table 2:** Effect of plant extracts on Maja fruit induced pyrexia in mice male.

| Groups | Treatment-dose, mg/kg p.o | Temperature at 1 h after induction | Rectal temperature (°C) at different hours after the treatment with the extract | | | | | p-Value Tukey's test |
|---|---|---|---|---|---|---|---|---|
| | | | +1 h | +2 h | +3 h | +4 h | Average | |
| I | Control-Ibuprofen 400 | 38.54 ± 1.40 | 37.42 ± 1.12 | 37.04 ± 0.38 | 36.54 ± 0.5 | 36.84 ± 0.30 | 36.96 ± 0.42* | 0.424* |
| II | Standard-Na CMC 1% | 38.76 ± 2.02 | 38.64 ± 0.12 | 38.54 ± 0.10 | 38.32 ± 0.22 | 38.60 ± 0.28 | 38.52 ± 0.04 | 0.038 |
| III | Extract-125 | 38.26 ± 1.42 | 37.50 ± 0.76 | 37.92 ± 0.42 | 38.08 ± 0.16 | 38.00 ± 0.08 | 37.87 ± 0.06 | 0.062 |
| IV | Extract-250 | 38.50 ± 1.36 | 38.02 ± 0.48 | 37.08 ± 0.94 | 38.08 ± 1 | 38.02 ± 0.06 | 37.80 ± 0.12 | 0.118 |
| V | Extract-500 | 39.06 ± 1.86 | 38.30 ± 0.76 | 37.78 ± 0.52 | 37.54 ± 0.24 | 36.90 ± 0.64 | 37.63 ± 0.54* | 0.538* |

*Significantly different when compared to Group II (Standard); III (Extract 125 mg/kg); IV (Extract 250 mg/kg). Statistically significant $p<0.05$ analyzed by *one-way* ANOVA followed by the Tukey test.

after the treatment was not the same for each mouse, even in the same treatment category. The antipyretic activity of the Maja (*C. cujete* L.) fruit extract has increased in quantity depending on the dosage. There was a greater antipyretic effect in Group V compared to Group I, II, III, and IV. However, antipyretic behavior was not substantially different from the statistical findings obtained from the control groups, I groups, and V groups.

Based on the results of the statistical analysis obtained, i.e., Group II was not significantly different from Group III and Group IV ($p \leq 0.05$) and was significantly different from Group I and Group V. Group I was significantly different from Group II, Group III, and Group IV and was not significantly different from Group V ($p \geq 0.05$). It could also be inferred that the results on the decrease of fever temperature in the mice revealed that there was a substantial difference between Group II, Group III, and Group IV on the reduce of fever temperature; on the other hand, Group I and Group V did not have a significant difference in the reduce of fever.

The next step was to observe the medication's average onset of action and the average duration of drug action obtained from the beginning of antipyretic therapy and the amount of time needed by the treatment to give therapeutic activity, as shown in Table 3.

It can be seen in Table 3 that Group III, IV, and V have the same average onset of action at minute 60. Duration of drug action was different; Group V had an overall duration of drug action that varied. Compared to Group III with a duration of drug action of 120 min and treatment Group IV with a duration of drug action of 180 min shorter than treatment Group I and V. This suggests that Group V had the best dose compared to Group III and IV as the median initiation and duration of action of the medicinal product were close to those of Group V.

**Table 3:** Onset and duration of action extract of Maja fruit induced pyrexia.

| Groups | Treatment-dose, mg/kg p.o | Onset, min | Durasi, min |
|---|---|---|---|
| I | Control-Ibuprofen 400 | 60′ | 240′ |
| II | Extract-125 | 60′ | 120′ |
| III | Extract-250 | 60′ | 180′ |
| IV | Extract-500 | 60′ | 240′ |

## Discussion

Several active compounds were believed to be responsible for the antipyretic activity caused by the Maja fruit ethanol extract (*C. cujete* L.), namely flavonoids, tannins, and saponins. Based on the antipyretic test findings, Maja fruit extract (*C. cujete* L.) produced antipyretic activity in DPT vaccine-induced mice. It also reported that Alkaloid [10, 16–19] saponin [16, 17, 20], tanin [16–18, 21], flavonoid [16–18, 21], steroid [18, 21], antrakuinon [8, 16], fenol [10, 16], cardenolides [16, 20], fitosterol [17], glikosida resin [18], apigenin, and nephtoquinon [21].

The results of this study were also supported by previous research, which proved that plants containing flavonoids (a compound of the phenolic group) could inhibit cyclooxygenase. The flavonoid mechanism functioned by inhibiting eicosanoids, which could induce cyclooxygenase pathway blockade to disrupt changes in endoperoxide arachidonic acid, contributing to the production of prostaglandin E2 in peripheral tissues that could not directly with the brain, resulting in a reduction in the set point in the hypothalamus [22]. Tannins may have antipyretic properties by inhibiting arachidonic acid in prostaglandin biosynthesis [23].

Antipyretic activity existed due to secondary alkaloid metabolites, which suppressed the COX of the enzyme [24], thereby disrupting the synthesis of prostaglandin. Alkaloids of the matrine form were thought to suppress dopamine release or block dopaminergic receptors to

interact with prostaglandin synthesis [25], revealed that the boldine alkaloid mechanism indicated that alkaloid was an important prostaglandin biosynthesis inhibitor. The study revealed that punarnavine, an alkaloid isolated from B *Diffusa*, decreased the rise in mouse levels of lipopolysaccharide-induced pro inflammatory cytokines such as TNF-, IL-1, and IL-6. Prostaglandin E2, the primary mediator of fever, was enhanced by releasing inflammatory mediators such as IL-1 and IL-6 in the body [26]. The mechanism of lowering the rectal temperature in fever-induced laboratory mice, the quality of flavonoids, hormones, tannins, and saponins was thought to function synergistically [27].

The use of vaccine-induced volumes of 0.1 and 0.2 cc DPT increased mice's body temperature within the 0.7–1.6 °C range [13, 14]. With the mice's temperature, the DPT pyrogen-induced vaccine could facilitate endogenous pyrogen production (IL-1 and TNF alpha) produced by polymorphonuclear cells. These pyrogens functioned to release arachidonic acid in the anterior hypothalamus's chemoreceptive region, inducing prostaglandin synthesis, which influenced the increase in body temperature [15]. Based on previous antipyretic, the amount and route of administration of 0.1 and 0.2 cc DPT vaccines were used. The use of DPT vaccine volumes of 0.1 and 0.2 cc allowed the increase in mice's body temperature within the range of 0.7–1.6 °C [28]. The DPT vaccine volume was 0.1 and 0.2 cc i.p, depending on these test results, with a temperature rise of 1.2–1.7 °C [27].

The drop in rectal temperature in each mouse after the treatment was not the same, even in one treatment category. Variables such as hormones, climate, and gastric condition were responsible for this decrease. Psychological causes, such as tension endured due to repetitive measurements in the rectum of mice, might have also induced it [29]. The dose-antipyretic activity of Maja fruit extract (*C. cujete* L.) improved as the dose increased. Group V showed more substantial antipyretic effects than Group I, II, III, and IV. However, there was no substantial difference in statistical findings between treatment Group I and V that provided antipyretic activity.

The isolation of alkaloid from *B. Diffusa* decreased lipopolysaccharide-induced elevated levels of TNF-1, IL-1, and IL-6 pro inflammatory cytokines in mice [26]. The release of inflammatory mediators such as IL-1 and IL-6 within the body facilitated the prostaglandin E2 synthesis, the primary fever mediator [26]. Alkaloids such as bolidine could minimize rising temperatures by inhibiting the synthesis of prostaglandin E2 [30]. Cyclooxygenase and 5-lipoxygenase inhibition pathways and eicosanoid biosynthesis inhibition along with their ability to block neutrophil degradation [25]. The inhibition of arachidonic acid peroxidation was also seen by flavonoids and their corresponding compounds, resulting in a decrease in prostaglandin levels, thereby minimizing fever and pain [31]. Saponin was involved in the mechanism of the opioid receptor [32] using antagonistic activity [33] by binding the sensory nerve terminals. Flavonoids, steroids, tannins, and saponin were the most important bioactive compounds of plants and were suspected to function synergistically in the process of rectal temperature reduction in fever-induced laboratory mice [34].

These compounds had a wide variety of pharmacological activities, such as anthelmintic [35], antidepressant and anti anxiety [36], anti inflammatory [7, 37], antibacterial [10, 38], antifertility [39], anti arthritis [40], and anti-diarrheal [6, 41]. The 500 mg/kg BW dosage of Maja fruit extract (*C. cujete* L) reduced fever. These results can be added to the study on Maja fruit's pharmacological activity and continued to be formulated as a novel drug, particularly as an antipyretic via the pharmacological mechanisms of the secondary metabolites present in Maja. However, for further research, the active components of the Maja fruit shall remain through the compound isolation stage to improve research results.

## Conclusions

The study showed that Maja fruit mice's antipyretic behavior at doses of 125, 250, and 500 mg/kg BW was confirmed as a result in reducing the body temperature of male mice. The 500 mg/kg BW dosage of Maja fruit extract (*C. cujete* L) effectively reduced fever. However, for further research, the active components of the Maja fruit shall remain through the compound isolation stage to improve research results.

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**Informed consent:** Not applicable.

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Yulistiani*, Claudia Tiffany, I. Dewa Gede Ugrasena and Mariyatul Qibtiyah

# Hydration effect on kidney function and serum electrolyte in children with tumor lysis syndrome (TLS) and risk of TLS

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**Abstract**

**Objectives:** Tumor lysis syndrome (TLS) is a life-threatening oncology emergency disorder, which may cause acute kidney injury (AKI), arrhythmias, seizures, and sudden death. Hydration is used to prevent TLS in medium-high risk patients, and treatment in TLS patients. According to the pediatric protocol in Dr. Soetomo District General and Teaching Hospital, close monitoring is required to prevent the progression of hematological malignancy towards TLS. The study aimed to analyze the hydration effect on potassium, calcium, and phosphate levels; serum creatinine (sCr); and blood urea nitrogen (BUN) level.

**Methods:** This was an observational and prospective study conducted at Dr. Soetomo District General and Teaching Hospital for four months on 15 pediatric hemato–oncology patients who got TLS and in risk of TLS. Laboratory parameters were observed in 11 days, pre and post hydration.

**Results:** Among the 15 patients who met the inclusion criteria, there were eight TLS patients and seven TLS risk patients. After hydration administration 67% of TLS patients achieved normal potassium level, 75% achieved normal phosphate level, 0% achieved normal calcium level, and 50% achieved normal sCr and BUN levels. Meanwhile, TLS risk patients reached normal level in all parameters. This difference in performance is caused by disease progression.

**Conclusions:** Hydration can maintain serum electrolytes and renal function in a normal range, preventing TLS in TLS risk patients. In TLS patients, hydration only tends slow the progression of the disease.

**Keywords:** blood urea nitrogen; creatinine serum; hyperhydration; serum electrolytes; tumor lysis syndrome.

## Introduction

Tumor lysis syndrome (TLS) is one of many complications that could occur on hematological malignancy. According to the Cairo–Bishop, TLS is classified into laboratory TLS (LTLS) and clinical TLS (CTLS). LTLS is defined as an increase in uric acid, potassium, and phosphate levels as much as 25% or the degradation of serum calcium level for more than 25% from the normal value, whereas clinical TLS could occur when clinical manifestations exist such as arrhythmia, seizure, and acute kidney injury (AKI) [1–4]. These clinical manifestations are the cause of the oncological emergency condition and has to be dealt with swiftly to reduce mortality, inpatient care duration, and costs that must be incurred [5, 6].

Increasing excess electrolyte and uric acid elimination when TLS occurs are important step to be done. Hydration or hyperhydration therapy plays an important role and is the main choice for TLS and risk of TLS patients [7–9]. Intravenous fluid used in hydration is: D5 with 0.225% normal saline (D5 ¼ NS), D5 with 0.45% normal saline (D5 ½ NS), and NaCl 0.9% with the volume of ±2,000–3,000 mL/m$^2$/day [5, 7, 9–11]. The hydration administration is started immediately after TLS risks are suspected to exist [7, 12].

Several studies have researched the effectivity of hydration towards kidney function and electrolyte serum on pediatric patients with TLS and with risks of TLS. The study of Burns et al. reported that the administration of intravenously on 2–3 L/m$^2$/day with or without furosemide added with rasburicase have shown the tendency of creatinine serum (Scr) decreased after 3 days of hydration therapy and the occurrence of uric acid degradation [13]. The study done by Mirrakhimov et al. [5] stated that the selection of intravenous fluid that could be used for hyperhydration is varied.

***Corresponding author: Yulistiani**, Department of Clinical Pharmacy, Airlangga University Hospital, Surabaya, Indonesia,
Phone: +628123013880, E-mail: yulist_r@yahoo.co.id
**Claudia Tiffany,** Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**I. Dewa Gede Ugrasena,** Faculty of Medicine, Department of Medical Pediatric, Airlangga University and Dr. Soetomo Hospital, Surabaya, Indonesia
**Mariyatul Qibtiyah,** Department of Pharmacy, Dr. Soetomo Hospital, Surabaya, Indonesia

However, several studies recommended the administration of D5 ¼ NS as the first and primary choice where NaCl 0.9% could also be administered if dehydration, hypovolemic, and hyponatremia conditions are found.

An overview of hydration effectivity on TLS patients and patients with TLS risks can be seen from the previous description. In Indonesia, the study(s) regarding hydration therapy on pediatric patients with hematological malignancy who suffered TLS are still limited, resulting in the unknown effectivity of hyperhydration administration towards kidney function (BUN, sCr) as well as electrolyte serum (potassium, phosphate, and calcium). This study aims to analyze the effect of hydration administration as prophylaxis and therapy of tumor TLS on children with hematological malignancy (ALL, AML, CML, NHL, and Hodgkin's lymphoma) based on serum electrolyte (potassium, phosphate, and calcium), and kidney function (BUN, sCr).

## Materials and methods

This was a prospective observational study and was declared ethical worthy by the health research ethics committee of Dr. Soetomo District General Hospital, Surabaya. The samples in this study were pediatric inpatients with hematological malignancies and experiencing TLS or at risk of TLS during the period of March–July 2020. The inclusion criteria including: (1) pediatric patients aged ≤16 years, (2) inpatients with a diagnosis of acute (ALL, AML) and chronic (CML) leukemia, and lymphoma (NHL, Hodgkin's lymphoma), (3) were at risk of experiencing TLS or having experienced TLS before or after chemotherapy, (4) getting hydration therapy along with other supporting therapies such as sodium bicarbonate, allopurinol, and furosemide, and (5) the patient's parents accept and sign an informed consent. These study's exclusion criteria were patients with electrolyte balance disorders, kidney disorders due to other causes, and patients with a history of chronic renal failure.

The research procedure includes: (1) research registration in the health research ethics committee of Dr. Soetomo District General Hospital, (2) patients recruitment by seeing the medical records and adjust to the inclusion criteria, (3) giving the information for consent and informed consent to the patient's family, and (4) blood draw before hydration therapy (baseline) and post hydration therapy, followed the patients schedule. Hydration was given to TLS and TLS risk patients following protocols at Dr. Soetomo District General Hospital: D5 with 0.225% normal saline (D5 ¼ NS), D5 with 0.45% normal saline (D5 ½ NS), or NaCl 0.9% with the volume of ±2,000–3,000 mL/m$^2$/day. Hydration given since the first day patients admitted to the hospital until the day before patients discharge from the hospital.

The laboratory parameters observed were BUN, sCr, and serum electrolytes such as potassium, phosphate, and calcium. These parameters were observed in 11 days, which is average length of stay for TLS and TLS risk patients in hospital. The observation period in this study may be varied with other studies, because TLS patients already in a terminal condition. But from other studies, it is known that there have been significant changes in serum electrolyte, BUN, and sCr after three days of hydration. Naem et al. [14] reported significant differences in Scr and phosphate on day three and potassium on day four after hydration. Burns et al. [13] reported the metabolic abnormalities in TLS patients started to reduce after three days hydration. Data analysis was performed by descriptively comparing the research data with the normal value of each parameter and by paired *t*-test (if the data were normally distributed) or Wilcoxon (if not normally distributed).

## Results

TLS occurred in eight of 15 pediatric patients with hematologic malignancy. Based on the mean ± SD leukocyte count of 15 patients (169,790.67 ± 259,604.02/mm$^3$), it was shown that patients tended to experience hyperleukocytosis (Table 1). Baseline serum electrolytes and renal function indicated pediatric patients with TLS tend to have decreased renal function, as indicated by an increase in mean Scr (1.35 ± 0.68 mg/dL), mean BUN (23.5 ± 16.82 mg/dL), and decreased mean creatinine clearance (77.95 ± 59.32 mL/min/1.73 m$^2$) (Table 2). The hydration fluid used in patients with TLS and risk of TLS in various types of hematological malignancies has the same type and volume. The fluids that can be used as a hydration in TLS and TLS risk patients are D5 with 0.225% normal saline (D5 ¼ NS), D5 with 0.45% normal saline (D5 ½ NS), or NaCl 0.9% [4]. These fluids are commonly used in pediatrics for maintenance or resuscitation [12]. Other fluids, such as ringer ascetate (R), electrolyte infusion, ringer lactate, were used in special conditions such as dehydration due to diarrhea (Table 3).

### Hydration effect on patient's potassium, calcium, and phosphate levels

The mean potassium, calcium, and phosphate levels were observed from day 0 (baseline) until day 11. At the end of this observation, the levels of potassium, calcium, and phosphate of TLS risk patients could return to normal range, on the other hand, TLS patients still fluctuate.

There was a tendency for hypokalemia in TLS patients and hyperkalemia in risk of TLS patients but not >5.9 mmol/l (LTLS criteria) (Figure 1). Mean ± SD calcium levels in TLS patients tend to be lower than patients with TLS risk (Figure 2) and mean ± SD phosphate levels in TLS patients tend to be higher than patients with TLS risk (Figure 3). High phosphate levels in TLS patients were associated with lysis of the tumor cells. Phosphate could bind calcium (ion) in the

**Table 1:** TLS and TLS risk patients characteristics (n=15).

| Variable | TLS patients (n=8) | | TLS risk patients (n=7) | | Total (n=15) | |
|---|---|---|---|---|---|---|
| | n, % | Mean ± SD/range | Frequency, % | Mean ± SD/Range | Frequency, % | Mean ± SD/Range |
| **Gender** | | | | | | |
| Male | 4 (50%) | | 6 (86%) | | 10 (67%) | |
| Female | 4 (50%) | | 1 (14%) | | 5 (33%) | |
| **Age, year** | | 11.6 ± 2.06 (9–15) | | 5.2 ± 3.87 (0.5–12) | | 8.6 ± 4.42 (0.5–15) |
| 0–4 | | | 2 (28%) | | 2 (14%) | |
| 5–11 | 4 (50%) | | 4 (57%) | | 8 (53%) | |
| 12–15 | 4 (50%) | | 1 (15%) | | 5 (33%) | |
| **Weight, kg** | | 38.1 ± 8.12 (30–51) | | 19.6 ± 18.57 (4.2–60) | | 29.4 ± 16.49 (4.2–60) |
| **Height, cm** | | 140.5 ± 11.15 (118–152) | | 104.4 ± 30.89 (62–155) | | 123.7 ± 28.60 (62–155) |
| **Body surface area, /m$^2$** | | 1.21 ± 0.16 (1–1.4) | | 0.73 ± 0.43 (0.26–1.6) | | 0.99 ± 0.39 (0.26–1.6) |
| **White blood cell (WBC), /mm$^3$** | | | | | | 169,790.67 ± 259,604.02 (10,730–847,180) |
| <50 × 10$^3$ | 5 (62%) | | | | 5 (33%) | |
| 50–100 × 10$^3$ | | | 2 (29%) | | 2 (14%) | |
| >100 × 10$^3$ | 3 (38%) | | 5 (71%) | | 8 (53%) | |
| **Diagnosis** | | | | | | |
| ALL | 6 (75%) | | 5 (71%) | | 11 (73%) | |
| AML | | | 2 (29%) | | 1 (7%) | |
| NHL | 1 (12.5%) | | | | 1 (7%) | |
| CML | 1 (12.5%) | | | | 2 (13%) | |

**Table 2:** Baseline serum electrolytes and kidney functions of TLS and TLS risk patients (n=15).

| Parameter | TLS patients (n=8) | | TLS risk patients (n=7) | |
|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range |
| Serum creatinine, mg/dL | 1.35 ± 0.68 | 0.5–2.1 | 0.44 ± 0.18 | 0.2–0.7 |
| eGFR[a] (mL/min/1.73 m$^2$) | 77.95 ± 59.32 | 36.93–165.2 | 142.17 ± 12.64 | 122.1–155.0 |
| BUN, mg/dL | 23.5 ± 16.82 | 12.0–48.0 | 13.0 ± 7.30 | 5.0–23.0 |
| Gallium, mmol/L | 4.1 ± 1.30 | 2.4–5.4 | 4.6 ± 0.90 | 3.5–5.6 |
| Calcium, mg/dL | 8.5 ± 1.34 | 6.8–10.0 | 9.2 ± 0.84 | 8.4–10.3 |
| Phosphate, mg/dL | 4.5 ± 2.05 | 2.1–6.4 | 3.5 ± 0.9 | 2.6–4.6 |

[a]eGFR, estimated glomerular filtration rate.

**Table 3:** The hydration fluid use in TLS and TLS risk patients.

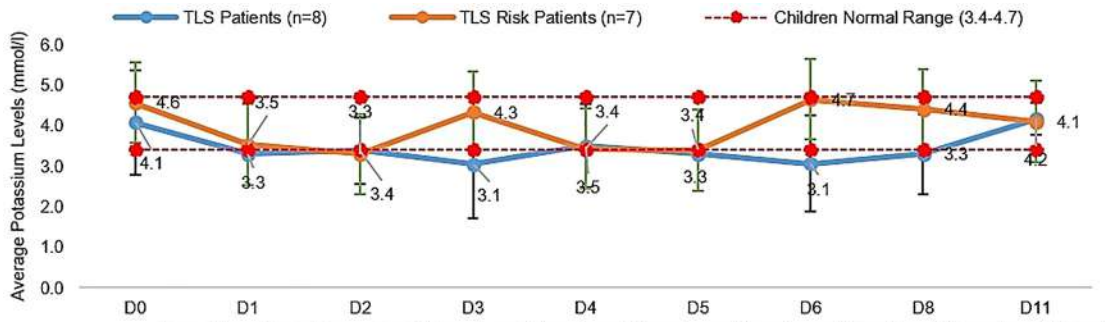
| Hydration fluid | TLS patients (n=8)[a] | TLS risk patients (n=7)[b] |
|---|---|---|
| D5 ½ NS | 8 | 5 |
| D5 ¼ NS | | 2 |
| NaCl 0.9% | 1 | |
| **Others** | | |
| RL | 3 | |
| Asering, R | 1 | |
| Electrolyte infusion | 2 | |

[a,b]One patient can use more than one hydration fluid, depends on the clinical condition.

blood to form calcium phosphate. This reaction caused a decrease in calcium levels of TLS patients [5].

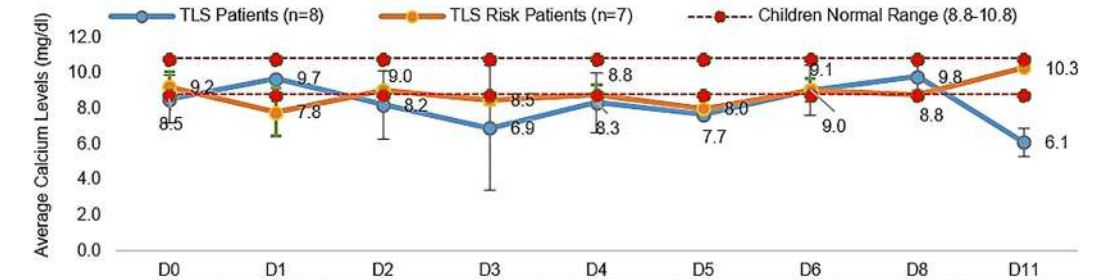
## Hydration effect on patient's BUN and Scr profiles

In Figure 4, it can be seen that the Scr of TLS patients is much higher and tends to be ≥1.5× the upper limit normal, and BUN looks fluctuating and tends to be above the normal limit of children (normal limit of children ≤18 mg/dL)




| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D8 | D11 |
|---|---|---|---|---|---|---|---|---|---|
| Average K+ of TLS Patients | 4.1±1.3 | 3.3±0.00 | 3.4±0.8 | 3.1±1.3 | 3.5±1.0 | 3.3±0.00 | 3.1±1.2 | 3.8±1.1 | 4.8±1.1 |
| Average K+ of TLS Risk Patients | 4.6±0.9 | 3.5±1.3 | 3.3±0.00 | 4.3±1.1 | 3.4±0.5 | 3.4±0.00 | 4.7±0.9 | 4.4±0.00 | 4.1±0.00 |
| Range K+ of TLS Patients | 2.4-5.4 | 3.3-3.3 | 2.1-4.3 | 2.1-4 | 2.2-5.2 | 3.3-3.3 | 1.9-4.3 | 2.6-4.7 | 3.8-6.0 |
| Range K+ of TLS Risk Patients | 3.5-5.6 | 2.7-5.0 | 3.3-3.3 | 3.4-5.6 | 2.9-3.7 | 3.4-3.4 | 3.8-5.9 | 4.4-4.4 | 4.1-4.1 |
| % Normal K+ of TLS Patients | 60% | 100% | 100% | 100% | 83% | 100% | 100% | 100% | 67% |
| % Normal K+ of TLS Risk Patients | 67% | 100% | 100% | 67% | 100% | 100% | 75% | 100% | 100% |

**Figure 1:** Potassium levels profile in TLS and TLS risk patients (n=15).




| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D8 | D11 |
|---|---|---|---|---|---|---|---|---|---|
| Average Ca2+ of TLS Patients | 8.5±1.34 | 9.7±0.00 | 8.2±1.91 | 6.9±3.54 | 8.3±1.68 | 7.7±0.00 | 9.0±1.41 | 9.8±0.75 | 6.1±0.80 |
| Average Ca2+of TLS Risk Patients | 9.2±0.84 | 7.8±1.32 | 9.0±0.00 | 8.5±0.26 | 8.8±0.53 | 8.0±0.00 | 9.1±0.62 | 8.8±0.00 | 10.3±0.00 |
| Range Ca2+ of TLS Patients | 6.8-10 | 9.7-9.7 | 4.9-10 | 4.4-9.4 | 5.5-10.3 | 7.7-7.7 | 7.9-10.6 | 9.3-10.3 | 5.4-6.9 |
| Range Ca2+ of TLS Risk Patients | 8.4-10.3 | 6.8-9.3 | 9.0-9.0 | 8.3-8.8 | 8.3-9.5 | 8.0-8.0 | 8.3-9.8 | 8.8-8.8 | 10.3-10.3 |
| % Normal Ca2+ of TLS Patients | 40% | 100% | 40% | 50% | 67% | 0% | 33% | 100% | 0% |
| % Normal Ca2+ of TLS Risk Patients | 60% | 67% | 100% | 67% | 50% | 0% | 75% | 100% | 100% |

**Figure 2:** Calcium levels profile in TLS and TLS risk patients (n=15).

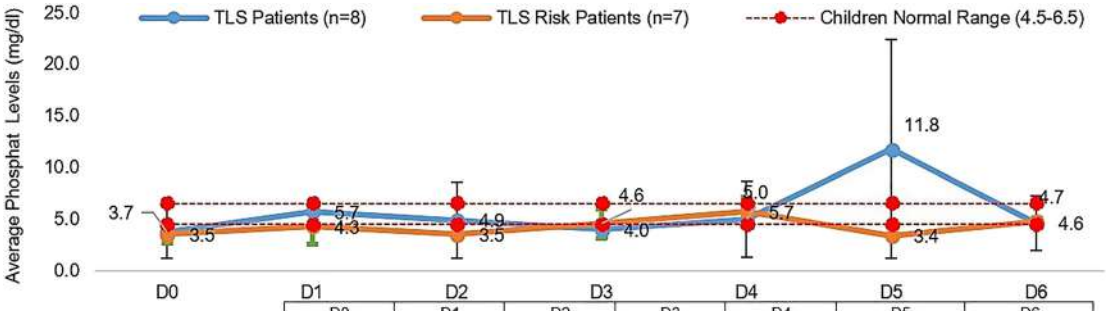
(Figure 5) which indicates that TLS patients have AKI, whereas patients at risk of TLS have sCr and BUN within normal limits.

## Comparison of mean serum electrolytes and kidney function in TLS and risk of TLS patients

In TLS patients (Table 4), there were no significant differences in all parameters ($p>0.05$). Day 1, 3, and 5 were not included in paired *t*-test because the amount of data is too small (1 to 2 data); whereas in TLS risk patient (Table 5), there was a significant difference in several parameters, starting from day 3 after hydration.

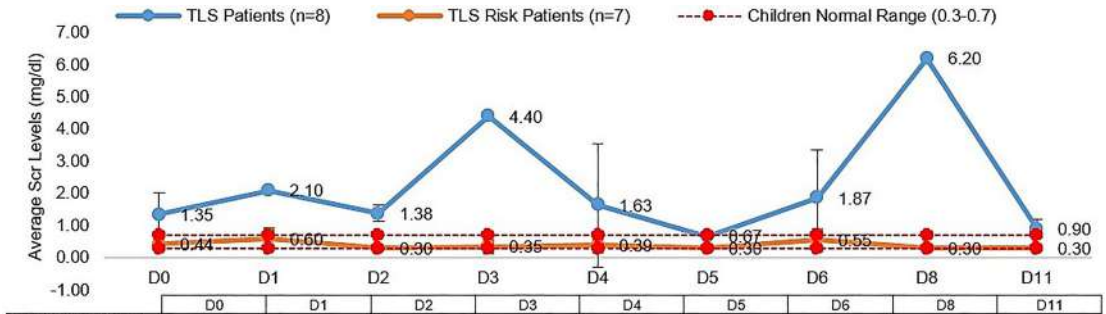
# Discussion

In this study, the number TLS risk patients experienced hyperkalemia was greater than TLS patients, mostly in high-risk TLS with WBC more than 200,000/mm$^3$. This is because hyperleukocytosis leading to an increased blast cell number and lysis [15, 16]. High potassium levels in TLS risk patients returned and remained within the overall normal range; whereas, in TLS patients, potassium levels cannot remain in



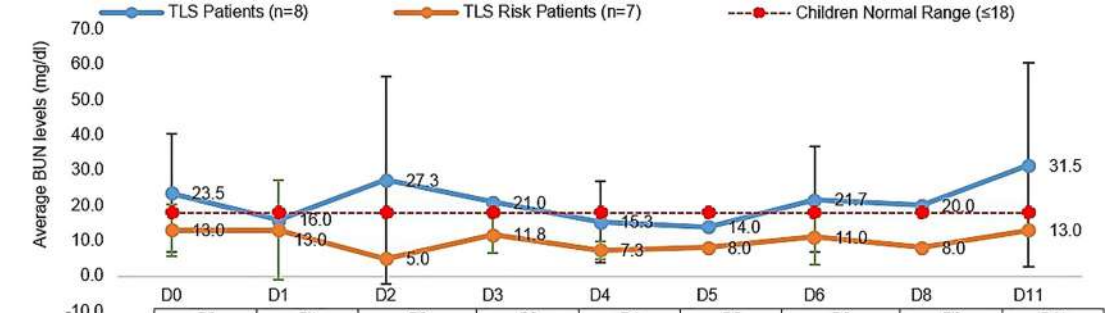

| | D0 | D1 | D2 | D3 | D4 | D5 | D6 |
|---|---|---|---|---|---|---|---|
| Average $PO_4^{3-}$ of TLS Patients | 3.7±2.55 | 5.7±0.00 | 4.9±3.73 | 4.0±0.85 | 5.0±3.71 | 11.8±10.60 | 4.6±2.69 |
| Average $PO_4^{3-}$ of TLS Risk Patients | 3.5±0.90 | 4.3±1.74 | 3.5±0.00 | 4.6±1.42 | 5.7±1.45 | 3.4±0.00 | 4.7±0.40 |
| Range $PO_4^{3-}$ of TLS Patients | 0.4-6.4 | 5.7-5.7 | 2.4-11.3 | 3.4-4.6 | 1.2-11.5 | 4.3-19.3 | 1.7-7 |
| Range $PO_4^{3-}$ of TLS Risk Patients | 2.6-4.6 | 2.3-5.6 | 3.5-3.5 | 3.6-6.2 | 4.3-7.2 | 3.4-3.4 | 4.3-5.1 |
| % Normal $PO_4^{3-}$ of TLS Patients | 100% | 100% | 80% | 100% | 67% | 50% | 75% |
| % Normal $PO_4^{3-}$ of TLS Risk Patients | 100% | 100% | 100% | 100% | 67% | 100% | 100% |

**Figure 3:** Phosphate levels profile in TLS and TLS risk patients (n=15).




| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D8 | D11 |
|---|---|---|---|---|---|---|---|---|---|
| Average Scr of TLS Patients | 1.35± 0.68 | 2.10± 0.00 | 1.38± 0.25 | 4.40± 0.00 | 1.63± 1.93 | 0.67± 0.00 | 1.87± 1.50 | 6.20± 0.00 | 0.90± 0.28 |
| Average Scr of TLS Risk Patients | 0.44± 0.18 | 0.60± 0.21 | 0.30± 0.00 | 0.35± 0.17 | 0.39± 0.19 | 0.30± 0.00 | 0.55± 0.17 | 0.30± 0.00 | 0.30± 0.00 |
| Range Scr of TLS Patients | 0.5-2.1 | 2.1-2.1 | 1.02-1.6 | 4.4-4.4 | 0.5-5.5 | 0.67-0.67 | 1.0-3.6 | 6.2-6.2 | 0.7-1.1 |
| Range Scr of TLS Risk Patients | 0.2-0.7 | 0.4-0.7 | 0.3-0.3 | 0.2-0.6 | 0.26-0.6 | 0.3-0.3 | 0.3-0.7 | 0.3-0.3 | 0.3-0.3 |
| % Normal Scr of TLS Patients | 25% | 0% | 0% | 0% | 67% | 100% | 25% | 0% | 50% |
| % Normal Scr of TLS Risk Patients | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |

**Figure 4:** Scr levels profile in TLS or risk of TLS patients (n=15).




| | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D8 | D11 |
|---|---|---|---|---|---|---|---|---|---|
| Average BUN of TLS Patients | 23.5± 16.82 | 16.0± 0.00 | 27.25± 29.35 | 21.0± 0.00 | 15.3± 11.50 | 14.0± 0.00 | 21.67± 15.01 | 20.00± 0.00 | 31.5± 28.99 |
| Average BUN of TLS Risk Patients | 13.0± 7.30 | 13.0± 14.14 | 5.0± 0.00 | 11.8± 5.12 | 7.3± 2.52 | 8.0± 0.00 | 11.0± 7.81 | 8.0± 0.00 | 13.0± 0.00 |
| Range BUN of TLS Patients | 12-48 | 16-16 | 9-71 | 21-21 | 3-37 | 14-14 | 7-37 | 20-20 | 11-52 |
| Range BUN of TLS Risk Patients | 5-23 | 3-23 | 5-5 | 7-19 | 5-10 | 8-8 | 6-20 | 8-8 | 13-13 |
| % Normal BUN of TLS Patients | 50% | 100% | 75% | 0% | 83% | 100% | 33% | 0% | 50% |
| % Normal BUN of TLS Risk Patients | 80% | 50% | 100% | 75% | 100% | 100% | 67% | 100% | 100% |

**Figure 5:** BUN levels profile in TLS or risk of TLS patients (n=15).

**Table 4:** Serum electrolytes and kidney functions in TLS patients (n=8).

| Day | Scr, mg/dL | BUN, mg/dL | $K^+$, mmol/L | $Ca^{2+}$, mg/dL | $PO_4^{3-}$, mg/dL |
|---|---|---|---|---|---|
| 0 | 1.35 ± 0.68 | 23.50 ± 16.82 | 4.10 ± 1.30 | 8.50 ± 1.34 | 3.70 ± 2.55 |
| 1 | 2.10 ± 0.00[a] | 16.00 ± 0.00[a] | 3.30 ± 0.00[a] | 9.70 ± 0.00[a] | 5.70 ± 0.00[a] |
| 2 | 1.38 ± 0.25 | 27.25 ± 29.35 | 3.40 ± 0.80 | 8.20 ± 1.91 | 4.90 ± 3.73 |
| 3 | 4.40 ± 0.00[a] | 21.00 ± 0.00[a] | 3.10 ± 1.30[a] | 6.90 ± 3.54[a] | 4.00 ± 0.85[a] |
| 4 | 1.63 ± 1.93 | 15.30 ± 11.50 | 3.50 ± 1.00 | 8.30 ± 1.68 | 5.00 ± 3.71 |
| 5 | 0.67 ± 0.00[a] | 14.00 ± 0.00[a] | 3.30 ± 0.00[a] | 7.70 ± 0.00[a] | 11.80 ± 10.60[a] |
| 6 | 1.87 ± 1.50 | 21.67 ± 15.01 | 3.10 ± 1.20 | 9.00 ± 1.41 | 4.60 ± 2.69 |

p-Value<0.05, *significant. [a]Paired *t*-test was not performed because there was only one to two data.

**Table 5:** Serum electrolytes and kidney functions in TLS risk patients (n=7).

| Day | Scr, mg/dL | BUN, mg/dL | $K^+$, mmol/L | $Ca^{2+}$, mg/dL | $PO_4^{3-}$, mg/dL |
|---|---|---|---|---|---|
| 0 | 0.44 ± 0.18* | 13.0 ± 7.30 | 4.6 ± 0.9* | 9.2 ± 0.84* | 3.5 ± 0.90* |
| 1 | 0.60 ± 0.21[a] | 13.0 ± 14.14[a] | 3.5 ± 1.3* | 7.8 ± 1.32* | 4.3 ± 1.74 |
| 2 | 0.30 ± 0.00[a] | 5.0 ± 0.00[a] | 3.3 ± 0.00[a] | 9.0 ± 0.00[a] | 3.5 ± 0.00[a] |
| 3 | 0.35 ± 0.17* | 11.8 ± 5.12* | 4.3 ± 1.1 | 8.5 ± 0.26* | 4.6 ± 1.42* |
| 4 | 0.39 ± 0.19 | 7.3 ± 2.52* | 3.4 ± 0.5* | 8.8 ± 0.53 | 5.7 ± 1.45* |
| 5 | 0.30 ± 0.00[a] | 8.0 ± 0.00[a] | 3.4 ± 0.00[a] | 8.0 ± 0.00[a] | 3.4 ± 0.00[a] |
| 6 | 0.55 ± 0.17 | 11.0 ± 7.81 | 4.7 ± 0.9 | 9.1 ± 0.62 | 4.7 ± 0.40* |

p-Value<0.05, *significant. [a]Paired *t*-test was not performed because there was only one to two data.

the normal range. These findings show the success of hydration as a prophylaxis in risk of TLS patients supported by normal renal function. Opposite to TLS risk patients who experience hyperkalemia, TLS patients experience more hypokalemia. Hypokalemia that occurs in TLS patients (5 of 8 TLS patients) can be caused by several factors such as: hydration which can increase potassium elimination, combined with the use of furosemide which is a strong diuretic, the occurrence of hypomagnesemia, metabolic alkalosis, diarrhea, and vomiting [17–20].

The calcium levels of TLS risk patients were fluctuating (Figure 2) but may return to normal values; whereas in TLS patients, an improvement in the calcium levels then followed by a decrease again. This was indicating the success of hydration therapy and correction of calcium gluconate in patients with hypocalcemia. Hydration and calcium correction give a positive impact on increasing calcium levels in TLS patients, but the disease progression of TLS patients is more rapid than TLS risk patients [21, 22]. Hypocalcemia in TLS patients is a secondary effect that arises because of hyperphosphatemia during the lysis of tumor cells [23]. The provision of hydration therapy and other supports such as furosemide have a positive impact on phosphate elimination. In TLS patients a decrease in phosphate levels was followed by an increase in phosphate levels again. Most of TLS patients have experienced AKI (7 patients), which affect phosphate elimination, combined with the severe progression of the patient's disease [24, 25].

Statistical analysis (Table 5) showed no significant differences ($p>0.05$) in all parameters, as hydration only slowed the progression of the disease in TLS patients. TLS patients still developed hyperkalemia, hyperphosphatemia, hypocalcemia, and AKI although hydration therapy has been administered [9, 26, 27]. This result was supported by the hospital discharge status of TLS patients that showed 75% of patients died. Otherwise, TLS risk patients (Table 5) had success prophylactic hydration therapy on all parameters (% normal day 11: 100%) and the patients are discharged from the hospital 100% alive. The results of this study have similarities with Cairo et al. [26] who stated giving hydration as prophylaxis in TLS risk patients can maintain the kidney function to prevent TLS. Burghi et al. [17] also stated that giving hydration is effective in preventing the occurrence of TLS in moderate-high risk patients, while in patients with TLS giving hydration is less effective than renal replacement therapy (example: dialysis). Therefore, early risk identification of TLS and parameter monitoring of TLS could be the better step to establish the appropriate time to administer hydration prophylaxis [19, 27, 28].

## Conclusions

Hydration can maintain serum electrolytes and renal function in a normal range, preventing TLS in TLS risk patients. In TLS patients, hydration tends to decrease the disease progressivity. However, TLS patients still have metabolic abnormalities such as hyperkalemia, hyperphosphatemia, and hypocalcemia. AKI occurs in most of TLS patients.

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Hasna Qatrunnada, Suharjono*, Siprianus Ugroseno Yudho Bintoro and Siti Wahyuni

# Drug utilization study and cost analysis of adult β-thalassemia major patient therapy at Dr. Soetomo General Hospital Surabaya

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**Abstract**

**Objectives:** The main therapy of β-thalassemia major are blood transfusion and iron chelation drugs. However, those therapies also have some adverse effects and problems such as iron overload, transfusion reactions, nutritional deficiencies, and patient compliance problems. Those arising problems also have an impact on therapy cost. Hence, this study was designed to analyze drug utilization study and cost of therapy in β-thalassemia major adult patients at Dr. Soetomo General Hospital Surabaya.

**Methods:** This research was conducted in descriptive observational-retrospective design using secondary data obtained from patient's medical records and billing registrations from January 1–December 31, 2019.

**Results:** There were 18 patients out of 233 patients that were analyzed. Deferasirox was the most administered drug with doses between 500 mg/day–1,500 mg/day while deferiprone was ranged between 1,500 and 4,500 mg/day. Patients also received transfusion reaction drugs with dexamethasone injection 5 mg/ml which was administered the most. The most administered supplement was folic acid 1 mg. Patients had an increase in serum ferritin due to low compliance. Deferasirox had the most adherence number of patients with decrease of serum ferritin. The two highest costs of direct medical components were top-up medicines and consumable medical supplies. Overall, the hospital gained profit from national health insurance claims.

**Conclusions:** The most administered chelating agent was deferasirox. Deferasirox also had the most adherence number of patients with decreased number of serum ferritin. However, deferasirox also yielded the highest cost. Yet, overall, the hospital gained profit from national health insurance claims.

**Keywords:** β-thalassemia major; compliance; cost analysis; drug utilization study.

## Introduction

Thalassemia is one of the blood diseases which inherited genetically from parent to their children [1]. This disease is classified into α, β, γ, δ, δβ, or εγδβ-thalassemia based on reduced or loss of normal globin chain synthesis. β-thalassemia major occurs when three or four α-genes or both β-genes are defective [3]. These conditions can lead to anemia, skeletal deformities, osteoporosis, and hepatosplenomegaly [2].

The established supportive therapy for thalassemia major patients (thalers) is blood transfusion and chelating agent. There is still no cure therapy for this condition yet [4]. In this case, thalers need a lifetime blood transfusion to treat their anemias. However, regular blood transfusions can develop iron overload. Therefore, thalers also receive iron chelating drugs, such as Deferoxamine (DFO), Deferiprone (DFP), and Deferasirox (DFX) [3, 5]. Other problems that also arise due to this periodic transfusion are transfusion reaction and nutritional deficiency. A transfusion reaction is any adverse reaction associated with a blood product transfusion. Transfusion reactions can be treated with certain medications such as loop diuretics, paracetamol, diphenhydramine, etc. [6, 7, 8]. While supplementation which is administered to thalers are vitamin C, folic acid, etc. [9, 10].

On the other hand, problems related to thalers adherence also arise due to the long-term use of chelating agents. A study stated that the average adherence of patients with chronic disease in developing countries was only 50%. Poor adherence to iron chelation therapy can lead to reduced chelation coverage. The presence of high labile plasma iron due to chelation coverage reduction can exacerbate tissue damage [11].

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +628121733877, E-mail: suharjono@ff.unair.ac.id
**Hasna Qatrunnada,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Siprianus Ugroseno Yudho Bintoro,** Department of Internal Medicine, Dr. Soetomo General Hospital, Surabaya, Indonesia
**Siti Wahyuni,** Department of Pharmacy, Dr. Soetomo General Hospital, Surabaya, Indonesia

The highest prevalence of major thalassemia and HbE β-thalassemia is located in malaria pandemic regions such as the Mediterranean, African, Middle East, Indian subcontinent, and Southeast Asia. There are more than 50,000 patients born with this condition [12]. In Indonesia, the number of thalassemia patients is more than 10,531 and there are around 2,500 babies born with thalassemia every year. This amount of thalassemia patients also contributes to the total cost of thalassemia therapy, which was ranked the fifth of the highest-cost disease in Indonesia [4]. In this study, the total amount of inpatient thalers in Dr. Soetomo General Hospital is around 233 patients during January-December 2019.

Besides, the high therapy cost was caused by the high price of iron-chelating drugs itself. Comparison of the detailed costs incurred by the Indonesian government to treat thalassemia with the UMR (regional minimum wage) in Surabaya, are 79.5% (2014), 132.0% (2015), 128.0% (2016), 127.7% (2017), and 88.4% (2018) [13]. The standard health service tariff of thalassemia has been established in the health insurance program, through the Badan Penyelenggara Jaminan Sosial Kesehatan (BPJS Kesehatan) or National Health Insurance. For advanced level health services (level II and III), the amount of claim payment by national health insurance is referred to the Indonesian-Case Based Groups (INA-CBG's) tariff, which is based on the classification of disease diagnoses and procedures [14]. There are two kinds of claims in national health insurance, health service claim and certain medicine claim (top-up medicine). This study was designed to analyze drug utilization study, including thalers adherence, and also therapy cost of β-thalassemia major adult patients at Dr. Soetomo General Hospital Surabaya.

# Materials and methods

This study was designed to comply with the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, with reference number 1853/KEPK/III/2020. This research was conducted in a descriptive observational manner with a retrospective design using secondary data obtained from patient's medical records and billing registrations from January 1–December 31, 2019.

The included data were adult β-thalassemia major patients with national health insurance class 1–3, including their membership types such as patient whose premium is paid by the government (PBI) and patients who pay their premium (Non-PBI), patients with complete medical records and billing registration data, and underwent hospitalization at least six times during the determined period of time. Most thalassemia patients were located in outpatient and inpatient installation in the internal medicine department. This study used inpatient data to observe full therapy including blood transfusion and its total cost. This study excluded patients who have comorbid and complications. Those data collections were recapitulated into a table, consisted of age, gender, domicile, diagnosis, statuses and classes of national health insurance, length of stay, clinical data, medical supporting data, therapy data, and all the direct costs. Data analysis was performed descriptively and presented in the form of tables.

# Results

## Patient demographic

There were 18 patients with β-thalassemia major diagnosis from 233 patient populations that have been analyzed with total visits 94 times. The data were collected from the Department of Internal Medicine of Dr. Soetomo Hospital Surabaya. Patients' demographics were distributed into sex, age, residence, and distance to the health facility. The data result is shown in Table 1. The β-thalassemia major patients consisted of males (55.5%) and females (44.4%). About 88.8% of them were 19–30 years old, and about 11.1% of them were above 31 years old. Patients mostly lived outside of Surabaya city (66.6%), and only 33.3% of them lived in Surabaya. According to distance numbers, about 61.1% of patients travel distance of 10–50 km, and only 38.8% of patients travel for more than 50 km. These patients who joined national health insurance were classified by membership type and healthcare service class. There were 61.1% patients as PBI and about 38.8% non-PBI. Most patients joined the third class of healthcare service, followed by the first class (11.1%), and none of them were in the second class (0.0%).

## Therapy profile which was administered to patients

Those inpatient β-thalassemia major patients were administered iron chelating drugs, supplements, and some of them have received transfusion reaction drugs (Table 2). The most administered iron-chelating drugs was DFX (73/4%) with a dosage range from 500–1,500 mg/day, followed by DFP with a dosage range of 1,500–4,500 mg/day which were administered 25 times (26.6%) from total of 94 times hospitalizations. The number of tablets which were administered to every patient within a month is based on the required doses/day for each patient ranged from 30 to 270 tablets. Dexamethasone injection 5 mg/ml was the most administered drugs (6.4%), then the second most administered transfusion reaction drugs were paracetamol 500 mg (4.2%) and Diphenhydramine HCl 10 mg/ml (4.2%). Unfortunately, we didn't observe further results according to biological

**Table 1:** Adult β-thalassemia major inpatients demographic at Dr. Soetomo general hospital.

| Demographic data | Category | n | Percentage, % |
|---|---|---|---|
| Sex | Male | 10 | 55.5 |
| | Female | 8 | 44.4 |
| Age | 19–30 | 16 | 88.8 |
| | >31 | 2 | 11.1 |
| Distance to health facility (Dr. Soetomo General Hospital) | <1–10 km | 4 | 22.2 |
| | 10–50 km | 7 | 38.8 |
| | 50–100 km | 5 | 27.8 |
| | >100 km | 2 | 11.1 |
| Domicile | Outside Surabaya | 12 | 66.6 |
| | Surabaya | 6 | 33.3 |
| Membership type of national health service | PBI (premium is paid by government) | 11 | 61.1 |
| | Non-PBI (people who pay their own premium) | 7 | 38.8 |
| Class of healthcare service | 1st class | 2 | 11.1 |
| | 2nd class | 0 | 0.0 |
| | 3rd class | 16 | 88.8 |

reactions for each patient. Thalers also received supplements as well as other drugs. The most administered supplement was folic acid 1 mg (40.4%).

## Patient adherence

According to Table 3, patients mostly consumed iron-chelating drugs less than six months (77.8%). Only 22.2% of patients underwent routine control for more than six months. Patients with the best adherence number were patients who consumed deferasirox (50.0%), and thus this might lead to the highest number of decreased serum ferritin in patients who consumed deferasirox (70.0%).

## Direct medical cost

The total direct medical cost of β-thalassemia major patients was IDR 929,685,354 and total national health insurance claim was IDR 988,404,780. The cost gap between total direct medical cost and national health insurance claim was IDR 58,719,426 (6.3%). The components which contributes the most were Top Up medication (Exjade®, DFX and Ferriprox®, DFP) with 81.0%. Then, followed by Consumable Medical Materials with 13.7%. These results can be observed through Tables 4 and 5.

# Discussion

There was no significanct difference between the number of male and female patients in Table 1. Yet, male patients have a greater number of 12% than female. However, this comparison is not always the case. This is because β-thalassemia is inherited in an autosomal recessive pattern, so that, the risk can happen to both males and females with equal probability [15]. Then related to age, most patients aged 19–30 years old were 88.8% (16 patients), while patients aged 31–40 were 11.1%. The number of patients over 30 years old is less than patients under 30 years old. This condition is theoretically caused by decreased life expectancy due to complications which were suffered by patients over 30 years

**Table 2:** Supplemental and drug treatments which were administered to β-thalassemia major patients.

| Treatments | List of treatments | Dosage | n[a] | Percentage, % |
|---|---|---|---|---|
| Iron-chelating drugs | DFP 500 mg (90–270 tab/month) (Ferriprox®) | 1500–4500 mg/day | 25 | 26.6 |
| | DFX 250 (60–150 tab/month) and 500 mg (30–90 tab/month) (Exjade®) | 500–1500 mg/day | 69 | 73.4 |
| Transfusion reaction drugs | Dexamethasone injection 5 mg/ml | 10–50 mg/day | 6 | 6.4 |
| | Paracetamol 500 mg | 1000–1500 mg/day | 4 | 4.2 |
| | Paracetamol infusion | 2000 mg/day | 1 | 1.1 |
| | Ranitidine 150 mg | 300 g/day | 1 | 1.1 |
| | Diphenhydramine HCl 10 mg/ml | 20–50 mg/day | 4 | 4.2 |
| | Metoclopramide injection 10 mg/2 ml | 40 mg/day | 1 | 1.1 |
| Supplementations | Ca Gluconate injection 10% | 1000 mg/day | 2 | 2.1 |
| | Folic acid 1 mg | 1–3 mg/day | 38 | 40.4 |
| | Vitamin B complex | 3 × 1 | 1 | 1.1 |
| | Vitamin C 50 mg | 150 mg/day | 1 | 1.1 |

DFP, deferiprone; DFX, deferasirox; tab, tablet. [a]Administration frequency from 94 times hospitalizations. Patients could receive more than one sort of treatments.

**Table 3:** Patient adherence based on patient routine control attendance and serum ferritin level.

| Description | Category | n | Percentage, % |
|---|---|---|---|
| Patient routine control during 1 period | Patients attendance: | | |
| | ≥6 months | 4 | 22.2 |
| | <6 months | 14 | 77.8 |
| Average of patient routine control based on their therapies[a] | Patients who consumed: | | |
| | DFX | 6 months | 50.0 |
| | DFP | 5 months | 41.7 |
| | DFX and DFP | 4 months | 33.3 |
| Patients with decreased number of serum ferritin based on their therapies | Patients who consumed: | | |
| | DFX (n=10) | 7 | 70.0 |
| | DFP (n=4) | 0 | 0.0 |
| | DFX and DFP (n=4) | 2 | 50.0 |

DFP, deferiprone; DFX, deferasirox. [a]Patients could receive more than one sort of treatments.

related to thalassemia. Another study stated that patients with age 26.9 years old only have a 50% survival chance from iron overload complications [16, 17].

The most administered iron-chelating drugs was DFX (73.4%), followed by DFP (26.6%) (Table 2). The goal of iron chelation treatment is to balance the rate of iron accumulation obtained from blood transfusions by increasing the iron excretion through urine and/or stool. The challenge of iron chelation administration is to balance iron excretion because iron is still needed for essential physiological purposes in our body. Hence, dose adjustment is necessary to avoid excess chelation as iron levels fall. Iron chelation dose adjustment is based on each patients' body weight [2].

From Table 2, we can observe that patients were given Exjade® (DFX) with a ranged dose of 500–1,500 mg/day. These doses were lower than the given ranged dose in Ferriprox® (DFP), which started from 1,500–4,500 mg/day. These results related to the advantage of DFX which is the longest half-time iron-chelating drug, 10–16 h. Other advantages of DFX are this drug can be consumed and mixed with water, apple juice, and orange juice to ease children and adults who have difficulty swallowing tablets. DFX also showed a decrease in liver iron concentration (LIC) significantly [2]. While DFP also has its own advantage in penetrating through cardiac myocytes and eliminates iron from those cells. DFP is the best iron remover in the myocardial among other iron-chelating drugs [18, 19]. In this case, patients received DFX and DFP based on drug availability in the hospital.

During the blood transfusion, some patients experienced transfusion reactions. Transfusion reactions can be classified into acute and delayed reactions. Acute transfusion reaction consists of acute hemolytic transfusion reaction, allergic and anaphylactic reactions, febrile nonhemolytic transfusion reaction, septic transfusion reactions, transfusion-associated acute lung injury (TRALI), and transfusion-associated circulatory overload (TACO). While, delayed transfusion reaction consists of delayed hemolytic transfusion reactions (DHTR), transfusion-associated graft vs. host disease (TA-GVHD), and post-transfusion purpura (PTP) [6]. From Table 2, patients received single and combination treatments prior to blood transfusion. The most administered transfusion reaction drug was dexamethasone injection 5 mg/ml. Dexamethasone as a corticosteroid was given in order to prevent mild allergy transfusion reactions with symptoms of urticarial, pruritus, flushing, and mild wheezing. Corticosteroids can be used to treat DHTR. Corticosteroids cannot be given in life-threatening situations and may be given if the patients are refractory to other forms of pre-medication [20, 21].

Patients also received supplements to balance essential substances within the body. The most administered supplement was folic acid 1 mg. Folic acid has an important role related to red blood cells (RBC) production. Folic acid works together with B12 to develop RBC and help iron optimization. Lack of any of these substances can lead to reduced erythrocyte production and reduced number of red blood cells in the circulation (anemia) [22, 23].

From table 3, only 22.2% of patients have attended ≥ six months. Based on their therapies, patients who consumed DFX have the best attendance average (50.0%), followed by DFP (41.7%) and a combination between DFX and DFP (33.3%). This might be caused by the DFX advantage which has a long half-time and easy to consume. Thus,

**Table 4:** Average of total direct medical cost and national health insurance claim.

| Description | Direct medical cost (IDR) | National health insurance claim (IDR) | Cost gap (IDR) | Cost gap, % |
|---|---|---|---|---|
| Total cost from 94 times hospitalizations | 929,685,354 | 988,404,780 | (58,719,426) | (6.3) |
| Average hospitalization cost | 9,890,270 | 10,514,944 | (624,674) | (6.3) |
| SD | 2,394,604 | 868,474 | – | – |

**Table 5:** Components of total direct medical cost.

| Cost components | Cost (IDR) | Cost average (IDR) | Cost, % |
|---|---|---|---|
| Administration | 990,000 | 10,532 | 0.1 |
| Accommodation | 13.685,000 | 145,585 | 1.5 |
| Referral ticket | 940,000 | 10,000 | 0.1 |
| Medical service | 14,186,500 | 150,920 | 1.5 |
| Medical Support service | 8,419,100 | 89,565 | 0.9 |
| Consumable medical equipment | 6,059,364 | 64,461 | 0.6 |
| Consumable medical material | 127,080,357 | 1,351,919 | 13.7 |
| Medications | 5,693,775 | 60,572 | 0.6 |
| Top-up medications | 752,631,258 | 8,006,716 | 81.0 |
| Exjade® (n=69) | 594,341,178 | 8,613,640 | (79.0) |
| Ferriprox® (n=25) | 158,290,080 | 6,331,603 | (21.0) |
| Total | 929,685,354 | 9,890,270 | 100.0 |

improvement in patient adherence gave a linear result in serum ferritin levels of patients who consume DFX. There were 70.0% of patients who consumed DFX have decreased the number of ferritin serum levels [2]. Low adherence of these thalassemia patients, theoretically associated with distance to the health facility. In this case, thalassemia patients need to control and receive DFX and/or DFP tablets in the health care facility (Dr. Soetomo hospital) every month. A number of tablets ranged from 30-270 tablets according to the dosage required by each patient within a month. About 66.6% of patients lived outside Surabaya city and only 22.2% of patients travel less than 10 Km. Therefore, patients who lived outside Surabaya city prone to have adherence problems. But distance is not the only factor that could affect patient adherence. Other factors such as regimen and illness (including infusion adverse effects), psychological and social, demographic, and health system need further investigation [11].

Poor adherence to iron chelation therapy can lead to iron overload complications, such as heart dysfunction and arrhythmia, endocrine complications (hypogonadism, hypothyroid, hypoparathyroidism, diabetes mellitus, and growth problem), osteoporosis, retinal pigment changes and abnormal electroretinography, and bacterial infections due to iron overload as the common cause of death and morbidity in thalassemia major patient. Besides, poor adherence to blood transfusion also causes problems such as extramedullary erythropoiesis [24]. However, this study didn't observe the presence of symptoms due to poor adherence for each patient.

Those treatments cost about IDR 929,685,354 as total direct medical cost, with the average hospitalization cost of about IDR 9,890,270. The comparison between average hospitalization cost and regional minimum wage in Surabaya 2019 (IDR 3,871,053) was 255%. This means that thalassemia therapy for each patient quite costly. National health insurance's grouping code for this treatment was D-14-13-I with diagnosing red blood cell disorders aside from mild sickle cell anemia crisis. International Classification of Disease (ICD)-10 code diagnose for primary and secondary diagnose is D56.1 [14, 25]. From national health insurance claim classification, it was obtained total claim for all BPJS class IDR 988,404,780. These calculations produced a cost gap of approximately IDR 58,719,426 (6.3%). Thus, the overall hospital gained a profit of around 6.3%. Two components which contribute the most to total direct medical cost were consumable medical material (13.7%) and (81.0%) top-up medicines, with DFX which cost the most (79.0% from the total top-up medicine cost) (Table 4–5).

# Conclusions

This study shows that the most administered iron-chelating drug was DFX with the highest number of adherence patients. This result linear with the decreased number of serum ferritin levels in patients with DFX. Patients also received transfusion reaction drugs as well as supplements. The overall hospital gained profit from national health insurance claims for total direct medical costs.

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Prihartini Widiyanti* and Purnomo Suryohudoyo

# The role of hyperbaric oxygen to platelet aggregation in noninsulin-dependent diabetes mellitus (NIDDM)

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**Abstract**

**Objectives:** Hyperglycemia in diabetes mellitus (DM) could cause rheological disorder, such as platelet aggregation and blood hyperviscosity. Hyperbaric oxygen (HBO) could decrease collagen as platelet aggregation agonist. This study aimed to explore the effect of HBO treatment to platelet aggregation parameters (latency time(LT), aggregation speed, aggregation index, and aggregation percentage) with the collagen aggregator in the noninsulin dependent diabetes mellitus (NIDDM).

**Methods:** The number of subjects in this study were 16 for each group normoxia normobaric (NONB) and HBO. NIDDM patients from DM polyclinic in Rumah Sakit Angkatan Laut (RSAL) Dr Ramelan Surabaya which was fulfilled inclusion criteria would receive HBO Therapy. Control Group/NONB were treated with NONB condition (20% $O_2$ 1 ATA) for 90 min and treatment group/HBO were treated with hyperoxia hyperbaric condition (100% $O_2$ 2.4 ATA) for $3 \times 30$ min with interval of $2 \times 5$ min for inhaling fresh air. Subject has been blood taken for platelet aggregation test before and after HBO Therapy. The length of treatment was 5 days for both condition (NONB and HBO).

**Results:** The data from both groups, NONB and HBO were tested first by normality test, homogenity test, correlation test, analysis of covariance, and paired t-test. Based on paired t-test, the decrease on platelet aggregation speed, aggregation index, and aggregation percentage after HBO treatment was showed significant difference on the LT and aggregation index while in aggregation speed and aggregation percentage was not significant. NONB group after 5 days was showed a significant difference on the aggregation speed and aggregation index while in LT and aggregation percentage was not significant.

**Conclusions:** The utilization of HBO 2.4 ATA 100% $O_2$ $3 \times 30$ min, once a day, for 5 days could decrease the platelet aggregation parameters (LT, aggregation speed, aggregation index, and aggregation percentage) in patients with NIDDM.

**Keywords:** hyperoxia hyperbaric oxygen (HBO); non-insulin dependent diabetes mellitus (NIDDM); normoxia normobaric oxygen (NONB); platelet aggregation.

## Introduction

Diabetes is crucial health problem due to its high morbidity and mortality [1]. Diabetes influences 285 million people worldwide, and the data tend to increase become 438 million by the year 2030, with two-thirds of all diabetes cases happening in low-to middle-income countries. The number of adults with impaired glucose tolerance will rise from 344 million in 2010 to an estimated 472 million by 2030. Indonesia as one of 10 countries which showed the largest numbers of diabetic cases [2]. Prevalence of diabetes mellitus (DM) case in Indonesia is 5.7%, and impaired glucose tolerance 10.2% [3]. DM is an endocrinal disease that an abnormal metabolic characteristic that will affect several organs in the long-term complication, such as eye, kidney, and vascular system. The diagnosis of DM is based on the osmotic diuretic symptoms and hyperglycemia [4]. Hyperglycemia for a long time could damage the component of organs. One of them was causing the rheological disorder, such as platelet, erythrocyte, leucocyte aggregation, and blood viscosity [5]. Patients with DM were found to have the hemorheological disorder and platelet hyperreactivity that influence hyperviscosity pathogenesis [6].

Hyperbaric oxygen therapy (HBOT) was a therapy by high concentrations of oxygen in the high-pressure chamber. In the therapeutic purposes, plasma partial pressure of oxygen might encompass levels greater than 20 times that of breathing room air at normal atmospheric

***Corresponding author: Prihartini Widiyanti**, Biomedical Engineering Study Program, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia; Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Universitas Airlangga, Campus C, Mulyorejo, Surabaya, 60115, Indonesia, Phone: +62 31 5992445, E-mail: pwidiyanti@fst.unair.ac.id
**Purnomo Suryohudoyo,** Biochemistry Department, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

pressure. Oxygen-rich plasma will be delivered to the hypoxic or ischemic tissue to encourage angiogenesis, diminish edema, and inflect the immune system response [7–9]. HBOT 100% $O_2$ 3 Atmosphere Absolute (ATA) for 2 h could help nitric oxide (NO) regeneration [10]. NO could activate the guanylate cyclase to stimulate production of cyclic guanosine monophosphate (c-GMP) [11]. c-GMP could inhibit platelet aggregation [12]. The study objective is to observe the effect of therapy by using 100% $O_2$ 2.4 ATA 3 × 30 min with an interval of 5 min for inhaling the fresh air towards the platelet aggregation parameters (latency time (LT), aggregation speed, aggregation index, and aggregation percentage) with the collagen aggregator in the noninsulin dependent diabetes mellitus (NIDDM) patients.

# Materials and methods

## Materials

The materials used in this study were one unit of high-pressure chamber, oxygen with concentration of 100 and 20%, DiaSTAT hemoglobin A1c reagent kit, plastic or silicone tube, plastic/silicone pipette, aggregometer, aggregation reagent "HELENA", collagen (Determine the collagen and dilution so that 0.5 mL of it will aggregate optimally with the normal plasma), Buffer Tris with pH 7.4, and 16 mL/100 mL hydrochloric acid (HCl).

## Methods

The number of subjects in this study were 16 for each group (normoxia normobaric (NONB) and HBO. Moderator variables were age, body weight, body height, fasting blood sugar, blood sugars 2 h post prandial, and HbA1c. The population of patients with NIDDM in the DM section of Rumah Sakit Angkatan Laut (RSAL) Dr Ramelan Surabaya was measured in term of their weight and height, physical examination, thoracic photograph, and electrocardiogram (ECG) as a prerequisite before performing HBOT. The length of treatment was 5 days for both condition (NONB and HBO). The result of the observations was consulted to a specialist doctor to get the patient who suited with inclusion criteria. Then, the sample was randomized with criteria age of 40–75 years old, normal physical examination, normal thoracic photograph, normal ear nose and throat (ENT), normal ECG, family line with DM, normal weight according the body mass index (BMI), glucose level no more than 400 mg/dl, NIDDM, normal hemoglobin A1c (HbA1c) level, and stop consuming antiplatelet, antidiabetes, and vitamin C and E drugs during the study. The protocol of this study was accepted by ethical committee of RSAL Dr Ramelan Surabaya no 19/EC/KERS/2011. The data from both groups, NONB and HBO were tested first by normality test, homogenity test, correlation test, analysis of covariance, and paired t-test.

**Platelet aggregation test (PAT):** When the aggregation agent was added to the thrombocyte-rich plasma, the form deformation and platelet aggregation (PA) affect the decrease of plasma optical density. The requirement of the examination was PAT should be performed in 1–3 h after the blood sample collection, stored in the incubator with a temperature of 37 °C, along with the examination of PAT, the blood sample should be shaken continuously to provide collision of platelet.

The blood was collected with a clean venipuncture. Ratio of blood volume: trisodium citrate 3.8% was 9:1 and was centrifuged at room temperature 10–12*x* for 5 min at 200–220 g's (g = 11.28 × 106 × centrifuge radius in cm × the speed in revolutions per minute (rpm) square)/ 3,000 rpm. The plastic pipette was used for transferring plasma into a plastic tube. The blood was centrifuged to obtain the plasma with low platelet level around 1,500 g's for 30 min. The plasma was stored at room temperature until the testing process. The complete test was performed in 3 h.

**Hyperbaric oxygen exposure:** The sample determination was performed by using a ballot. Before the treatment, the subjects filled the agreement form and questionnaire. The subjects who have been fulfilled the inclusion criteria, undergone all the examination, stated an agreement to be a subject in this study were randomized based on the random number. The sample that picked randomly was divided into two groups, control group (NONB) which was subject group that were treated with normobaric normocytic condition (20% $O_2$ 1 ATA for 90 min and treatment group (HBOT) which was subject group that were treated with hyperoxic hyperbaric condition (100% $O_2$ 2.4 ATA) for 3 × 30 min with interval of 2 × 5 min for inhaling fresh air as seen in Figure 1.

# Results

The parameters of platelet aggregation were including:

a. Latent period: Time/time in seconds between the introduction of an initiator and the first start of the aggregation reaction/starting to appear on the graph curve.

**Figure 1:** Hyperbaric chamber in Lembaga Kesehatan Kelautan RSAL Dr Ramelan Surabaya.

Normal value: Collagen = 35–50

b. Aggregation velocity: the angle between the abscissa of the curve and the tangent (base line) drawn through the starting point of the curve and the curve ($\alpha$).

Normal value: Collagen = 52–80

c. Aggregation index: The ratio between the vertical height of the plateau point vs. the abscissa distance between the starting point of the trigger until the plateau is reached. ($dh/dt$), Normal value: Collagen = 0.3–0.7

d. Percent aggregation: The ratio in percent (%) between the plateau curve and the base of the graph curve vs. the height of the deviation between the PKT point and the PMT point.

Normal value: Collagen = 50–75

From Table 1, there is a difference in the final latent time between the HBO and NONB groups (p=0.001; p<0.05) with a significant effect of HbA1c (p=0.039, p<0.05).

From Table 2, there was no significant difference in the final aggregation speed between the HBO and NONB groups (p=0.439; p>0.05) with a significant effect of GD 2 J PP (p=0.010; p<0.05).

From Table 3, there is a significant difference in the index of aggregation between the OHB and NONB groups (p=0.043; p<0.05). The Moderator variable has no effect.

From Table 4, there was no significant difference in the percentage of aggregation between the OHB and NONB groups (p=0.545; p>0.05). The Moderator variable has no effect.

**Table 1:** Analysis of covariance result between moderator variables vs. latent time in HBO and NONB group (dependent variable: latency time).

| Source | Type III sum of squares | df | Mean square | F | p-Value |
|---|---|---|---|---|---|
| LT 1 | 172.557 | 1 | 172.557 | 12.342 | 0.002 |
| Age | 27.059 | 1 | 27.059 | 1.935 | 0.177 |
| BMI | 0.272 | 1 | 0.272 | 0.019 | 0.890 |
| FBS | 9.690 | 1 | 9.690 | 0.693 | 0.414 |
| BS 2H PP | 41.688 | 1 | 41.688 | 2.982 | 0.098 |
| HbA1c | 67.250 | 1 | 67.250 | 4.810 | 0.039 |
| Group | 211.581 | 1 | 211.581 | 15.133 | 0.001 |
| Total | 26,928.000 | 32 | | | |

R squared = 0.656 (Adjusted R squared = 0.537). LLT, latent time; BMI, body mass index; FBS, fasting blood sugars; BS 2H PP, blood sugar 2 hours post pandrial.

**Table 2:** Analysis of covariance result between moderator variables vs. speed of aggregation in HBO and NONB group (dependent variable: speed of aggregation).

| Source | Type III sum of squares | df | Mean square | F | p-Value |
|---|---|---|---|---|---|
| SA 1 | 56.880 | 1 | 56.880 | 2.608 | 0.120 |
| Age | 85.413 | 1 | 85.413 | 3.916 | 0.060 |
| BMI | 0.116 | 1 | 0.116 | 0.005 | 0.943 |
| FBS | 20.037 | 1 | 20.037 | 0.919 | 0.348 |
| BS 2H PP | 171.787 | 1 | 171.787 | 7.877 | 0.010 |
| HbA1c | 7.938 | 1 | 7.938 | 0.364 | 0.552 |
| Group | 13.540 | 1 | 13.540 | 0.621 | 0.439 |
| Total | 126,443.0 | 32 | | | |

R squared = 0.386 (Adjusted R squared = 0.173). SA, Speed of Aggregation; BMI, Body Mass Index; FBS, Fasting Blood Sugars; BS 2H PP, Blood Sugar 2 Hours Post Pandrial.

**Table 3:** Analysis of covariance result between moderator variables vs. index of aggregation in HBO and NONB group (dependent variable: index of aggregation).

| Source | Type III sum of squares | df | Mean square | F | p-Value |
|---|---|---|---|---|---|
| IA 1 | 0.147 | 1 | 0.147 | 13.561 | 0.001 |
| Age | 0.006 | 1 | 0.006 | 0.564 | 0.460 |
| BMI | 0.000 | 1 | 0.000 | 0.041 | 0.842 |
| FBS | 0.000 | 1 | 0.000 | 0.001 | 0.981 |
| BS 2H PP | 0.004 | 1 | 0.004 | 0.392 | 0.538 |
| HbA1c | 0.005 | 1 | 0.005 | 0.438 | 0.514 |
| Group | 0.050 | 1 | 0.050 | 4.598 | 0.043 |
| Total | 12.150 | 32 | | | |

R squared = 0.510 (Adjusted R squared = 0.339). IA, index of aggregation; BMI, body mass index; FBS, fasting blood sugars; BS 2H PP, blood sugar 2 hours post pandrial.

**Table 4:** Analysis of covariance result between moderator variables vs. percent of aggregation in HBO and NONB group (dependent variable: percent of aggregation).

| Source | Type III sum of squares | df | Mean square | F | p-Value |
|---|---|---|---|---|---|
| P 1 | 138.887 | 1 | 138.887 | 2.312 | 0.142 |
| Age | 81.971 | 1 | 81.971 | 1.365 | 0.255 |
| BMI | 4.049 | 1 | 4.049 | 0.067 | 0.797 |
| FBS | 26.567 | 1 | 26.567 | 0.442 | 0.513 |
| BS 2H PP | 141.794 | 1 | 141.794 | 2.361 | 0.138 |
| HbA1c | 0.659 | 1 | 0.659 | 0.011 | 0.918 |
| Group | 22.674 | 1 | 22.674 | 0.378 | 0.545 |
| Total | 157,839.0 | 32 | | | |

R squared = 0.315 (Adjusted R squared = 0.077). PA, percent of aggregation; BMI, body mass index; FBS, fasting blood sugars; BS 2H PP, blood sugar 2 hours post pandrial.

## Discussion

The decrease of LT on platelet aggregation of HBOT group after out of high-pressure chamber (HPC) at the fifth day compared to the LT of platelet aggregation before going inside the HPC on the first day, which was from 28.75 ± 3.87 to 25.75 ± 2.82. Meanwhile, The NONB group showed an increase in LT of platelet aggregation on the first day, which was from 30.00 ± 5.16 to 31.25 ± 6.19.

The decrease in LT , aggregation speed, aggregation index, and aggregation percentage of platelet that has been occurred after 5 days HBOT $O_2$ 100% 2.4 ATA 3 × 30 min with an interval of 3 × 5 min of inhaling the fresh air showed a significant difference for LT (p=0.001) and aggregation index (p=0.000). Meanwhile, the aggregation speed (p=0.022) and aggregation percentage (p=0.013) did not show a significant difference. The NONB group after 5 days showed a significant difference for aggregation speed (p=0.000) and aggregation index (p=0.005). On the other hand, the LT (p=0.362) and aggregation percentage (p=0.118) did not show a significant difference. The NONB group showed an increase in term of platelet aggregation speed on the fifth day compared to the platelet aggregation speed on the first day, which was from 68.19 ± 3.37 to 62.81 ± 6.52. Besides that, there was a decrease in the platelet aggregation index on the HBOT Group after going out from the HPC on the fifth day compared to the platelet aggregation index before going in the HPC on the first day, which was from 0.763 ± 7.188E-02 to 0.581 ± 8.342E-02. The NONB indicated an increase in the platelet aggregation index on the first day, which was from 0.706 ± 0.118 to 0.625 ± 0.161. There was decrease in the platelet aggregation percentage in the HBOT group after going out from HPC on the fifth day compared to the platelet aggregation percentage before going in the HPC on the first day, which was from 76.56 ± 8.06 to 69.13 ± 6.03. The NONB Group showed an increase in the platelet aggregation percentage on the fifth day compared to the platelet aggregation percentage on the first day, which was from 73.94 ± 9.45 to 70.44 ± 9.86.

Based on paired t-test, the decrease on the speed of aggregation (SA), index of aggregation (IA), and percent of aggregation (PA) after the exposure of 5 days HBOT 100% $O_2$ 3 × 30 min with interval of 5 min for inhaling fresh air could show a significant difference on the LT (p=0.001) and index of aggregation (p=0.000). The speed of aggregation (p=0.022) and percent of aggregation (p=0.013) did not show a significant difference. On the other hand, the NONB group after 5 days showed a significant difference on the speed of aggregation (p=0.000) and index of aggregation (p=0.005). The LT (p=0.362) and percent of aggregation (p=0.118) did not show a significant difference.

From the results stated above, it was indicated that HBOT 100% $O_2$ 3 × 30 min with an interval of 5 min for inhaling fresh air for 5 days on the patients with NIDDM could decrease the LT, aggregation speed, aggregation index, and aggregation percentage of thrombocyte. The exposure of HBOT 100% $O_2$ 3 × 30 min with an interval of 3 × 5 min of inhaling fresh air for 5 days could decrease adenosine diphosphate (ADP) and collagen which was an aggregator (platelet aggregation trigger) [13]. Reactive oxygen species caused a concentration-dependent inhibition of whole blood aggregation (WBA) in blood. This was obviously exposed in response to the biologically pertinent agonists, collagen, and ADP [14]. Based on the study of Kemeny SF et al. [15], DM could decrease the production and action of NO, which was an interaction with glycosylation end products. NO as a free radical substance and an important molecule of endothelia could react and produce a cellular response [16]. The beneficial action of NO towards vascular was NO could inhibit the platelet aggregation and adhesion, inhibit leukocyte adhesion towards activated endothelium, inhibit the migration and proliferation of vascular smooth muscle cell migration. NO could stimulate the production of c-GMP [17]. NO can inhibit the platelet aggregation [18]. NO activated the guanylate cyclase to control c-GMP [19]. NO could inhibit the platelet aggregation through c-GMP mechanism [20].

The conditions of platelet aggregation status in diabetic patients were influenced by exercise, stress, and diet factor. Doing exercise in the patients with NIDDM has benefited as glycemic control, losing weight, overcoming the iatrogenic complication, blood lipid problem, increasing the blood pressure, and hypercoagulation [21]. Hypocaloric diet could improve the short-term glycemic level and increase long-term metabolic control [22]. Emotional stress in the patients with NIDDM consisted of dietary and psychiatric disorders which could give a negative effect on the blood glucose level and lead to rheological problems [23].

## Conclusions

The use of HBOT 2.4 ATA 100% $O_2$ 3 × 30 min, once a day, for 5 days continuously could decrease the aggregation parameters (LT, aggregation speed, aggregation index, and aggregation percentage) of platelet in patients with NIDDM.

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Dwi Setyawan*, Firdaus Rendra Adyaksa, Hanny Lystia Sari, Diajeng Putri Paramita and Retno Sari

# Cocrystal formation of loratadine-succinic acid and its improved solubility

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**Abstract**

**Objectives:** Loratadine belongs to Class II compound of biopharmaceutics classification system (BCS) due its low solubility and high membrane permeability. Cocrystal is a system of multicomponent crystalline that mostly employed to improve solubility. Succinic acid is one of common coformer in cocrystal modification. This research aims to investigate cocrystal formation between loratadine and succinic acid and its effect on solubility property of loratadine.

**Methods:** Cocrystal of loratadine-succinic acid was prepared by solution method using methanol as the solvent. Cocrystal formation was identified under observation of polarization microscope and analysis of the binary phase diagram. The cocrystal phase was characterized by differential thermal analysis (DTA), powder X-ray diffraction (PXRD), Fourier transform infrared (FTIR), and scanning electron microscopy (SEM). Solubility study was conducted in phosphate-citrate buffer pH 7.0 ± 0.5 at 30 ± 0.5 °C.

**Results:** Loratadine is known to form cocrystal with succinic acid in 1:1 M ratio. Cocrystal phase has lower melting point at 110.9 °C. Powder diffractograms exhibited new diffraction peaks at $2\theta$ of 5.28, 10.09, 12.06, 15.74, 21.89, and 28.59° for cocrystal phase. IR spectra showed that there was a shift in C=O and O–H vibration, indicating intermolecular hydrogen bond between loratadine and succinic acid. SEM microphotographs showed different morphology for cocrystal phase. Loratadine solubility in cocrystal phase was increased up to 2-fold compared to loratadine alone.

**Conclusions:** Cocrystal of loratadine and succinic acid is formed by stoichiometry of 1:1 via C=O and H–O interaction. Cocrystal phase shows different physicochemical properties and responding to those properties, it shows improved loratadine solubility as well.

**Keywords:** cocrystal; ensuring health; loratadine; solubility; succinic acid.

## Introduction

Cocrystal is a multicomponent crystalline consisting of two or more different molecules in a stoichiometric ratio. Cocrystal offers an improvement in physicochemical properties of an active pharmaceutical ingredient (API) and becomes an alternative approach to salification in recent years to overcome manufacturing problems related to API properties. Cocrystal is mostly employed to improve solubility/dissolution rate, but it shows advantage in stability profile, hygroscopicity, mechanical properties, and drug bioavailability as well [1–3]. Instead of ionic interaction involved in salification, cocrystal formation is associated with weaker interaction, mainly hydrogen bond, between API and cocrystal former (or coformer) molecules. Common coformer is a pharmaceutically inactive compound and does not require an ionized state. Therefore, there are more available options for coformer than counterion, adding plus point to cocrystal over a salt [4, 5].

Loratadine is a second generation of antihistamine from piperidine derivative that works selectively to inhibit H1-receptors [6]. Loratadine belongs to the Class II compound of biopharmaceutics classification system (BCS) due its low aqueous solubility but high membrane permeability. Loratadine solubility depends on the pH of the medium, so that loratadine exhibits solubility-limited absorption profile leading to variable bioavailability [7, 8]. Loratadine has a carbonyl group and an aromatic imine in its structure that can act as hydrogen bond acceptor [9]. It makes loratadine an interesting compound to be subjected to cocrystal modification in order to improve the solubility in water.

Succinic acid has been extensively used as coformer in cocrystal formation and reported to successfully improve solubility profile of API(s). Succinic acid is dicarboxylic acid that abundantly found in plant and animal tissues. Succinic

***Corresponding author: Dwi Setyawan**, Faculty of Pharmacy, Department of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 315 933 150, E-mail: dwisetyawan-90@ff.unair.ac.id

**Firdaus Rendra Adyaksa, Hanny Lystia Sari, Diajeng Putri Paramita and Retno Sari,** Faculty of Pharmacy, Department of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, Indonesia

acid is generally regarded as safe (GRAS) compound that is commonly used in food industry [10, 11]. Succinic acid has two carboxylic groups at the alternate end of its carbon chain that can act as either hydrogen bond donor or acceptor. Therefore, it is predicted that succinic acid is able to form cocrystal with loratadine through two possible interactions: carboxylic acid-carbonyl group or carboxylic acid-aromatic imine hydrogen bonding [12, 13].

Various methods have been accessed to prepare a cocrystal that could be classified into the solution and the solid-state method [14, 15]. Solution method is most common and basically performed by dissolving cocrystal components (API and coformer) in a common solvent with an appropriate stoichiometric ratio. Then, cocrystal solid is obtained by evaporating the solvent or cooling the solution. Solution method allows API to interact with coformer in molecular state, suggesting complete formation of cocrystal phase. Solvent selection plays a crucial point in this method where both API and coformer need to have similar solubility in the selected solvent. If their solubilities are not similar, the less soluble compound will precipitate first [16, 17].

This research was conducted to interact loratadine with succinic acid to form a cocrystal phase. Solution method was used with methanol as the solvent. Methanol was selected since loratadine and succinic acid has been known to have similar solubility in methanol [18, 19]. Cocrystal formation was first identified via observation under polarization microscope, then further analyzed from the binary phase diagram. Physical properties of the cocrystal phase were characterized using differential thermal analysis (DTA), powder X-ray diffraction (PXRD), infrared spectroscopy, and scanning electron microscopy (SEM). Solubility study was performed as well, to acknowledge solubility modification of poorly soluble loratadine by cocrystal formation.

# Materials and methods

## Materials

Loratadine (L) was obtained from Vasudha Pharma Chem Limited, India. Succinic acid (SA) and pro-analytical methanol were purchased from Merck KGA, Germany.

## Identification of cocrystal formation

The identification was conducted using polarization microscope (Optika, Italy) equipped with a camera, which L and SA were interacted via cold contact method. Small amount of SA was placed on an object glass and was dripped with methanol to dissolve the solid. Then, the solvent was evaporated to recrystallize SA. L was placed on the other side of object glass and some drops of methanol was added on it. The solution was allowed to contact with SA and dissolve some of the solid to form a contact zone as methanol evaporated. The contact zone between L and SA was observed under a polarization microscope for growth of new crystalline phase at 40x magnification.

## Binary phase diagram study

Binary phase diagram was constructed from DTA thermograms of L-SA physical mixture samples (instrumentation of Mettler Toledo FP85 TA Cell, Switzerland). Those were prepared in various molar ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10. Endothermic peak(s) resulted from each sample was plotted against the molar ratio to obtain the phase diagram.

## Preparation of L-SA cocrystal

L and SA were weighed according to 1:1 M ratio. Each compound was dissolved in separate methanol at each saturated solubility, then two solutions were mixed homogeneously with a magnetic stirrer. The solution was evaporated at room temperature under continuous stirring. New crystalline formed then was stored in a desiccator. Physical mixture of L and SA was prepared in the same molar ratio as the cocrystal for comparison.

## Characterization of L-SA cocrystal

**Thermal analysis by differential thermal analysis (DTA):** Thermal analysis was performed using Mettler Toledo FP85 TA Cell differential thermal analyzer (Switzerland). Each sample weighed 1–3 mg was placed in a sealed aluminum pan and heated from 30 to 230 °C with 10 °C/min heating rate.

**Powder X-ray diffraction (PXRD):** Powder diffraction patterns were collected using Philips XPert MPD X-ray diffraction (Netherland) with Cu radiation. Sample was placed on a sample holder and flattened to prevent particle orientation during measurement. The condition was set as follows: 40 kV, 30 mA, divergence slit size 0.2500°, step size 0.017°, and $2\theta$ range of 5°–40°.

**Fourier transform infrared (FTIR) spectroscopy:** FTIR spectra were produced using Jasco 5300 FTIR spectrophotometer (England). Sample powder was ground homogeneously with potassium bromide (KBr, spectroscopic grade), then compacted in a hydraulic press to form a transparent disc. The disc was placed in a sample holder and scanned at wavenumber range of 4,000–400 $cm^{-1}$.

**Scanning electron microscopy (SEM):** Morphology and crystal habit of samples were characterized using Jeol JSM 840a scanning electron microscope (Japan). Sample powder was placed in a sample holder and coated with 10 nm-thickness of gold. Microscopic observation was carried out at magnification of 2,500 and in electrical condition of 20 kV and 12 mA.

### Solubility study

Solubility study was carried out using a buffer solution of citric-phosphate pH 7.0 ± 0.5 at 30 ± 0.5 °C. Each sample was weighed equivalent for 10 mg of loratadine and stirred in 40 ml buffer solution using a magnetic stirrer for 360 min. Approximately 5 ml aliquots were drawn at the time interval of 5, 10, 15, 30, 60, 180, and 360 min and filtered using a 0.45 μm membrane filter. The filtrates were analyzed using a UV–Vis spectrophotometer (Hitachi UH5300, Japan) at maximum wavelength of 247 nm to measure loratadine concentration.

## Result and discussion

Observation using a polarization microscope is one of simple ways to identify cocrystal formation in early stage. API is interacted with coformer on object glass and the zone where both compounds meet a.k.a. the contact zone is observed. If cocrystal is formed, coformer molecule is involved in the crystal lattice of API with hydrogen bonding and it will generate a new crystalline phase [10]. Cocrystal phase has different crystal from its starting compounds, as well as the way it directs polarized light. Hence, one can expect to see a change of crystal habit in the contact zone compound under polarization microscope. The original method utilizes heat to melt the lower melting compound so that the molten phase makes contact with recrystallized solid of the higher melting compound. The method is called *Koffler's hot stage* or *hot contact method* [20]. Later, modification is suggested to accommodate cocrystal identification of thermolabile compounds. Instead of heating, solvent is used to dissolve the solid of starting compounds so they interact to each other in solution state prior to recrystallization. This modified version is known as the cold *contact method* [21].

Loratadine didn't undergo recrystallization into crystalline form after melting in our laboratory. Due to this unusual behavior, identification of cocrystal formation was conducted using the cold contact method with methanol as the solvent. Recrystallized loratadine and succinic acid exhibited distinctive crystal habit as depicted in Figure 1. Drop-like shape was observed for loratadine and branched structure for succinic acid. Different habit and color of needle structure appeared in the contact zone. This new crystal habit indicates that loratadine is capable to form cocrystal with succinic acid [22].

If the microscope preparation is subsequently heated, it will show two black regions flanking the cocrystal solid that gradually spreads at rising temperature. Black region occurs as the solid melts first and liquid/molten phases allow the polarized light to pass through unchanged. Such regions represent eutectic events. Cocrystal formation is characterized with two eutectic points separating three melting point maxima [20, 23]. It is depicted in the binary phase diagram and for loratadine-succinic acid is given in Figure 2. The phase diagram exhibited melting points of pure loratadine and succinic acid at temperature of 137 and 189 °C, respectively, whilst a new phase (cocrystal) melted at approximately 120 °C. Two eutectic points were observed at 100.1 and 112.4 °C. According to the phase diagram, it is predicted that cocrystal between loratadine and succinic acid is formed in 1:1 stoichiometric ratio with melting point possibly below the starting compounds.

First characterization was performed using DTA instrumentation to analyze thermal behavior of each compound. Figure 3 shows thermograms of loratadine, succinic acid, physical mixture, and cocrystal phase. Thermogram of pure loratadine and succinic acid exhibited a single endothermic peak at temperature of 137.5 °C and 189.3 °C, respectively, due to the melting event [24–26]. Physical mixture of loratadine-succinic acid revealed two broad endothermic peaks at 120.9 and 176.6 °C. It has been known that physical mixture of cocrystal components has specific thermogram containing two endothermic peaks attributed to eutectic and cocrystal melting [27, 28]. In addition, heating rate can influence cocrystal formation in the rate and extent. Since the cocrystal phase melts in close range to both eutectic points, it is suggested that their melting events

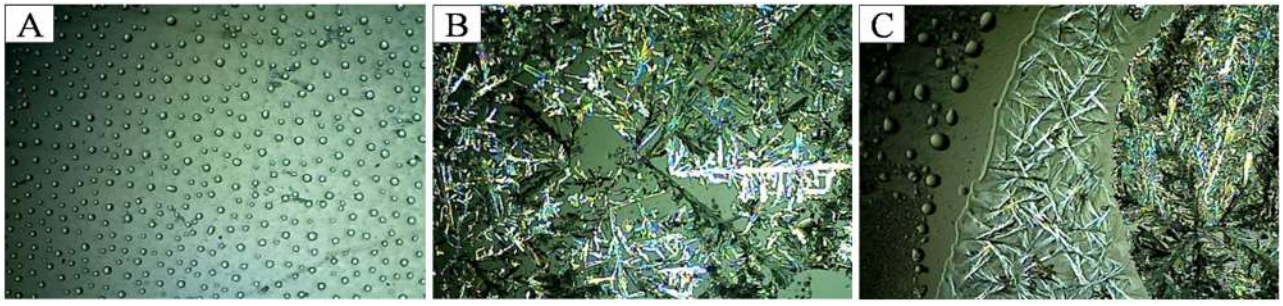


**Figure 1:** Photomicrographs of (A) loratadine, (B) succinic acid, and (C) the contact zone of loratadine-succinic acid under polarization microscope observation.

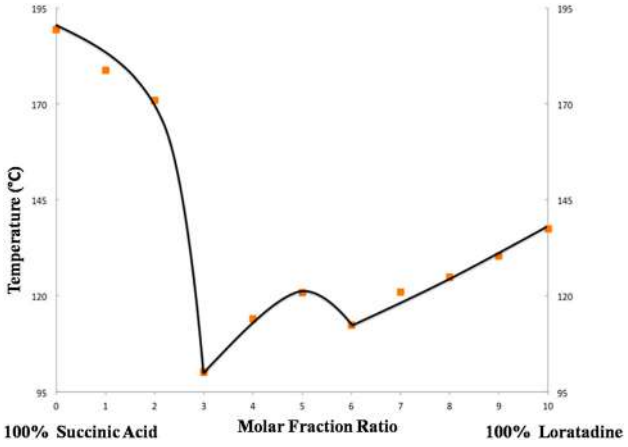


**Figure 2:** The binary phase diagram of loratadine and succinic acid.

occur almost at the same time and their endothermic peaks become one broad peak observed around 120 °C. Considerably fast heating rate may also contribute to the result and the appearance of succinic acid's endothermic peak [27]. The second peak at higher temperature is given by succinic acid that shifts to lower temperature due to the existence of cocrystal phase. On the other hand, the cocrystal sample exhibited one new sharp endothermic peak at 110.9 °C. Melting point is related to crystal lattice energy, so any change in melting point indicates an alteration to lattice structure [29]. It is relevant with the cocrystal case because interaction between API and coformer molecule in crystal lattice affects its internal structure.

X-ray diffraction was used to characterize crystal lattices that are affected in cocrystal formation. Powder diffractograms of loratadine, succinic acid, physical mixture, and cocrystal phase are presented in Figure 4. Loratadine has been recognized for specific diffraction peaks at 2$\theta$ of 6.37°, 7.53°, 12.70°, 15.02°, 16.41°, 19.48°, 21.22°, and 24.34° [30]. As for succinic acid, the diffractogram showed distinctive peaks at 2$\theta$ of 20.12°, 26.24°, and 31.62° [31]. Diffraction pattern of both loratadine and succinic acid was observed to be superimposed to each other in the diffractogram of physical mixture, suggesting no interaction between these compounds. On the contrary, there were noticeable new peaks in the diffractogram of cocrystal phase, at 2$\theta$ of 5.28°, 10.09°, 12.06°, 15.74°, 21.89°, and 28.59°. Each diffraction peak at 2$\theta$ value represents a certain crystal lattice inside of molecule's structure. Therefore, any appearance (or disappearance) of diffraction peaks is responsible for alteration in the crystal lattice. It is plausible in cocrystal formation as interaction takes place between API and coformer molecule [32].

To identify the interaction involved in cocrystal formation of loratadine and succinic acid, IR analysis was carried out. IR spectra are given in Figure 5 for loratadine, succinic acid, physical mixture, and cocrystal phase. Loratadine has a carbonyl and an aromatic imine group which is characterized by C=O and C=N vibration at 1,703 and 1,643 $cm^{-1}$, respectively [33]. Succinic acid exhibits IR spectrum with broad peak at 3,000–2,500 and sharp one at 1,695 $cm^{-1}$ due to O–H stretching and carbonyl group, respectively [22]. Superimposition of absorbance of both compounds occurred in the physical mixture spectrum, whereas in the cocrystal spectrum, C=O vibration of loratadine shifted to 1722 $cm^{-1}$ and absorbance band of the O–H region changed in shape. The result suggests that hydrogen bond is involved in cocrystal formation between loratadine and succinic acid. The hydrogen bonding is

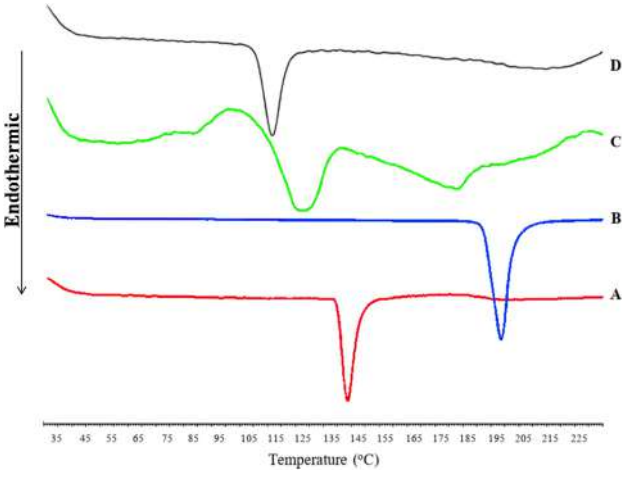


**Figure 3:** Thermograms of (A) loratadine, (B) succinic acid, (C) physical mixture, and (D) cocrystal phase.

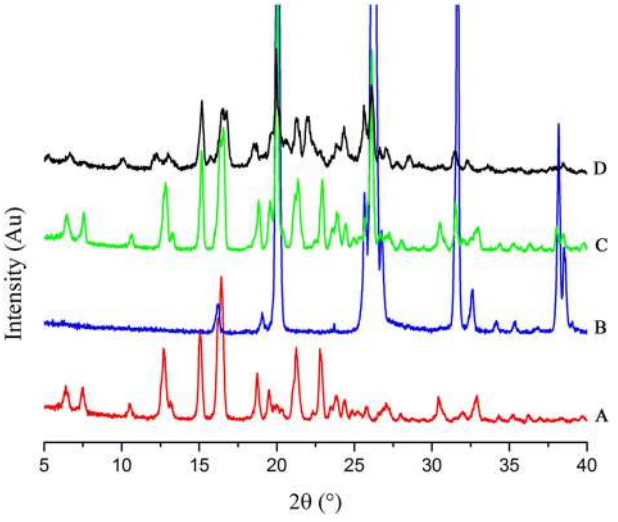


**Figure 4:** Powder diffractograms of (A) loratadine, (B) succinic acid, (C) physical mixture, and (D) cocrystal phase.

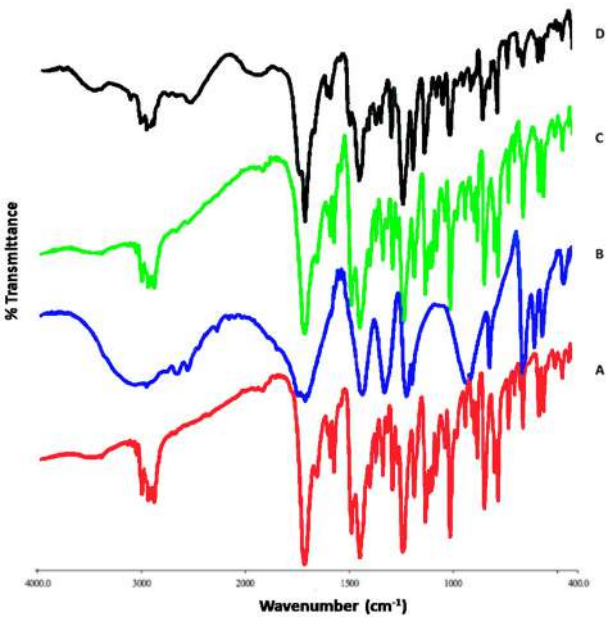


**Figure 5:** IR spectra of (A) loratadine, (B) succinic acid, (C) physical mixture, and (D) cocrystal phase.

predicted to occur through interaction of carbonyl group of loratadine and hydroxyl group of succinic acid with the latter acts as hydrogen bond donor to the former (a hydrogen bond acceptor).

Microscopic observation using SEM was conducted to evaluate morphology properties of loratadine, succinic acid, and cocrystal phase. It was observed that loratadine had a columnar shape [30] while succinic acid exhibited layered-structure [34], as depicted in Figure 6. Cocrystal phase itself showed a small prismatic shape as aggregated particles that was possibly contributed by continuous stirring during solvent evaporation. It is not uncommon to obtain cocrystal solid in a smaller particle size [35, 36]. Rather, it is desirable in cocrystal formation for its solubility advantage. Every compound has specific crystal packing which is expressed in different crystal habits. Hence, morphological alteration can be used as complementary evidence toward cocrystal formation.

According to characterization results above, it is known that a cocrystal phase has lower melting point even

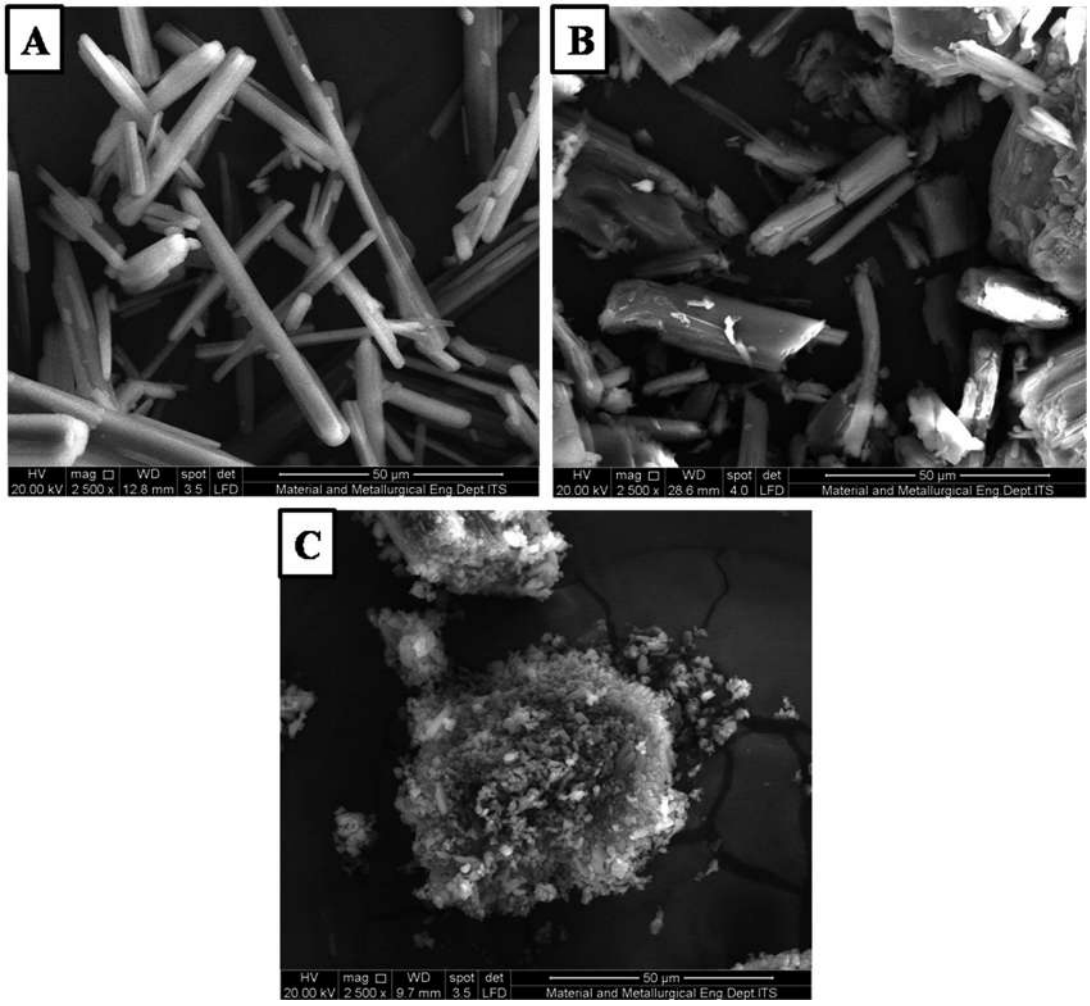


**Figure 6:** Photomicrographs of (A) loratadine, (B) succinic acid, (C) cocrystal phase.

in comparison to pure loratadine. Melting point is often associated with energy level of crystal lattice: the lower the melting point, the lower energy needed to break the lattice itself. Therefore, alongside with smaller particle size, the cocrystal phase of loratadine-succinic acid is expected to dissolve easily in water [37–39]. Our solubility study (pH 7) showed an improvement in loratadine solubility profile in the cocrystal phase. Constant solubility of loratadine was reached at 2.46 mg/l after 360 min. By the end of study, loratadine solubility from physical mixture and cocrystal phase fell around the value which cocrystal phase was slightly higher at 2.74 mg/l. However, they yielded a very different profile as shown in Figure 7. Unlike pure loratadine that reached its constant solubility within 15 min, physical mixture and cocrystal phase increased loratadine solubility up to 2-fold in the same time interval. Physical mixture dropped loratadine solubility drastically at 60 min, but the cocrystal phase was able to maintain loratadine solubility at higher level in longer time until it decreased gradually to the constant value.

Solubility profile of physical mixture sample may be described by the existence of more soluble compound of succinic acid. Loratadine interacts with succinic acid by unspecified interaction limited to the surface area where both compounds are in contact. Thereby, higher solubility of loratadine is followed by sudden drop to the constant value. On the other hand, specific hydrogen bonding links loratadine to succinic acid so that solubility improvement of the cocrystal phase follows the "spring and parachute" concept [40]. Cocrystal phase is proposed to generate transient highly soluble loratadine due to weak hydrogen bonding that also contributes to fast dissociation of loratadine and succinic acid molecules. As the more soluble compound, succinic is extracted out first into the aqueous medium, causing the crystal lattice to collapse to an amorphous state. Amorphous loratadine provides higher aqueous solubility (the spring) yet quickly undergoes transformation to a metastable polymorph then to a stable polymorph following the Ostwald's Law of Stage. Succinic acid acts as an inhibitor toward the transformation process to maintain high solubility of loratadine in long period of time (the parachute). Maintaining loratadine solubility in high level for long enough time leads to complete absorption in the gastrointestinal tract and eventually will improve the bioavailability of loratadine [40, 41]. This will increase loratadine activity thereby ensuring health and promote well-being for all people.

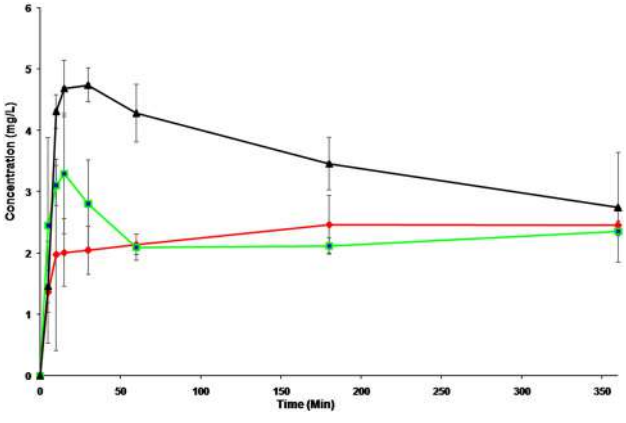


**Figure 7:** Solubility profile of loratadine, physical mixture, and cocrystal phase of loratadine-succinic acid.

## Conclusion

Cocrystal is a promising approach to modify poor solubility of loratadine. It has been confirmed that loratadine interacts with succinic acid to form cocrystal phase in 1:1 stoichiometric ratio. Hydrogen bonding is involved between the carbonyl group of loratadine and the carboxyl group of succinic acid and in C=O and H–O interaction. Cocrystal phase exhibits different crystal lattice with lower melting point and smaller particle size. Responding to those properties, cocrystal phase shows improved loratadine solubility as well. The solubility improvement can be illustrated by "the spring and parachute" concept that the spring is achieved by amorphous state of loratadine and the parachute is when high solubility of loratadine is maintained long enough by succinic acid.

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Herry Wibowo*, Prihartini Widiyanti and Syaifullah Asmiragani

# The role of chondroitin sulfate to bone healing indicators and compressive strength

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**Abstract**

**Objectives:** The function of bone is to protect the vital organs of the body. Mechanical strength, especially compressive strength, plays an important role in fulfilling its function. Fracture healing depends on several substances, such as collagen, glucosaminoglycane and proteoglycan. Chondroitin sulfate as part of proteoglycane is an important component in the formation of callus in fracture healing. The aim of this study is to prove chondroitin sulfate role in supporting fracture healing.

**Methods:** The *in vivo* experiment has been performed to *Rattus novergicus* which met the inclusion criteria (age 3 months, 200–300 g weight), 18 males of *R. norvegicus*, Wistar strain, were divided into three equal groups of six rats each. After being anesthetized, fracturation was performed in a sterile manner to get simple fracture. The area of dissection is in half length of tibial bone and the fracture incision is about 1 cm. Then it followed by immobilization of the lower leg bone on one side with a cast. The first group was given chondroitin sulfate 7 mg in 2 mL distilled water/200 g weight for 2 weeks. The second group was given chondroitin sulfate 7 mg in 2 mL distilled water/200 g weight for 4 weeks. The third group was given distilled water. This research was focused on treatment of cartilage. The callus position is in half length of tibial bone.

**Results:** There were significant differences in the increase of TGF-β, the number of osteoblasts and callus compressive strength in the groups with chondroitin sulfate treatment for 2 and 4 weeks, compared to the control group ($p<0.01$).

**Conclusions:** Administering chondroitin sulfate in a dose of 7 mg in 2 mL distilled water for 2 and 4 weeks may increase production of TGF-β, the osteoblast numbers and the callus compressive strength in fracture healing.

**Keywords:** callus compressive strength; chondroitin sulfate; osteoblast number; *Rattus novergicus*; TGF-β.

***Corresponding author: Herry Wibowo**, Laboratory of Physiology, Department of Biomedical, Faculty of Medicine, Universitas Surabaya, Raya Rungkut, Kalirungkut, Surabaya, 60293, Indonesia, E-mail: drherrywibowo@staff.ubaya.ac.id
**Prihartini Widiyanti,** Biomedical Engineering Study Program, Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia; and Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
**Syaifullah Asmiragani,** Department of Orthopaedic and Traumatology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

## Introduction

The high number of traffic accidents in modern life results in an increasing incidence of bone fractures, the healing of which can include complications, such as malunion, delayed union and nonunion. Handling fractures cannot succeed without a full understanding of the cellular mechanisms of bone healing. There are several factors that can affect the healing of broken bones on which to base a rationalization of fracture therapy. Things which play important role in fracture healing consist of local and systemic factors. The local factors are including degree of local trauma and bone loss, type of bone affected, degree of immobilization and local pathologic conditions while the systemic factors are including age, hormones, local stress and electric currents.

One of the factors influencing healing is indicated by the presence of collagen type 1, type 2, type 3, glycosaminoglycans (GAGs) and proteoglycans deposition. Heparan sulfate, dermatan sulfate and chondroitin sulfate proteoglycans are three of the important components of callus formation in the first 1–2 weeks of fracture healing [1, 2].

Supplementation of oral chondroitin sulfate may reduce cartilage matrix degradation process components, especially collagen II, GAGs and other proteoglycans [3, 4].

Karacal N et al. [5] found that chondroitin sulfate plays a role in accelerating bone healing. In this study, oral administration of chondroitin sulfate has the same effect on bone fracture healing and callus formation as local therapy directly [3, 4]. The healing process of bone, called regeneration in fractures, is a complex process, and multifactorial. Changes that occur in fracture healing are a series of phases which include the instantaneous phase of the inflammatory reaction, the development of the osteogenic tissue formation of callus, and the soft and hard callus remodeling phase [6].

Chondroitin sulfate proteoglycans act as a major contributor in the early phase of biochemical bone healing.

Previous research by Liang [7] on the mechanical characteristics, such as modulus of elasticity, compressive strength and tensile strength to the scaffold, found that the role of chondroitin sulfate in forming callus strength and compressive strength *in vivo* is an unclear mechanism. Associated with the bone healing, chondroitin sulfate appeared to accelerate the rate of bony repair [8] but did not affect the ultimate quantity or quality of bone produced. In this research, we examine the effect of chondroitin sulfate on the bone healing parameter process including the number of osteoblasts, TGF-β and callus compressive strength. Osteoblast as bone forming cell play important role in bone healing process. TGF-β which has physiological role in the processes of proliferation, differentiation and synthesis of cartilage and bone tissue, collectively known as the bone healing process. Callus as bony and cartilaginous material forming a connecting bridge across a bone fracture during repair is the marker of bone healing. Within 1–2 weeks after injury, a provisional callus forms, enveloping the fracture site. Callus compressive strength could illustrate the condition in fracture site whether it is leading to bone healing.

# Materials and methods

Eighteen rats *Rattus novergicus* strain Wistar were anesthetized with ether solution using a hood and titration method. After sedation and minimal movement of the rats, hair removal was performed in the lower leg area. After showing the skin, then disinfection with povidone-iodine and narrowing the field of operation with sterile Doek. Fracturation was performed in a sterile manner, using sharp scissors to get a simple fracture, followed by immobilization of the lower leg bone on one side with a cast. Chondroitin sulfate was administered in 7 mg/200 g b.w./day via Nasogastric (NG) tube in the treatment groups. The duration of time for group 1 is 14, 28 days for group 2 and group 3 was given a placebo in the form of a solution of 0.5% Sodium/Natrium Carboxymethyl Cellulose (CMC Na) via NG tube with a volume equal to that of the solution of chondroitin sulfate. On day 28, the rats were anesthetized with ether and 5 cc of blood taken directly from the heart. Blood samples were placed in EDTA tubes and sent to the laboratory for examination of physiology levels of TGF-β. TGF-β levels in the control group and both treatment groups were measured by the ELISA technique. The lower leg bones containing bone callus were examined by the anatomical pathology lab, and the strength of the callus was determined using the Shimadzu Autograf. Quantifications were performed at 40× magnification to determine the number of osteoblast cells, the levels of TGF-β in the serum were measured, and the compressive strength of the callus was measured with a three-point bending test method using a Shimadzu Autograf on the incipient fracture healing, and these values were used in a descriptive analysis. The number of osteoblasts, the levels of TGF-β in blood serum and the callus compressive strength of each group were used to perform the statistical test, an F-test statistical analysis by ANOVA using SPSS. Significance was established based on the value of p compared with α (5%). All of the research treatments in animal model has been approved by Ethical Committee of Health Research Rumah Sakit Umum Daerah Dr Saiful Anwar Malang, East Java, Indonesia with number of certificate 400/CVVIII/K.3/302/2013.

# Results

The body weight of rats in all groups showed values greater than the significance level of p≥0.05 meaning the data are homogeneous and appropriate for use in the research hypotheses by using a one-way ANOVA test.

## Levels of TGF-β

TGF-β levels in the control group and both treatment groups were measured by the ELISA technique. TGF-β attachments levels measured in the control group and the groups receiving chondroitin sulfate administration for 2 and 4 weeks are shown in Figure 1.

## Osteoblast cell count

The number of osteoblasts in the control group and the treatment groups receiving chondroitin sulfate for 2 and 4 weeks were analyzed histologically at 40× magnification. The number of osteoblasts for the three groups is shown in Figure 2.

## Compressive strength of callus

The compressive strength of callus in the control group and both treatment groups was measured with a Shimadzu Autograf. The results for the three groups are shown in Figure 3.

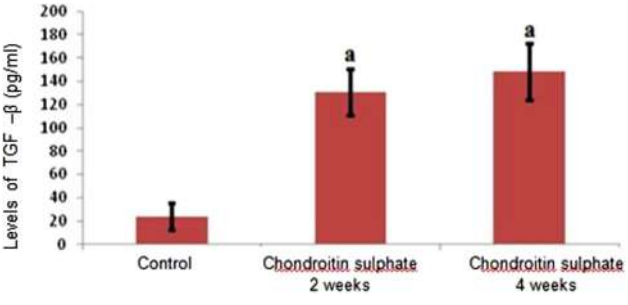


**Figure 1:** Diagram of stem histogram levels of TGF-β in the control and treatment groups.
Description: p<0.01 compared to control group. Remarks: pg/mL (picogram/mL).

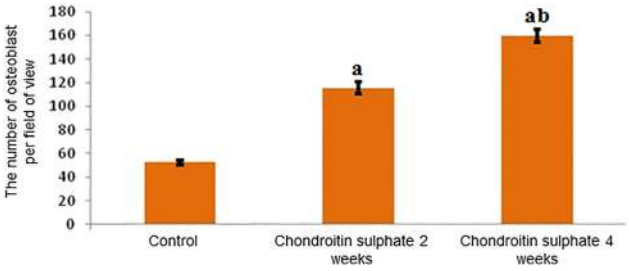


**Figure 2:** Diagram of the stem (histogram). The number of osteoblasts in the control and treatment groups.
Description: ap<0.01 compared to control group; bp<0.01 compared to group administered chondroitin sulfate for 2 weeks.

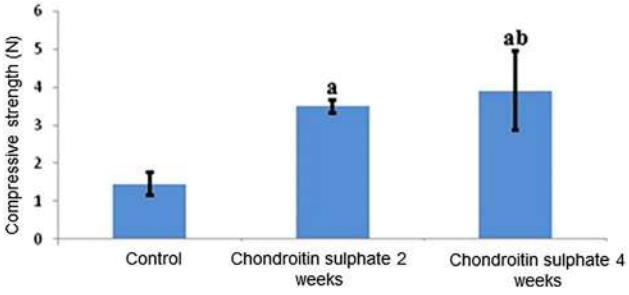


**Figure 3:** Diagram of the stem (histogram) compressive strength values of callus in the control and treatment groups.
Description: ap<0.01 compared to control group; bp<0.01 compared to group treated with chondroitin sulfate for 2 weeks. Description: Newton (N).

## Discussion

Fracture healing is a complex process consisting of a variety of cellular events in a highly coordinated sequence of acute inflammation, resolution of inflammation, soft callus formation (cartilage) and hard callus (woven bone), angiogenesis, remodeling callus and laminar bone formation to bridge a gap. Woven bone supports forming the callus that bridges the fracture gap providing stability during the early healing process. Wovenbone is always remodeled and replaced by normal lamellar bone, which provides better mechanical stability [9].

TGF-β is a molecule that is responsible for the quality of the matrix [10]. Increasing levels of TGF-β in the treatment groups given chondroitin sulfate for 2 and 4 weeks as compared to the control group is very significant (p<0.01), as shown in Figure 1. This indicates that chondroitin sulfate can modulate the synthesis of TGF-β in the fracture healing process. TGF-β is secreted by platelets in a hematoma in the early stages by osteoblasts on intramembranous ossification, and again when the last chondrogenesis chondrocytes and endochondral ossification takes place [11]. The biological activity of chondroitin sulfate involves a variety of growth factors and chemokines, and the function is highly correlated with the sulfation pattern and characteristics of the polysaccharide chain [12]. TGF-β is also bound by chondroitin sulfate-containing proteoglycans such as biglycan and decorin. Pathway of chondroitin in stimulating TGF-β release through the role of decorin and biglycan which can modulate TGF-β-dependent cellular responses, apparently in a tissue-specific manner [13].

There is also evidence in the treatment groups given chondroitin sulfate of increasing osteoblasts, and this is extremely meaningful compared to the control group (p<0.01), as shown in Figure 2. This is consistent with the theory that chondroitin sulfate is involved in the adhesion of osteoblasts [14, 15]. Chondroitin sulfate is a major component of cartilage proteoglycans such as aggrecan. These proteoglycans, decorated with GAG chains, are well known for their mechanical and biological functions in articular cartilage [16].

Precursors of osteoblasts are multipotent mesenchymal stem cells such as bone marrow stromal cells, chondrocytes, muscle cells and adipocytes [17, 18]. Osteoblast progenitors not only of the original mesenchymal stromal progenitors in the marrow, but also mesenchymal cells attached to the endothelial lining of blood vessels. Osteoblast precursors reach bone from the circulation, and have an average lifespan of about 3 weeks. Osteoblasts produce and secrete matrix proteins fully differentiated from bone. Furthermore the mineralized matrix will be under the control of osteoblasts [17].

Chondroitin sulfate in both treatment groups increased the compressive strength of callus significantly compared to the control group (p<0.01) (Figure 3). Chondroitin sulfate can stimulate proliferation and differentiation of osteoblasts involved in fracture healing. In addition, there is the possibility that chondroitin sulfate can increase the lifespan of osteoblasts [18, 19]. Bone matrix is a mixture of fibers (fibrils of collagen type I) to withstand the force of the pull, and solid particles (hydroxyapatite crystals) to withstand compression [20]. Between the 2nd and 3rd weeks of fracture healing, callus stiffness will increase and angular displacement to the point of failure decreases. The result of this research is in accordance with the research of Schneiders W [21] which showed an increase in compressive strength by 15% by addition of chondroitin sulfate. Chondroitin sulfate is binding to the core protein through *N*- and *O*-linkages leads to aggregates of monomers with high molecular weights. The proteoglycan aggregate showed viscoelastic and hydration properties [22].

Karaçal N et al. [5] found that chondroitin sulfate plays a role in accelerating bone healing. Therefore, 3 weeks is the time when the early healing callus formed a bridge, and

removed cartilage [6]. In this study, the compressive strength of the callus increased when chondroitin sulfate was administered for a longer period of time (4 weeks). This indicates that chondroitin sulfate is capable of improving the transition of cartilage in the center of the callus into a bony callus bridge. Thus, chondroitin sulfate can accelerate mineralization and bone repair [23].

Chondroitin, better known as chondroitin sulfate, is a glycosaminoglycan (GAG) composed of a sulfated chain of branched sugars (*N*-acetylgalactosamine and glucuronic acid). It is usually found attached to proteins as part of a proteoglycan compound. A chondroitin chain can have over 100 individual sugars that can sulfated in every part of the variable. Chondroitin sulfate is an important structural component of cartilage and tissue with a constituent role in increasing resistance to stress [24]. Along with glucosamine, chondroitin sulfate is widely used as a dietary supplement to prevent osteoarthritis [25].

The oral dose of chondroitin for use in human clinical trials is 800–1,200 mg per day. Some other sources such as shark cartilage, fish and poultry have been used. Due to chondroitin not being a uniform substance and naturally arising in many variations and forms, the composition of each supplement can differ markedly. This can be caused by supplement manufacturers not having to meet the Good Manufacturing Process (GMP) standards required for human food nor the standards for the manufacture of pharmaceuticals. No significant effects of overdoses of chondroitin have been reported for long-term use. The European League Against Rheumatism (EULAR) has confirmed that chondroitin sulfate is one of the safest drugs for osteoarthritis [26]. The provision of chondroitin sulfate for 2 or 4 weeks on healing fractures in the treatment groups was shown to increase the production of TGF-β, the number of osteoblasts and the compressive strength of the fracture healing callus.

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**Competing interests:** No conflict of interest.

**Informed consent:** This research do not use human subject. There is no need to have informed consent.

**Ethical approval:** This research has been approved by Health Ethic Committee Saiful Anwar General Hospital Malang, East Java, Indonesia with number 400/CVVIII/K.3/302/2013.

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Jamal Nasser Saleh Al-maamari, Mahardian Rahmadi*, Sisca Melani Panggono, Devita Ardina Prameswari, Eka Dewi Pratiwi, Chrismawan Ardianto, Santhra Segaran Balan and Budi Suprapti

# The effects of quercetin on the expression of SREBP-1c mRNA in high-fat diet-induced NAFLD in mice

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**Abstract**

**Objectives:** The study aimed to determine the effect of quercetin on the expression of primary regulator gene involved in lipogenesis and triglycerides synthesis in the liver, and the sterol regulatory binding protein-1c (SREBP-1c) mRNA in non-alcoholic fatty liver disease (NAFLD) with a high-fat diet (HFD) model.

**Methods:** Fifty-six Balb/c mice were divided into seven groups: standard feed; HFD; HFD and quercetin 50 mg/kg for 28 days; HFD and quercetin 100 mg/kg BW for 28 days; HFD and quercetin 50 mg/kg for 14 days; HFD and quercetin 100 mg/kg for 14 days; HFD and repaired fed for 14 days. Quercetin was administered intraperitoneally. The animals were sacrificed 24 h after the last treatment; the liver was taken for macroscopic, histopathological staining using hematoxylin–eosin and reverse transcription-PCR analysis sample.

**Results:** HFD significantly increased the expression of SREBP-1c mRNA; meanwhile, quercetin and repaired feed significantly reduced the expression of SREBP-1c mRNA in the liver. Quercetin at a dose of 50 mg/kg and 100 mg/kg also improved liver cells' pathological profile in high-fat diet NAFLD.

**Conclusions:** The present study suggests that quercetin has an inhibitory effect on SREBP-1c expression and improved liver pathology in NAFLD mice.

***Corresponding author: Mahardian Rahmadi,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +6281224656516,
E-mail: mahardianr@ff.unair.ac.id
**Jamal Nasser Saleh Al-maamari,** Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, E-mail: jamal-2019@ff.unair.ac.id
**Sisca Melani Panggono, Devita Ardina Prameswari, Eka Dewi Pratiwi, Chrismawan Ardianto, Santhra Segaran Balan and Budi Suprapti,** Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

**Keywords:** non-alcoholic fatty liver disease; quercetin; healthy lifestyle; high-fat diet; SREBP-1c.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) has been the primary trigger of chronic liver disease in Western countries. It is predicted to become the most frequent cause of liver transplantation by 2030 [1]. NAFLD affects approximately 30% of the United States, Middle East, and South America population. Meanwhile, the prevalence of the disease in European and Asian countries is about 24 and 27% with the highest incidence in East Asia [2]. It shows that 20% of steatosis is due to metabolic causes rather than alcohol intake in Shanghai. NAFLD spread in South Asia and Southeast Asia, namely India, Sri Lanka, Malaysia, Singapore, and Indonesia, differing from 9 to 45%. The lowest estimations (8.7–18%) usually come from more impoverished and physically active rural areas [3].

NAFLD is a liver disorder that appears in patients with an accumulation of lipids in hepatocytes weighing more than 5% of the liver weight, without the hepatitis B virus, hepatitis C virus, and excessive ethanol intake (less than 20 g of ethanol per week) [4]. While NAFLD's pathogenesis remains unclear, a "two-hit" hypothesis has been proposed to explain the NAFLD development. In this hypothesis, the "first hit" is hepatic steatosis due to excess fat accumulation in hepatocytes conjugated by increased lipogenesis and fatty acid absorption, inhibition of β-oxidation, impaired triglyceride (TG) clearance, excessive fat buildup, and lipid homeostasis disruption in the liver. All of these contribute to the pathologic condition of NAFLD [5]. Furthermore, the liver's oxidative stress and inflammation are two crucial factors of the "second hit" that cause tremendous liver cell damage. Several attacks, including genetic mutations and gut microbiota, develop NAFLD, which progresses to non-alcoholic steatohepatitis (NASH) and cirrhosis [6]. ROS's excessive production affects antioxidants and decrease oxidative stress's clearance, which stimulates the production of sterol regulatory element-binding protein-1c (SREBP-1c) and lipogenesis [7].

SREBP-1c is one of the primary regulators of gene expression involved in hepatic triglyceride synthesis. In different ways, an excessive SREBP-1c activity results in a pathological increase in fat synthesis and leads to hepatic fat accumulation (steatosis) [8]. SREBP-1c is a critical transcription factor that regulates hepatic TG synthesis by inducing enzymes involved in hepatic lipogeneses, such as fatty acid synthase, stearoyl-coenzyme a desaturase 1 (SCD-1), and acetyl-CoA carboxylase, which then, will encourage the synthesis of triglycerides in the liver [9]. Furthermore, lipogenesis is regulated by several transcription factors, including sterol regulatory element-binding protein 1 (SREBP-1c) [8].

To date, there are no drugs receiving FDA approval for NAFLD or NASH [6]. Clinical therapeutic approaches mainly focus on dietary change, lifestyle and physical activities, hyperglycemia correction, insulin tolerance, and hyperlipidemia, which are NAFLD-related metabolic disorders. It can improve therapeutic approaches to reduce NAFLD risk by understanding the molecular mechanisms responsible for lipid accumulation, impaired oxidative balance, mitochondrial dysfunction, and fibrosis in the liver. A promising emerging therapeutic strategy for patients suffering from hepatic steatosis is antioxidant compounds that modulate lipogenesis, lipid oxidation and peroxidation, and inflammation [10].

Quercetin is a flavonoid compound found in lettuce, chili, cranberries, onions, tomatoes, broccoli, and apples [11]. Research conducted on the NAFLD model type 2 diabetes shows that quercetin can be avoided or cure many diseases caused by oxidative stress. Because of its antioxidant properties, quercetin can eliminate ROS, such as peroxyl radicals, hydrogen peroxide, superoxide, and oxygen [12]. It is also known that quercetin has hepatoprotective effects and can reduce fat accumulation in the liver (steatosis) in diabetic rats [13]. Therefore, quercetin has the potential to be developed as a pharmacological agent to treat NAFLD.

This study aims to determine the effect of quercetin administration on the expression of SREBP-1c in the liver of NAFLD mice with a high-fat diet (HFD) model and to provide an overview regarding the mechanism of quercetin in treating and preventing the progression of NAFLD. Furthermore, this study is expected to contribute to the pharmaceutical sector in the formulation of different quercetin dosage forms as a drug to treat NAFLD and complement existing therapies.

# Materials and methods

## Materials

All the materials used in the experiment were of analytical grade. The quercetin was purchased from Tokyo Chemical Industry (Japan). Quercetin was given to treatment groups (3, 4, 5, and 6) at a dose of 50, 100 mg/kg body weight intraperitoneal with a total volume of not more than 10 ml/kg body weight mice. Quercetin is prepared every day by dissolving 20 mg of quercetin in 2 ml of the vehicle (5% tween 80 of the total volume). The composition of the HFD feed consists of 60% beef fat, which has been melted at 65 °C using a hotplate that is sufficient (mixed) to 100% with a standard feed that has been mashed.

## Animal and diets

Fifty-six Balb/c male mice adaptation was carried out for one week before being used in the study. Mice were placed in groups in cages measuring 20 × 20 × 15 cm and covered with 6 mm gauze. All test animals were reared in the same way and given the same amount of food. All of them were kept in a well-ventilated room whose temperature is around 22 ± 2 °C. Their cages were cleaned every day and replaced with new husks every three times a week and lighting is regulated on a 12-h light/dark cycle. The composition of the HFD consists of 60% of beef tallow (902 kcal/100 g) and 40% of standard feed (306.20 kcal/100 g). All experiments were performed at the Animal Research Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia. The protocol was approved by the Ethical Committee (Animal Care and Use Committee) of the Faculty of Veterinary Medicine, Universitas Airlangga (No. 2.KE.076.05.2019).

## Treatment protocol

In this experimental study, 56 mice were used and randomly divided into seven groups (n=8), as the following: (1) mice fed with a standard feed and treated with 5% polysorbate 80 (vehicle) for 28 days (standard feed group); (2) mice fed with an HFD and treated with vehicle for 28 days (HFD group); (3) mice fed with an HFD and treated with quercetin (quer) 50 mg/kg BW i.p. for 28 days; (4) mice fed with an HFD and treated with quer 100 mg/kg BW i.p. for 28 days; (5) mice fed with an HFD for 28 days and treated with a quer 50 mg/kg BW i.p. from the 15th day to the 28th day; (6) mice fed with an HFD for 28 days and treated with a quer 100 mg/kg BW i.p. from the 15th day to the 28th day; and (7) mice fed with an HFD for 14 days and switched to standard feed from the 15th day to the 28th day (HFD + repaired feed). In brief, the division of treatment groups in experimental animals is present in Table 1. At the end of the study, after 28 days of treatment and 24 h of the last treatment, all mice in each group were euthanized and the liver was extracted and put in the freezer −80° for further analysis.

## Liver macroscopic evaluation and histopathological examination

The macroscopic evaluation of the liver was carried out visually. To evaluating hepatic histopathological steatosis, hematoxylin-eosin (H&E) staining was used. In short, liver tissues were fixed and stored in a sample container at 4 °C in 4% paraformaldehyde, routinely processed and embedded in paraffin, prepared into 5 μm thick sections and stained for 8 min hematoxylin, washed for 60 min in flowing tap water, counterstaining at room temperature with eosin for 60 s. The steatosis can be characterized by the presence of lipid droplets, ballooning, and inflammation on the histopathological of liver tissue under a light microscope (magnification, ×400; Nikon Company, Tokyo, Japan).

**Table 1:** Grouping of experimental animals and their treatment.

| Group | Diet | Treatment |
|---|---|---|
| Group 1 | Standard feed (28 days) | Vehicle (5% polysorbate 80) for 28 days (from 1st day of feeding) |
| Group 2 | High-fat diet (28 days) | Vehicle (5% polysorbate 80) for 28 days (from 1st day of feeding) |
| Group 3 | High-fat diet (28 days) | Quercetin 50 mg/kg BW i.p. for 28 days (from 1st day of feeding) |
| Group 4 | High-fat diet (28 days) | Quercetin 100 mg/kg BW i.p. for 28 days (from 1st day of feeding) |
| Group 5 | High-fat diet (28 days) | Quercetin 50 mg/kg BW i.p. for 14 days (from 15th day of feeding) |
| Group 6 | High-fat diet (28 days) | Quercetin 100 mg/kg BW i.p. for 14 days (from 15th day of feeding) |
| Group 7 | High-fat diet (14 days) | Repaired feed for 14 days (HFD was discontinued and switched with standard feed from 15th day of feeding) |

### RNA extraction and reverse transcription-PCR analyses

Measurement of SREBP-1c mRNA relative expression was obtained from the liver tissue measurement using the reverse transcription-polymerase chain reaction (PCR) method. The animals were sacrificed and the largest lobe was taken from the liver. Total RNA was isolated from samples using the PureLink® RNA Mini Kit. The results of RNA isolation were calculated with the Quantus tool. SuperScript® III First-Strand Synthesis System is used in the Reverse Transcription stage using reverse transcription-PCR. The following primers were used: SREBP-1c (Forward: 5′-CGCTTCTTACAGCACAGC-3′; Reverse: 3′-TGCCCAAGGACAAGGGGCTA-5′) and β-actin (Forward: 5′-TGTTACCAACTGGGACGACA-3′; Reverse: 3′-AAGGAGGCTGGAAAAG AGC-5′). The PCR standard used is CSL-JDNA.

### Data analyses

The data obtained from this study were analyzed using the GraphPad Prism program version six through the following analysis: Data on the relative expression of SREBP-1c mRNA were analyzed for differences between all groups using the one-way analysis of variance (ANOVA) method with a 95% degree of confidence with $p<0.05$ used to determine statistical significance and followed by a follow-up post hoc test (Tukey's test for multiple comparisons).

# Results

## Effect of quercetin on bodyweight profile of experimental animals

All mice were weighed every three days. Figure 1 below shows that during the 28 days of treatment, there was an increase in body weight of about 5% in the group of mice fed standard and a decreased bodyweight of about 25% in the group of mice given HFD. The group of mice given HFD and quer 50 or 100 mg/kg BW for 28 days experienced a weight loss of about 25%. While the group of mice given HFD for 28 days and treated with a quer 50 mg/kg BW from day 15 to day 28 experienced a weight loss of more than 25% and the group of mice given HFD for 28 days and treated with a quer 100 mg/kg BW from day 15 to day 28 experienced a weight loss of about 20%. Moreover, in the group of mice that were given HFD for 14 days, it was observed that until the 14th day they experienced a weight loss of more than 15%, the treatment was continued with standard feeding (switched) without quercetin administration until day 28. This group of mice experienced a weight gain body until it returns to the baseline. Thus, based on all of these results, it shows that giving HFD can reduce body weight in mice (Figure 1).

## Effect of quercetin on the profile of experimental animal food intake

The amount of food intake in the group of mice receiving HFD (group 2) and the group of mice receiving HFD and quercetin (group 3, 4, 5, and 6) was lower than that of mice given standard feed (group 1) in weeks I, II, III, and IV. Whereas in the group that received HFD for 14 days and then, switched to standard feed from day 15–28 (group 7) experienced an increase in food intake in grams at weeks III and IV. However, there was no significant difference in the experimental animal food intake in grams in those two groups at the week I, II, III, and IV (Figure 2A).

The caloric consumption in the group of HFD (group 2) and in the groups of HFD + quercetin (group 3, 4, 5, and 6) was greater than the intake in normal mice in weeks I, II, III, and IV. Whereas, in the mice in group 7 that were given

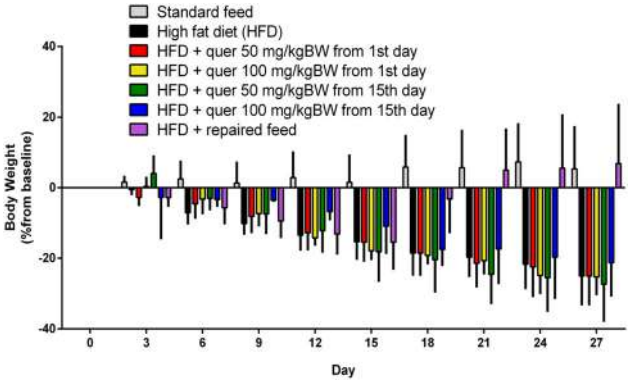


**Figure 1:** Bodyweight profile of each group of tested animals. Each bar represents the mean ± standard error of the mean (SEM) of the six experimental animals per group.

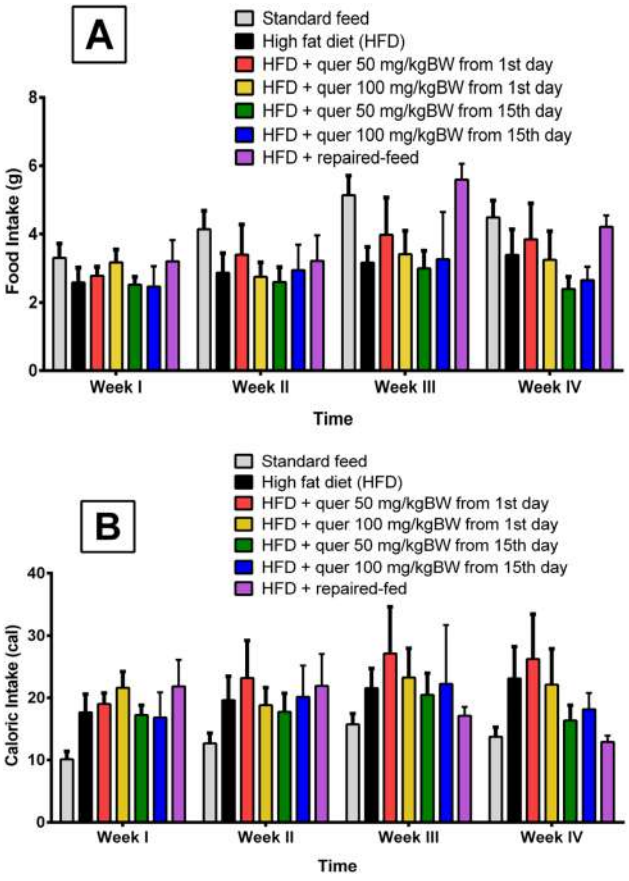


**Figure 2:** Food intake profile of each group of tested animals for 28 days (units of grams [A]; units of calories [B]). Each bar represents the mean ± SEM of the six experimental animals per group.

HFD for 14 days, the calorie consumption in week III and IV declined from day 15–28 on standard feed. Nevertheless, there were no major variations in the calorie consumption of experimental animal feed among the groups of weeks I, II, III, and IV (Figure 2B).

## Effect of quercetin on the macroscopic liver of experimental animals

The results of the macroscopic observation showed that the test animals receiving the standard feed produced dark red liver tissue with a smooth surface (Figure 3A), while the test animals receiving the high-fat diet produced pale liver tissues with a granular surface (Figure 3B). The test animal group receiving HFD and quercetin 50 mg/kg BW and the test animal group receiving HFD and quercetin 100 mg/kg BW for 28 days showed red liver tissues (Figures 3C and D). The liver tissues of the test animal group receiving 28 days of HFD and quercetin 50 mg/kg BW from day 15–28 were

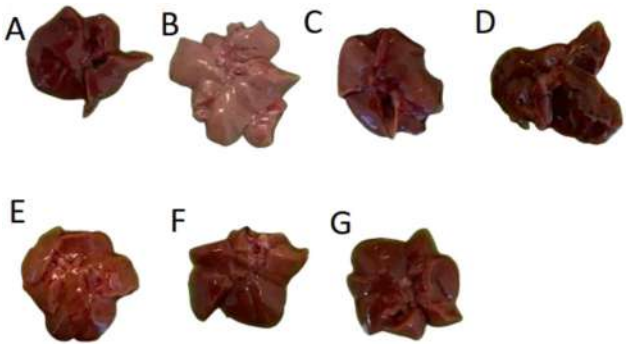


**Figure 3:** Macroscopic liver of mice. (A) Standard feed; (B) high fat diet (HFD); (C) HFD + quer 50 mg/kg BW for 28 days; (D) HFD + quer 100 mg/kg BW for 28 days; (E) HFD + quer 50 mg/kg BW for 14 days (from the 15th day to the 28th day); (F) HFD + quer 100 mg/kg BW for 14 days (from the 15th day to the 28th day); (G) HFD + repaired-feed.

slightly pale red (Figure 3E), but not as pale as the group that only received HFD. While the test animal group was given 28 days of HFD and quercetin 100 mg/kg BW from day 15–28, the liver tissues were as red as the normal ones (Figure 3F). Likewise, the test animals that were given HFD for 14 days and, then switched to standard feeding from the 15th day until the 28th day produced red liver tissues with a smooth surface (Figure 3G).

## Effect of quercetin on liver histopathology of experimental animals

NAFLD can be characterized by the presence of lipid droplets, ballooning, and inflammation on the histology of liver tissues. In the standard feed group, the histopathological features show hepatocytes with normal cell structure with a clear sinusoid image. No lipid droplets, inflammation, and ballooning were seen (Figure 4A). While in a group that is given the HFD for 28 days, the picture shows hepatocytes with abnormal cell structures. There was inflammation in several areas, ballooning, and lipid droplets in almost all areas (Figure 4B).

In the group receiving HFD and quercetin 50 mg/kg BW i.p. for 28 days, the histopathological features showed hepatocytes with abnormal cell structures with ballooning in hepatocytes. Quercetin administration improved the condition, namely by reducing the macrovesicular lipid droplet when compared to the group that only received HFD for 28 days (Figure 4C). The group receiving HFD and quercetin 100 mg/kg BW i.p. for 28 days (Figure 4D) also showed hepatocytes with abnormal cell structures with ballooning in the hepatocytes but improved when compared to groups receiving quercetin at a dose of 50 mg/kg BB i.p. for 28 days

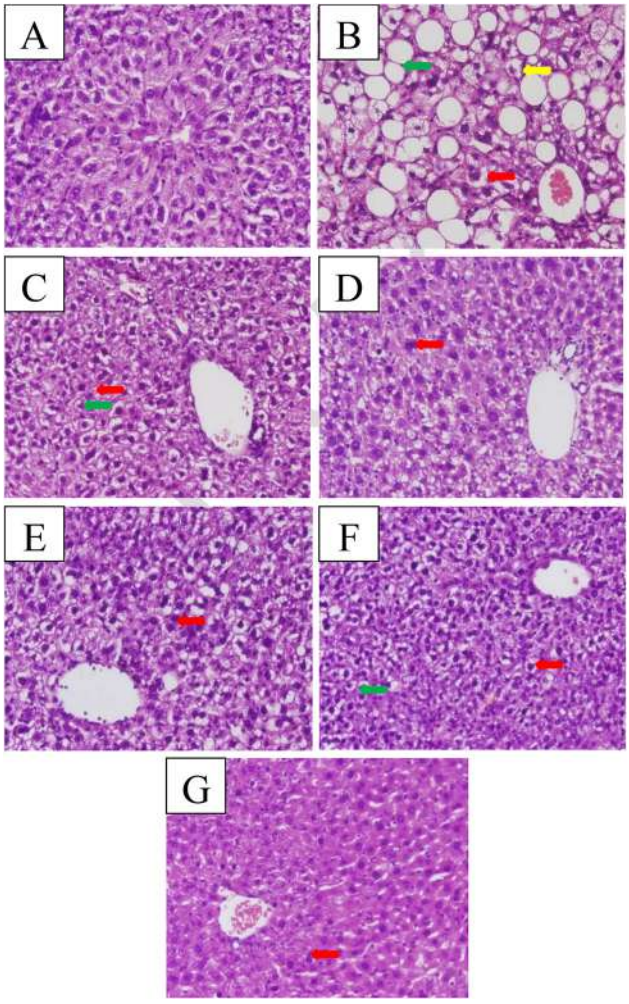


**Figure 4:** Histopathological features of mouse liver. (A) Standard feed; (B) high fat diet (HFD); (C) HFD + quer 50 mg/kg BW for 28 days; (D) HFD + quer 100 mg/kg BW for 28 days; (E) HFD + quer 50 mg/kg BW for 14 days (from the 15th day to the 28th day); (F) HFD + quer 100 mg/kg BW for 14 days (from the 15th day to the 28th day); (G) HFD + repaired-feed. Preparations were made using H&E staining, then observed under a microscope at 400× magnification. Red arrows indicate lipid droplets, green arrows indicate ballooning, and yellow arrows indicate inflammation.

(Figure 4C). The same results were presented in Figures 4E and F, namely, the group given HFD for 28 days and quercetin 50 and 100 mg/kg BW for 14 days. The picture showed hepatocytes with abnormal cell structures with ballooning, but the group receiving quercetin at a dose of 100 mg/kg BW for 14 days improved when compared to the group given quercetin at a dose of 50 mg/kg BB for 14 days. Furthermore, in the group that was given HFD for 14 days then switched to standard feeding from the 15th day to the 28th day (Figure 4G) showed the reduced appearance of lipid droplets and inflammatory cells, although it is still visible. There were also some cells that experience ballooning. This showed an improvement compared to the group receiving HFD alone for 28 days. This could be caused by the regeneration process of hepatocytes which had been well regulated by these cells because the liver had excellent regenerating properties.

These results indicate that quercetin administration has a repair effect on the histopathology of the hepatocyte structure of NAFLD mice HFD models. There were no visible lipid droplets, which is one of the hallmarks of liver steatosis. Therefore, ballooning is still present, but it is better than the HFD group.

## Effect of quercetin on the relative expression of SREBP-1c mRNA

Measurement of the relative expression of SREBP-1c mRNA in mice's liver tissue was carried out using the reverse transcriptase PCR method. The results showed an increase in SREBP-1c mRNA expression in the HFD group of mice. The group of mice receiving HFD and quercetin at a dose of 50 and 100 mg/kg BW for 28 days showed a significant decrease in SREBP-1c mRNA expression than the mice that were given HFD only. The results above tend to be the same as the group of mice given HFD for 28 days, then getting quercetin 50 and 100 mg/kg BW from the 15th day to the 28th day. The examination of the group showed that there was a significant

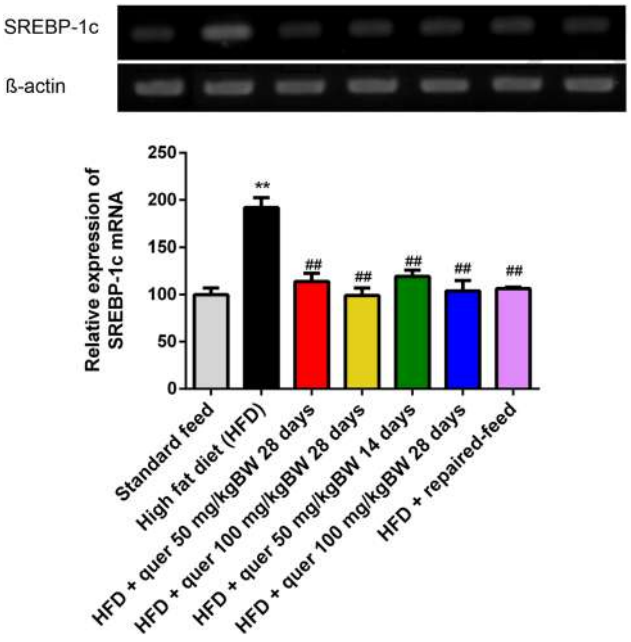


**Figure 5:** Relative expression of SREBP-1c in the liver of experimental animals analyzed using reverse transcription PCR. The data analysis was done using one way-ANOVA. **p<0.05 vs. standard feed group, ##p<0.05 vs. high-fat diet (HFD) group. Each bar represents the mean ± SEM of the six experimental animals per group.

decrease in SREBP-1c mRNA compared to the group of mice that were only fed with HFD. The group of mice was given HFD for 14 days and, then switched to standard feeding from the 15th day to the 28th day, also showed a significant decrease in SREBP-1c mRNA expression compared to the group of mice that were only given HFD (Figure 5).

## Discussion

This study was conducted on NAFLD mice models by giving high-fat content and low carbohydrates. Diets that are high in fat and low in carbohydrates have greater effectiveness in losing weight and increasing metabolism. Although this diet is popular for treating obesity, it has been shown to cause hepatic steatosis in mice [14]. Quercetin administration on mice with fat due to the HFD diet can help reduce body weight, thereby preventing the occurrence of hepatic steatosis in mice. The profile of each mouse's food intake showed no significant difference in the group receiving HFD compared to the group receiving HFD and quercetin. This indicates that the test animals' food intake does not influence the weight loss in mice consuming HFD.

Quercetin appears to be the most potent flavonoid for protecting the body from reactive oxygen species (ROS) produced during normal oxygen metabolism or induced by exogenous damage. Quercetin prevents tissue damage caused by free radicals in various ways; one way is to directly eradicate free radicals, by inhibiting low-density lipoprotein oxidation *in vitro* [15]. Quercetin is known to have an activity to eliminate ROS, such as peroxyl radicals, hydrogen peroxide, superoxide, and oxygen, because of its antioxidant properties to prevent oxidative stress [10].

According to macroscopic liver evaluation, the group of mice that were given HFD for 28 days showed pale yellow tissue with a granular surface. This may be due to the accumulation of excess lipids. A study has confirmed that liver discoloration is one of the clinical signs of NAFLD [16]. Another study revealed that the occurrence of discoloration and the increase in the size of the liver are caused by high levels of bilirubin in the blood and tissues. This condition is also known as hyperbilirubinemia, which indicates liver damage [17]. Interestingly, our study found that giving HFD combined with quercetin showed a macroscopic improvement, particularly in the color and surface texture of the liver compared to the HFD-only group. This may be due to the repair effect of quercetin which can lower hepatic TG levels in rodents, which is closely associated with the hepatoprotective effect of quercetin [16, 17].

The effect of quercetin on the liver histopathology of NAFLD mice showed an improvement in hepatocyte structure. No lipid droplets were seen, which is the main feature of liver steatosis and NAFLD. However, the quercetin groups still showed some ballooning, but it was better than the HFD group. Quercetin administration can reduce lipid accumulation in HFD-induced NAFLD [18]. In addition, one study found that decreased lipid accumulation in NAFLD prevented hepatocytes from degenerating fat. Furthermore, if lipid metabolism is irregular, reactive oxygen species (ROS) can increase [19]. Excessively produced ROS causes oxidative stress, triggering SREBP-1c activation [7]. SREBP-1c is one of the main regulators of gene expression involved in lipogenesis and hepatic TG synthesis [8].

The relative expression of SREBP-1c mRNA decreased in the group receiving quercetin compared to the HFD group. SREBP-1c induced an enzyme involved in hepatic lipogenesis, encouraging TG synthesis in the liver [9]. Excessive SREBP-1c activity increased the pathological fat synthesis and leads to hepatic fat accumulation (steatosis) [8]. After the treatment with quercetin, SREBP-1c expression was inhibited in the NAFLD mice, indicating that quercetin improved hepatic lipid accumulation by influencing fatty acid synthesis mediated by inhibition of SREBP1c expression (Figure 5) [20].

Previous studies found that quercetin prevents HFD-induced steatosis through its modulation effects on hepatic gene expression related to lipid metabolism [21]. Furthermore, adenosine monophosphate – activated protein kinase (AMPK) is known to have a crucial role in regulating lipid metabolism by integrating signaling circuits between peripheral tissues and the hypothalamus to regulate energy intake and expenditure throughout the body in order to maintain balance [22]. Another study found that quercetin can increase AMPK activation. The increase of activated AMPK contributes to inhibit the activity of Liver X Receptor α (LXRα) in mouse hepatocytes [15]. Under NAFLD conditions, overexpression of LXRα was found. LXRα is one of the crucial factors related to the development of steatosis in NAFLD, specifically related to its role in increasing lipogenesis. Moreover, one study confirmed that inhibition of LXRα was associated with decreased liver SREBP-1c expression. Thus, the effect of quercetin in decreasing SREBP-1c expression in NAFLD mice liver occurs via the AMPK and LXRα signaling pathways [15, 22].

Finally, our present study found the potential effect of quercetin in overcoming NAFLD in HFD-induced mice. This is proven by an improvement in the macroscopic picture of the liver, histopathological features of the liver, and a decrease in the relative expression of SREBP-1c mRNA. This finding is in line with previous studies that developed several compounds to treat fatty liver disease, which found that

improvement in fatty liver disease was closely associated with improved liver histopathology and decreased relative expression of SREBP-1c mRNA [23, 24].

This study also found that the expression of SREBP-1c mRNA in mice induced with HFD and treated with quercetin was not significantly different compared to mice that were induced with HFD and given repaired-diet. These findings do not diminish the potential effect of quercetin in treating NAFLD since changing diets in patients suffering from NAFLD is not an easy task [25]. Therefore, quercetin administration remains a promising alternative pharmacological therapy. However, based on the current results, it is also concluded that the mice model of NAFLD we developed is still reversible because when the mice were given the repaired diet, it shows potential liver repair effects. On this basis, it is interesting to develop further irreversible experimental animal models of NAFLD to investigate the potential effects of quercetin in overcoming NAFLD further.

## Conclusion

Based on the current study, it is concluded that the administration of quercetin at a dose of 50 mg/kg BW and 100 mg/kg BW has the potential to provide pharmacological effects in overcoming NAFLD, especially in improving the macroscopic picture of the liver, histopathological features of the liver, and reducing SREBP-1c mRNA expression on the liver of HFD-induced NAFLD mice.

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**Informed consent:** Not applicable.

**Ethical approval:** All experiments performed at the Laboratory of Animal Research, Faculty of Pharmacy, Universitas Airlangga were approved by the Ethical Committee, Faculty of Veterinary, Universitas Airlangga, No: 2.KE.076.05.2019, in accordance with the Guidelines for Care and Use of Laboratory Animals issued by National Institutes of Health revised in 1985.

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Dhani Wijaya, Suharjono*, Fendy Matulatan and Elfri Padolo

# Analysis of stress ulcer prophylaxis drug regimentation in surgical patients

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**Abstract**

**Objectives:** The World Health Organization (WHO) estimated that more than 50% of drugs were prescribed incorrectly, including stress ulcer prophylaxis (SUP) drugs. Prescribing SUP drugs in incorrect doses and frequencies are considered irrational, and may affects to the effectivity of the therapy. This research aimed to assess the appropriateness of the SUP drugs regimentation in the inpatient surgery room at Dr. Soetomo Hospital, Surabaya, Indonesia.

**Methods:** This research was cross-sectional study and conducted for 4 weeks in 2019 in the inpatient surgery room of Dr. Soetomo Hospital. The population was SUP drugs that were prescribed in inpatient surgery room. Those SUP drugs with indications for the prevention of stress-induced ulcers that complied to the terms listed on the American Society of Health-System Pharmacists (ASHP) were included as the samples, and vice versa. The samples then assessed for their regimentation appropriateness using the dose and frequency standard of ASHP.

**Results:** There were 224 dose units taken as sample, from the total population of 1,404 SUP drugs. The result showed that as much as 48.2% of SUP medications were given to the patients in inappropriate regimentation. Of that number, all ranitidine injection were inappropriately regimented. On the contrary all omeprazole injection dose units were appropriately regimented, meanwhile the amount of appropriate regimentation of sucralfate suspension were 74.6%.

**Conclusions:** According to ASHP standard, the SUP drugs in the inpatient surgery room at Dr. Soetomo Hospital were mostly given in inappropriate regimentation. Further research is needed to explore how will those inappropriate regimentation affect on the efficacy of therapy in the patients.

**Keywords:** dose; frequency; regimentation; stress ulcer prophylaxis; ulcer.

## Introduction

Stress ulcer prophylaxis (SUP) drugs can reduce the patient's risk of gastrointestinal bleeding [1]. Among classes of SUP drugs, proton pump inhibitor (PPI), histamine 2 receptor antagonist (H2RA), and a cytoprotective sucralfate suspension are the most commonly used agents in ulcer therapies [2]. Those drugs as any other drug should be administered rationally in conformity to patient's clinical conditions [3]. Irrational administration of drug dose and frequency may leads to failure in achieving goal of therapy and reduce the health quality [4]. As in patients undergoing surgical procedures, they are prone to ulcers due to stress and physical trauma of surgeries [5]. Therefore these patients need to be given SUP drugs with a correct dose and frequency.

This regimentation appropriateness issue becomes significant since the World Health Organization (WHO) estimated that >50% of drugs were prescribed incorrectly [6]. Some researches had reported similar results, emphasized the incorrectness of drug doses and frequencies, as a study reported in 2016 [7]. However, there has never been any study regarding the appropriateness of SUP drugs regimentation in inpatient surgery room at Dr. Soetomo General Hospital. The analysis of SUP drugs regimentation is important to provide an overview of doses and frequencies in SUP drug administration. This research was conducted to analyze the appropriateness of SUP drugs regimentation administered in inpatient surgery room at Dr. Soetomo General Hospital.

## Materials and methods

We conducted a cross-sectional study for 4 weeks in 2019 to obtain the information, by observing patient's medical records in the inpatient

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 031 5033710, E-mail: suharjono@ff.unair.ac.id
**Dhani Wijaya,** Department of Pharmacy, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Kota Malang, Indonesia; and Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Fendy Matulatan,** Department of General Surgery, Dr. Soetomo General Hospital, Surabaya, Indonesia
**Elfri Padolo,** Department of Pharmacy, Dr. Soetomo General Hospital, Surabaya, Indonesia

surgery room of Dr. Soetomo General Hospital. The population were the total of SUP drugs regimented to the patients in inpatient surgery room in the hospital. We then chose the samples by inclusion criterias, which in compliance to the standard of American Society of Health-System Pharmacists (ASHP). ASHP guideline is evidence based, compiled and composed from largest quantities of references compared to other SUP guidelines [8].

According to ASHP, SUP drugs should only be prescribed to the patient with 1 absolute indication or minimum 2 relative indications. Absolute indications refer to patient with the usage of mechanical ventilation over 48 h with or without coagulopathy. Meanwhile those included as relative indications are patient with: burns more than 35%; partial hepatectomy; Injury Severity Score (ISS) of 16 or greater; liver or kidney transplantion; number Glasgow Coma Scale (GCS) score of 10 or less; spinal cord injury; liver dysfunction; kidney disorders; history of gastric ulcers; and/or gastrointestinal bleeding in the past one year; sepsis; intensive care unit treatment for more than seven days; and use of corticosteroid drugs with high doses [9].

The number of SUP drugs (population and sample) were specified as dose units. For ranitidine injection, a dosage form of 50 mg/2 mL ampoule was counted as a dose unit, meanwhile for omeprazole injection, a dosage form of 40 mg/10 mL vial. The other dose units were 500 mg/5 mL of sucralfate suspension. The appropriateness of each dose unit then assessed by the conformity to the information of regimentation listed in ASHP. As recommended in ASHP, rantidine injection is indicated for use in adult by the dose of 50 mg for every 6–8 h, while for children is 0.13–0.8 mg/kgBW for every 6 h. Suggested regimentation for omeprazole is 40 mg (loading dose) followed by dose of 20–40 mg daily per oral (PO) or by naso gastric tube (NGT). The last dose unit, sucralfate suspension is recommended to be regimented as 1 g dose, four times daily for adult, and 40–80 mg/kg/day in 4 divided doses in children [9].

# Results

During the phase of sampling in inpatient surgery room there were a total of 152 patients recorded, varied in ages and gender. Patients middle aged adult with ages between 31 and 50 years old were the most commonly found (38.82%), and with ages over 50 years old (senior adult) come in second (38.16%). Meanwhile children patient (0–15 years old) were the least (5.92%). Male patients were more frequently found than female by 55.3% percentages of the total patient number (Table 1).

**Table 1:** Patient characteristics in the study of analysis of stress ulcer prophylaxis drug regimentation in surgical patients.

| Classification | Category | ∑ Patient |
|---|---|---|
| Age, year | 0–15 | 9 |
| | 15–30 | 26 |
| | 31–50 | 59 |
| | >50 | 58 |
| Gender | Female | 68 |
| | Male | 84 |

There were only 224 dose units taken as samples from a total population of 1,404, due to their conformity to ASHP standard (Figure 1).

## Assessment of the accuracy of SUP drugs regimentation

There were three variations of ranitidine injection regimentation recorded during the study, 50 mg twice daily (BID), 20 mg BID, and 12.5 mg BID, which all three of them were categorized as inappropriate. For omeprazole injection we noted there were two types of regimentations, which were 40 mg once daily (OD) and 20 mg BID, both of them complied to ASHP recommendation. Meanwhile for sucralfate suspension, there were 88 unit doses regimented 4 times a day that appropriate to ASHP recommendation, and the rest 30 unit doses (regimented 3 times a day) were on the contrary (Table 2).

# Discussion

From a total of 1,404 drug dose units, only 224 dosage units were included as samples. This result showed that only 15.95% of SUP drugs were administered to patients in conformity to the ASHP criteria. However, it is possible that the rest 84.05% of population were utilized for purposes other than prophylaxis for stress ulcer. Gastric acid inhibitor and cytoprotector can also be indicated for pulmonary aspiration prevention, hypersensitivity reactions, and prevention of ulcers due to NSAID use [10–12].

Ranitidine injection's half-time is 2–3 h [13], therefore, daily ranitidine injection should be given at 3–4 times a day frequency. A shorter interval of administration (6–8 h) would result in a lower drug fluctuation amount between

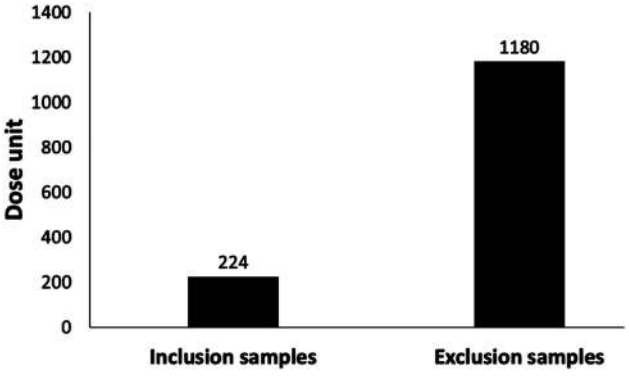


**Figure 1:** Number of samples in the study of analysis of stress ulcer prophylaxis drug regimentation in surgical patient.

**Table 2:** Assessment of the appropriateness of SUP drugs regimentation in the study of analysis of stress ulcer prophylaxis drug regimentation in surgical patients.

| Name of drug | Patient dosage regimentation | Appropriate regimentation | Inappropriate regimentation | Note |
|---|---|---|---|---|
| Ranitidine injection | 2 × 12.5 mg | 0 | 8 | Prescription in children, 22 kg |
| | 2 × 20 mg | 0 | 2 | Prescription in children, 20 kg |
| | 2 × 50 mg | 0 | 68 | |
| Omeprazole injection | 1 × 40 mg | 20 | 0 | |
| | 2 × 20 mg | 8 | 0 | |
| Sucralfate suspension | 3 × 500 mg | 0 | 12 | Prescription in children, 20 kg |
| | 3 × 1 g | 0 | 18 | Prescription in children, 20 kg |
| | 4 × 1 g | 88 | 0 | Prescription in children, 20 kg |
| Total | | 116 | 108 | |

the maximum concentration and the minimum concentration in the blood stream, compared to a longer interval (i.e. 12 h). A low fluctuation value would increase the safety aspect of a drug. In addition, the level of steady state concentration at 12-h administration interval would be lower compared to the shorter one (i.e. 8 h). Therefore administering ranitidine injections twice daily are less potential to provide the desired therapeutic effect. Intravenous ranitidine at interval 8 h is capable of maintaining gastric pH at value of >4 longer, compared to 12 h interval [14]. The efficacy of ranitidine injection at 8 h interval as prophylaxis of stress ulcer has already been proven in a SUP group patients where 96% of patients did not experience GI bleeding [15]. Therefore, as much as 78 dose units of ranitidine injection in this study, which were given at frequency of 2 times a day was categorized as inappropriate regimentation

In some other cases, we concluded that administering ranitidine injections to children every 12 h was considered inappropriate. Regimenting ranitidine injections to 20–22 kgBW children should be done with 10.4–70.4 mg every 6 h. A cohort study stated that in order to maintain a gastric pH of ≥4 in patients less than 9 years old, the ranitidine injection had to be given at the frequency of less than 8 h [16], a reduced doses would result in less success in controlling the gastric fluid acidity.

At a same dose there would not be any difference between intravenous and oral PPI in maintaining intragastric pH of >6 for as long as 72 h [17]. In addition intravenous and also oral PPIs both have a short half-lives and similar effect on gastric acid secretion, so the dosage and frequency are likely to be interchangeable [18]. Therefore ASHP can still be used for appropriateness analysis of omeprazole injection, which refers to 20–40 mg daily for its dose and frequency.

There were no significant difference of recurrent bleeding rates between OD and BID PPI [19]. Even so the recommended regimentation of PPI is once daily, due to the inhibitory effect of stomach acid by omeprazole which lasted approximately 72 h after administration. Intravenous omeprazole 40 mg once a day reach daily gastric pH of 4.68 ± 0.18. With the daily use of drug, the effect was stable for 4 days [20–22]. The frequency of omeprazole usage could be increased in patients diagnosed with gastro esophageal reflux disease or as a combination of *Helicobacter pylori* eradication therapy to improve the control of gastric acidity [23].

The regimentation of sucralfate suspension in patients weighing 20 kg was 800–1,600 mg given at 6 h intervals. As much as 1 g of sucralfate was able to neutralize 14–16 mEq of acid [24]. Sucralfate formed a protective layer of gastric mucose with 6 h duration of therapy. Therefore, only 88 units of 4 × 1 g sucralfate suspension were categorized as correct regimentation.

This study showed that almost half of total samples of SUP drugs were regimented inappropriately. Inappropriate use of SUP drugs can occur due to a lack of information on correct regimentation of SUP drugs, or can also caused by the lack of particular guidance regulating the usage of SUP drugs at the hospital of study. Therefore guidelines for the usage of SUP drugs are necessarily needed to support the rational medication. This study also confirms the need for interventions to optimize the safety of patient care in hospital.

This research was conducted in the largest hospital in eastern part of Indonesia, therefore the results of this study may illustrate the SUP drugs regimentation profile in Indonesia. However, this study only evaluates the regimentation of SUP drugs use without observing the effectiveness of SUP drugs, which becomes a limitation of this

study. Further research is needed to evaluate the relationship of effectiveness and regimentation of SUP drugs.

## Conclusions

The results showed that there were cases of inappropriateness of SUP drug regimentation in terms of the frequencies and doses of administration in the inpatient room of Dr. Soetomo General Hospital, Surabaya. A study focusing on the correlation of drug regimentation and therapeutic effectiveness is recommended to be conducted. Furthermore, a policy regarding to the use of SUP drugs at the hospital is expected to be drafted based on the study, in order to improve the safety and efficacy aspect of the therapy.

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Dini Retnowati, Retno Sari*, Esti Hendradi and Septiani Septiani

# The stability and irritability study of the chitosan–*Aloe vera* spray gel as wound healing

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**Abstract**

**Objectives:** Chitosan is a natural polysaccharide widely used in various clinical applications including regeneration of skin tissue. *Aloe vera* has properties in healing burns on the skin, anti-inflammatory effect, and leaves a protective layer on the skin after drying so it provides protection to the wound. The spray gel of chitosan–*A. vera* was developed as a wound healing that has combined of effect of both component and easy to use. The purpose of this study was to determine the physical stability and irritability of chitosan–*A. vera* spray gel.

**Methods:** The spray gel stability test was conducted using thermal cycling and centrifugation methods. The organoleptic, viscosity, and pH of the spray were evaluated. The irritation test was performed by Draize Rabbit Test method.

**Results:** Chitosan (0.5%)–*A. vera* (1%) spray gel characteristics has a weak yellow color, clear, and a strong *A. vera* odor. The pH of the spray gel was 4.88 ± 0.01; and the viscosity was 36.50 ± 0.23 cps. The result from the chitosan (0.5%)–*A. vera* (1%) spray gel stability test using thermal cycling method showed a decrease of viscosity, but remained stable when evaluated using centrifugation method. There was no difference in the pH and organoleptic observation from both tests. Based on the scoring and analysis of the reaction in rabbit skin, the Primary Irritation Index (PII) obtained was 0.56.

**Conclusions:** The spray gel of chitosan (0.5%)–*A. vera* (1%) was stable and according to response category from the acute dermal irritation test, it can be concluded that chitosan (0.5%)–*A. vera* (1%) spray gel had a slightly irritating effect.

**Keywords:** *Aloe vera*; chitosan; irritability study; stability.

***Corresponding author: Retno Sari**, Department of Pharmaceutics, Faculty of Pharmacy, University of Airlangga, Kampus C, UNAIR. Jl. Mulyerejo, Surabaya, 60115, Indonesia, E-mail: retno-s@ff.unair.ac.id
**Dini Retnowati, Esti Hendradi and Septiani Septiani,** Department of Pharmaceutics, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia

## Introduction

Chitosan is a polysaccharide derived from the deacetylation of chitin biopolymers, consisting of glucosamine and *N*-acetyl-glucosamine. Chitosan has low toxicity, good biocompatibility and biodegradability, so it is widely used in biomedical and pharmaceutical applications, one of which is wound healing [1]. Chitosan is widely used as a wound healing because it has antimicrobial activity, analgesic activity and hemostatic properties [2]. Chitosan can stimulate cell proliferation, increase collagenization and accelerate cell regeneration in injured skin. Chitosan stimulates polymorphonuclear (PMN) cell migration, activates macrophages, and mediates the phagocytosis process [3].

To increase the effectiveness of wound healing, chitosan can be combined with several ingredients that have antibacterial, anti-inflammatory and growth factors effects [4]. One natural ingredient that has anti-inflammatory and antibacterial effects is *Aloe vera*. *A. vera* accelerates the wound healing process by providing a moisturizing, antibacterial, anti-inflammatory, and epithelial effect. The acemanan (mannose-6-phosphate) content of speed up stimulates fibroblasts, thereby increasing collagen synthesis and epithelialization. *A. vera* leaves a protective layer after drying on the skin and provides a protective effect against wounds [5, 6].

Topical treatment can quicken the wound healing process in patients with open wounds because it is more effective than oral or parenteral administration. Gel is an ideal topical preparation to use as a wound dressing because it is nonsticky, provides moisture and its cool effect [7]. Spray gel is a development of gel-form preparations for wound healing which has the advantage of reducing the possibility of contamination or infection and trauma to the patient because it can be delivered directly to the wound without contact with cotton swabs or hands [8]. Based on Yulia's (2018) research, chitosan gel spray preparation (0.5%)–*A. vera* (1%) can accelerate wound healing process with good clinical descriptions and provide 98.73% wound healing [9].

Pharmaceutical preparations are said to be stable if during the storage and use period, their properties and characteristics are the same as when they were initially

made [10]. The stability of a dosage form can be affected by the stability of the active ingredient, the interaction of the active ingredient with excipients, the manufacturing process, the packaging process and environmental conditions during shipping and storage. To assess the stability of a preparation, testing is necessary [11]. Stability tests are carried out to determine the effect of environmental factors on product quality, determine appropriate storage conditions, and determine storage instructions on product labels [12].

Stability tests can be performed in real time and accelerated conditions. In the accelerated stability test, the product is stored under stress conditions so that the rate of physical change will increase [13, 14]. The accelerated stability test is a fast and reliable way to compare different formulas and perform formula selection based on physical stability [15]. Based on research by Sukmawati (2017), real time stability tests were carried out on chitosan gel spray preparations – green betel leaf extract, during storage for 28 days at room temperature, the preparation was declared stable [16]. Another stability test to see the physical stability of spray gel can be done by using the cycling test and centrifugation test methods [10].

Besides being stable, a pharmaceutical preparation must meet the criteria of being safe, effective, and efficient. The safety parameter that needs to be considered when administering topical preparations is the possibility of irritation when the preparations are applied. Irritation is a reaction that occurs directly on the first use of the preparation because one or more of the ingredients it contains are irritating [17]. The acute dermal irritation test is performed to determine the presence of an irritating effect on the skin as well as to assess and evaluate the characteristics of the preparation when exposed to the skin. The irritation test was carried out *in vivo* with the Draize Rabbit Test method on albino rabbits [18].

Based on the description above, the physical stability test of chitosan–*A. vera* spray gel preparation will be carried out including organoleptic, viscosity, and pH using the thermal cycling method and centrifugation test. In addition, an acute dermal irritation test was also performed using the Draize Rabbit Test method on albino rabbits and then the primary irritation index of the preparation was determined.

# Materials and methods

## Materials

**Chemical material:** Materials used in this research were Chitosan 50 cps (obtained from Biotech. Co. Ltd, Korea); *A. vera* powder (obtained from Haldin Pasific Semesta, Indonesia); Propylene glycol (obtained from CV. Tristar Chemical); HPMC 5 cps; Acetic acid pro analysis; Aquadest.

**Experimental animal:** The animal used was male albino rabbit, adult, healthy, and no visible abnormalities on the body, weighing about 2 kg. This study was designed to comply the criteria for ethical concerns and was approved by the Animal Care and Use Committee (ACUC) of Faculty of Veterinary Universitas Airlangga, with reference number 2.KE.046.04.2019.

## Methods

**Spray gel preparation:** Chitosan (0.5%) was dissolved into 0.5% acetic acid solution with a speed of 300 rpm for 10 min, the pH of chitosan was checked with universal indicator. *A. vera* (1%) solution was prepared by dissolving *A. vera* powder into aquades with a speed of 400 rpm 10 min. Then chitosan was mixed with *A. vera* solution with a speed of 300 rpm for 10 min. The HPMC (0.8%) was dissolved in aquadest in a different container with a speed of 600 rpm for 10 min. Chitosan–*A. vera* solution was mixed with HPMC solution at a speed of 250 rpm for 10 min, then the propylenglycol was added at a speed of 200 rpm for 10 min. Aquadest was added until it reached a 100 mL preparation volume while being stirred until homogenous at a speed of 200 rpm for 15 min.

### Evaluation of spray gel preparation

#### *Physical characteristic*

*Organoleptic:* Organoleptic examination was done by looking at the physical appearance of the preparation such as consistency, color, and odor of preparation.

*Viscosity:* Capillary viscosimeter (Ostwald) was used in this viscosity test. Water was measured as a comparison before the preparation was put in the viscosimeter. The time needed for water to pass through two lines was noted. Then the preparation that was about to get measured was inserted into the viscosimeter, the time was noted.

*pH:* pH measurement of the preparation was done using a Schot CG 842 pH meter. The electrode was washed with distilled water and wiped with tissue, pH meter calibration was performed using standard buffer solution pH = 7.0 and then the electrode was rinsed with distilled water and wiped with tissue again. The preparation was put into the beaker glass then the electrode was inserted into the preparation. The number that was shown in the machine was noted.

### Stability test

***Thermal cycling test:*** The spray gel was stored at 2–8 °C for 48 h, then stored at 40 °C for 48 h (1 cycle). The test was carried out in three cycles. Physical characteristic such as organoleptic, viscosity, and pH of the spray gel was evaluated for each cycle.

***Centrifugation test:*** Spray gel was put in the centrifugator with a rotating speed of 1,500, 2,500, and 3,500 rpm for 30 min. Physical characteristic of the spray gel including organoleptic, viscosity, and pH were evaluated.

***Irritability test:*** The irritability test was perform using Draize Rabbit Test method. The animal used was male albino rabbit, adult, healthy, and no visible abnormalities on the body, weighing about 2 kg. Rabbit hair was shaved on their dorsal area of approximately 10 × 15 cm, 24 h before testing. 0.5 mL gel was exposed to the skin area of then covered with gauze and plastered with a nonirritant plaster. After 4 h of exposure, the patch was opened and sign of erythema or edema were observed at 1, 24, 48, and 72 h after opening the patch. The visual changes in the skin were scored. The score obtained was calculated using formula and then the irritation category was determined.

## Results

The results of organoleptic examination on chitosan–*A. vera* spray gel Thermal Cycling test before and after three cycles can be seen in Figure 1. It can be observed that from all the three thermal cycle, chitosan–*A. vera* spray gel gave clear pale yellow color, strong *A. vera* and weak acetic acid odor.

From the viscosity evaluation, the result of chitosan–*A. vera* spray gel in the 0, 1, 2, and 3 thermal cycling cycles were 36.50 ± 0.23; 27.59 ± 0.87; 25.86 ± 0.57; 24.62 ± 0.26 cps as shown in Figure 2. For pH evaluation, the average pH values for spray gel chitosan–*A. vera* in the 0, 1, 2, and 3 thermal cycling cycles were 4,879 ± 0.010; 4,864 ± 0.025; 4.852 ± 0.025; 4,849 ± 0.012, respectively (see Figure 3).

From chitosan–*A. vera* spray gel centrifugation test result, it can be concluded that chitosan–*A. vera* spray gel gave clear pale yellow color, strong *A. vera* and weak acetic acid odor. The viscosity value of the chitosan–*A. vera* spray gel preparation in the centrifugation test with a rotating speed of 0; 1,500; 2,500; and 3,500 rpm were 36.50 ± 0.23; 36.30 ± 0.35; 36.35 ± 0.12; 35.55 ± 0.63 cps, respectively (see Figure 4). The average pH value of chitosan–*A. vera* spray gel preparation in the centrifugation test with a rotating speed of 0; 1,500; 2,500; and 3,500 rpm were 4.879 ± 0.010; 4,845 ± 0.007; 4,842 ± 0.002; 4,847 ± 0.004 (see Figure 5).

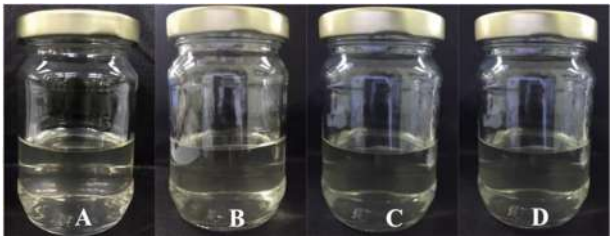


**Figure 1:** Organoleptic examination results for chitosan–*A. vera* spray gel preparation in cycles 0 (A), 1 (B), 2 (C), and 3 (D) on the thermal cycling stability test.

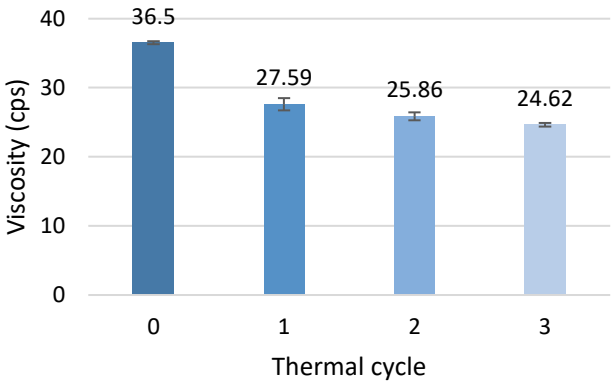


**Figure 2:** The histogram of chitosan–*A. vera* spray gel viscosity evaluation on the 0, 1, 2, and 3 cycles on the thermal cycling stability test. Data represent the mean of three replications ± SD.

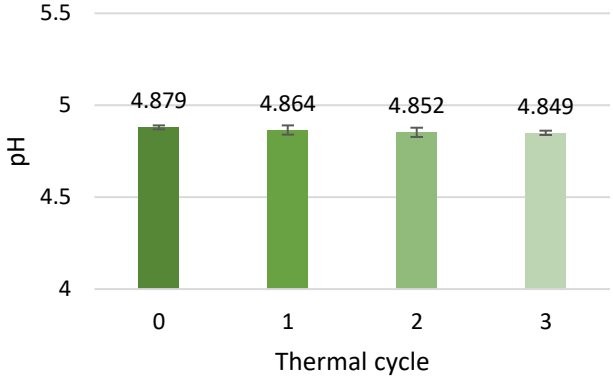


**Figure 3:** The histogram of chitosan–*A. vera* spray gel pH evaluation on the 0, 1, 2, and 3 cycles on the thermal cycling stability test. Data represent the mean of three replications ± SD.

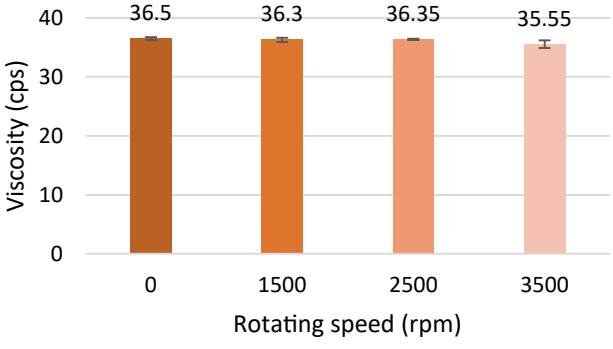


**Figure 4:** The histogram of chitosan–*A. vera* spray gel viscosity evaluation in the centrifugation test with a rotating speed of 0; 1,500; 2,500 rpm. Data represent the mean of three replications ± SD.

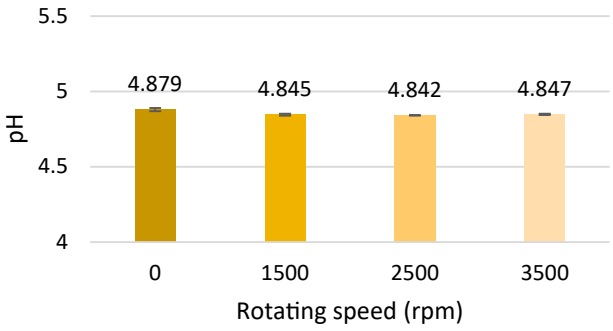


**Figure 5:** The histogram of chitosan–*A. vera* spray gel pH evaluation in the centrifugation test with a rotating speed of 0; 1,500; 2,500 rpm. Data represent the mean of three replications ± SD.

The Draize Rabbit test is an acute skin irritation evaluated *in vivo* in rabbits after they have been shaved (see Figure 6). Rabbit skin reaction after exposure to chitosan–*A. vera* spray gel can be seen in Table 1.

## Discussion

The stability test for chitosan–*A. vera* spray gel was prepared using the thermal cycling method and the centrifugation test. In the stability test using the thermal cycling method, there was no organoleptic change in color, odor, or clarity of the preparation for all three cycles. In the viscosity evaluation, the preparation experienced a decrease in viscosity after one thermal cycling cycle. The preparation continued to experience a decrease in viscosity in the second and third cycles in the thermal cycling test. To see the effect of the cycle on the thermal cycling stability test on viscosity, an analysis was performed statistics using the One Way ANOVA method with a confidence degree of 95%. Based on the results of statistical analysis, there was a significant decrease in the viscosity of the preparation after thermal cycling on cycles 1, 2, and 3. The decrease in viscosity can be due to depolymerization of chitosan at high temperatures (40 °C). Depolymerization can cause a decrease in the molecular weight of chitosan, causing a decrease in the viscosity of the preparation [19]. On pH examination, the spray gel has a pH of about 4.8 both before the thermal cycling stability test and after the thermal cycling stability test for three cycles. Even though there was a slight decrease, the pH of the preparation was still by the skin pH range, namely 4.5–6.5.

In the centrifugation test with a speed of 1,500, 2,500, and 3,500 rpm, there was no change in the organoleptic preparation either in color, clarity, or odor. It can be concluded that the spray gel was stable during the

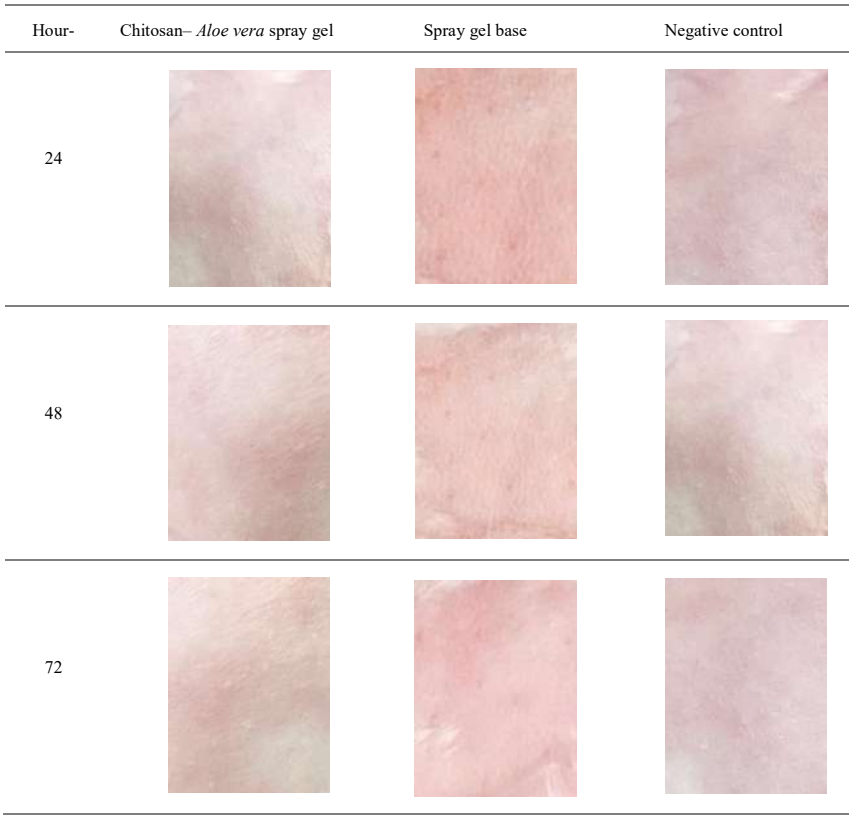


**Figure 6:** Skin reaction result from Draize Rabbit Test for chitosan–*A. vera* spray gel irritability study.

**Table 1:** Primary Irritation Index (PII) categories in a rabbit.

| Mean score | Response category |
|---|---|
| 0 to 0.4 | Negligible |
| 0.5 to 1.9 | Slight |
| 2 to 4.9 | Moderate |
| 5 to 8 | Severe |

centrifugation test. In the viscosity measurement, the preparation experienced a decrease in viscosity after centrifugation at a speed of 1,500, 2,500, and 3,500 rpm. To see the effect of centrifugation speed on the viscosity of the preparation, a statistical analysis was performed using the One Way ANOVA method with a 95% degree of confidence. Based on the results of statistical analysis, there was a significant decrease in the viscosity of the preparation after centrifugation. The decrease in viscosity may be caused by the increase in mechanical energy which can also cause an increase in temperature in the centrifugation test. With an increase in temperature, chitosan undergoes a depolymerization causes a decrease in the molecular weight of chitosan and causes a decrease in the viscosity of the preparation [19]. However, the increase in centrifugation speed did not provide a significant difference in viscosity. In the pH examination, based on the results of statistical tests using the One Way ANOVA method with a 95% degree of confidence there was no significant change in pH so that it can be said that the pH of the preparation was stable during the centrifugation test.

Based from the observation of irritability study using Draize Rabbit test, there was very small erythema and almost indistinguishable from the negative control. After scoring and analysis, the IIP value for spray gel chitosan–*A. vera* was 0.56 (slight irritation), while for spray gel base it was 0.78 (mild irritation). On a gel base, irritation can be caused by propylenglycol, which is mildly irritating. The spray gel base has a pH of 4, which is thought to cause irritation because the pH is outside the pH range of the skin, namely 4.5–6.5 [17, 20]. Based on the results of nonparametric statistical tests with Kruskal Wallis, there was no significant difference in IIP.

It can be concluded that although there were changes in the physical characteristics of the spray gel chitosan–*A. vera* after the stability test was carried out with the thermal cycling method and the centrifugation test, the preparation still met the requirements for a good spray gel in terms of viscosity and pH. To ensure that the preparation meets the aspects of chemical and microbiological stability during storage, it is necessary to carry out further observations on the chemical and microbiological stability of the spray gel chitosan–*A. vera*.

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**Informed consent:** Not applicable.
**Ethical approval:** Research involving animals complied with all relevant national regulations and institutional policies (Animal Care and Use Committee of Faculty of Veterinary Universitas Airlangga, Surabaya, Indonesia) for the care and use of animals.

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Rozalina Loebis*, Bambang Subakti Zulkarnain and Fitri Amalia Siswanto

# Effectiveness of citicoline in pediatric patients with refractive amblyopia in Surabaya, East Java, Indonesia

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**Abstract**

**Objectives:** Amblyopia is a decrease of visual acuity that cannot be attributed to any structural abnormality of the eye or visual system, causing a partial or complete loss of vision due to inadequate stimulation in early life. Citicoline has been reported to improve visual acuity in amblyopic eyes as adjuvant treatment. This study was aimed to determine the effectiveness of citicoline in pediatric patients with refractive amblyopia in ophthalmology daily practices.

**Methods:** This was a retrospective–descriptive study with a time limited sampling method. This study was conducted at Surabaya Eye Clinic, East Java, Indonesia, by reviewing medical records for the period of January 2018 to December 2019.

**Results:** A total of 34 eyes were included in the study with the majority aged five years (41.2%) and six years (35.3%). The severity of amblyopia varied among patients, 21 eyes (61.76%) had mild amblyopia, seven eyes (20.59%) had moderate amblyopia, and two eyes (5.88%) had severe amblyopia. The duration of given therapy also varied, 18 eyes (52.94%) were given 3 months therapy, two eyes were given 4 months therapy, 12 eyes were given 6 months therapy, and two eyes were given 8 months therapy. Citicoline was found effective in mild and moderate amblyopia and for the duration of 3 and 6 months ($p<0.05$). In others group who did not showed statistically significant improvement was due to inadequate samples but clinically significant improvement was noted.

**Conclusions:** Citicoline therapy resulted in a clinically and statistically improvement in refractive amblyopia patients.

**Keywords:** amblyopia; citicoline; pediatric; refractive error.

## Introduction

Amblyopia is a disease of decreased visual acuity that cannot be associated with structural abnormalities of the eye or visual system and causes partial vision loss or blindness due to insufficient stimulation at an early age [1]. The prevalence of amblyopia at the age of less than five years is 5.66%, at the age of 5–15 years is 2.24%, and at the age of 16–25 years is 4.20%. It stated that 13.49% of amblyopia was caused by strabismus case, 69.84% was caused by refractive anomaly, and 16.67% of cases were caused by mixed abnormalities (strabismus/anisometropia). Thus, most amblyopia was caused by refractive anomaly [2].

Amblyopia is one of the causes of decreased vision in children [3]. The impact of amblyopia is a decrease in visual acuity. The presence of amblyopia can reduce the ability of vision function in children and if not well treated will last until adulthood [4]. The impact of visual disturbances on amblyopia causes the need for appropriate amblyopia treatment. Treatment of amblyopia involves several stages, namely elimination of visual axis obstructions such as cataracts, correction of refractive anomalies, and improving amblyopia eye function. In addition, a research showed that citicoline has the ability to improve visual acuity in amblyopia patients [5]

Citicoline (cytidine-5-diphosphocholine) is an intermediate of phosphatidylcholine, a phospholipid in cell membranes. Citicoline is used as a supporting treatment in traumatic, ischemic, and degenerative diseases, for neurological and eye disorders [6]. In amblyopia eyes, citicoline works to improve the visual pathways of the postretina and retina by stimulating the dopaminergic system, thereby increasing visual acuity [7]. Administration of citicoline can improve visual acuity in 46 of 50 adult amblyopia patients for at least 4 months of therapeutic

***Corresponding author: Rozalina Loebis**, Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, Phone: +628113419344,
E-mail: rozalinaloebis2512@gmail.com
**Bambang Subakti Zulkarnain,** Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Fitri Amalia Siswanto,** Bachelor of Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

effect after administration of 1,000 mg of citicoline intramuscularly for 15 days [5]. Other studies have shown that oral administration of 800 mg or 1,200 mg of citicoline combined with patching can provide a more stable therapeutic effect of increasing visual acuity than using patching alone [6]. Furthermore, in other study showed that giving 250 mg or 500 mg citicoline once a day combined with patching can improve visual acuity more significantly than patching alone [8].

Citicoline has been used as common adjuvant therapy in refractive amblyopia for some time by several ophthalmologists in their daily practices, however, analysis of its effectiveness in pediatric patients have not been carried out in Surabaya, East Java, Indonesia. Thus, this study was aimed to analyze pediatric patients' medical records that had been prescribed citicoline therapy to determine its effectiveness.

## Materials and methods

This was retrospective observational study using medical records from January 1, 2018 to December 31, 2019. This study was designed to comply with the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Faculty of Pharmacy, Universitas Airlangga, with reference number 13/LE/2020. The inclusion criteria for this study were: aged up to eight years old and amblyopia patient receiving citicoline as adjuvant therapy. Whereas, the exclusion criteria was documented side effect while using citicoline therapy in medical record and do not continue its therapy. Furthermore, the severity of amblyopia, duration of citicoline therapy, and best corrected visual acuity (BCVA) for every visit to their ophthalmologist was noted. BCVA was measured with Snellen Chart Projector and Kay Pictures Cards. Snellen Chart Projector was used for children who can read letters and numbers whereas Kay Pictures Cards for children who cannot read.

The amblyopia severity was classified as follows [9]: Mild amblyopia is visual acuity in the range 6/9 to 6/12. Moderate amblyopia, which is visual acuity in the range 6/12 to 6/36. Severe amblyopia is visual acuity greater than 6/36.

The visual improvement of this study after citicoline therapy was defined as: improve relative visual acuity that is if the visual acuity measurement is 6/9 to 6/12 or it is close to the normal visual acuity of 6/6); improved functional visual acuity that is if there is a change in visual acuity by two to three lines on the Snellen chart (0.2 LogMAR units).

All BCVA data was converted to LogMAR units. Data analysis was performed using the Statistical Package for the Social Sciences Data (SPSS) version 25. Mann Whitney comparison test was used. The test result is significant if the p value <0.05.

## Results

A total of 17 patients (34 eyes) were included in this study. Demographic data include gender and age. Male and female patients were almost equal in number whereas mostly patients aged five years (41.2%) and aged six years (Table 1).

The amblyopia severity varied among patients which 21 eyes had mild amblyopia (61.76%), seven eyes had moderate amblyopia (20.59%), and two eyes had severe amblyopia (5.88%). This classification of amblyopia severity was based on William [9] (Table 2). Moreover, the duration of citicoline therapy was also varied in which 18 eyes were given 3 months (52.94%), two eyes were given 4 months (5.88%), 12 eyes were given 6 months (35.29%), and two eyes were given 8 months of citicoline therapy (5.88%) (Table 3). The varied duration of citicoline therapy was based on the patients' follow up to their ophthalmologist.

Table 4 shows the effectiveness of citicoline therapy based on amblyopia severity. In mild and moderate amblyopia, citicoline therapy significantly improved visual acuity ($p<0.001$) whereas the similar result was not found in severe amblyopia ($p = 0.102$).

Based on the duration of citicoline therapy, it showed that 3 and 6 months therapy with citicoline has significant improvement in visual acuity ($p<0.001$) whereas 4 and 8 months was not showed statistically significant different (Table 5).

## Discussion

The selection of patients with a maximum age of 8 years was carried out because in that age range the patient is still in a critical period of vision development that is until the first 7–8 years of life [10]. It is known that early detection and therapy have high effectiveness in treating amblyopia [11].

**Table 1:** Demographic data pediatric patients with refractive amblyopia receiving citicoline therapy.

| Demographic data | Number of patients | Percentage, % |
|---|---|---|
| **Gender** | | |
| Male | 9 | 52.94% |
| Female | 8 | 47.06% |
| Total | 17 | 100% |
| **Age** | | |
| 4 years old | 1 | 11.8 |
| 5 years old | 7 | 41.2 |
| 6 years old | 6 | 35.3 |
| 7 years old | 1 | 5.9 |
| 8 years old | 1 | 5.9 |
| Total | 17 | 100 |

**Table 2:** Severity of amblyopia in pediatric patients with refractive amblyopia.

| Severity of amblyopia | Number of eyes | Percentage, % |
|---|---|---|
| Mild | 21 | 61.76 |
| Moderate | 7 | 20.59 |
| Severe | 2 | 5.88 |
| Normal | 4 | 11.76 |
| Total | 34 | 100 |

**Table 3:** Duration of citicoline therapy in pediatric patients with refractive amblyopia.

| Duration of therapy | Number of eyes | Percentage, % |
|---|---|---|
| 3 months | 18 | 52.94 |
| 4 months | 2 | 5.88 |
| 6 months | 12 | 35.29 |
| 8 months | 2 | 5.88 |
| Total | 34 | 100 |

**Table 4:** Mean visual acuity (LogMAR) before and after citicoline therapy based on severity of amblyopia group.

| Severity of amblyopia | Mean visual Acuity (LogMAR) | | Success rate[a] | Sig. |
|---|---|---|---|---|
| | Before | After | | |
| Mild (n = 21) | 0.19 ± 0.02 | 0.04 ± 0.01 | 21 eyes (100%) | 0.000[b] |
| Moderate (n = 7) | 0.48 ± 0.04 | 0.16 ± 0,03 | 7 eyes (100%) | 0.001[b] |
| Severe (n = 2) | 0.92 ± 0.00 | 0.35 ± 0.05 | 2 eyes (100%) | 0.102 |

[a]Functional or relative visual acuity improvement. [b]Significant, LogMAR: Log (minimum angle of resolution).

BCVA data used in this study were converted into LogMAR using conversion tables. This was because the LogMAR method follows the standard recommendations for measuring visual acuity adapted by the British Standards Institutions [12]. In addition, the use of LogMAR facilitates data assessment and analysis [13]. The results of BCVA data collection obtained from medical records were processed using the Statistical Product and Service Solutions (SPSS) program. The Kolmogorov–Smirnov and Shapiro–Wilk normality test showed that the data was not normally distributed and thus the data were processed using the Mann Whitney test.

The severity of amblyopia in this study was classified based on the classification from Williams [9] and those 34 eyes varied from mild amblyopia to severe amblyopia. Mild amblyopia was the commonest form of amblyopia in this study (21 eyes/61.76%). Based on Table 4, it showed clinically improvement in visual acuity both either relative or functional. Relative improvement visual acuity is defined as achievement of 6/9 to 6/12 visual acuity or close to normal visual acuity 6/6. In addition, functional improvement was defined as improvement of visual acuity of two to three lines on the Snellen chart (0.2 LogMAR units) [14].

In the mild and moderate amblyopia groups there were significant differences in the use of citicoline ($p < 0.05$) and they showed significant improvement (Table 4). These results were consistent with previous studies which showed therapeutic success in mild and moderate amblyopia despite different methods of therapy. In the research of Woodruff et al. [15], investigators prescribed occlusion therapy for each sample and demonstrated therapeutic efficacy at all levels of amblyopia severity. Meanwhile, in the research of Hussein et al. [16], researchers prescribed patching therapy or pharmacological atropine therapy and demonstrated the same therapeutic efficacy.

**Table 5:** Mean visual acuity (LogMAR) before and after citicoline therapy based on duration of citicoline therapy group.

| Duration of therapy | Mean visual Acuity (LogMAR) at *x*th month observation | | | | Success rate[a] | Sig. |
|---|---|---|---|---|---|---|
| 3 months (P) n = 18 eyes | P0 | 0.15 ± 0.03 | P3 | 0.04 ± 0.02 | 18 eyes (100%) | 0.002[b] |
| 4 months (Q) n = 2 eyes | Q0 | 0.30 ± 0.10 | Q3 | 0.30 ± 0.10 | 2 eyes (100%) | 1.000 |
| | | | Q6 | 0.10 ± 0.00 | | 0.102 |
| 6 months (R) n = 12 eyes | R0 | 0.39 ± 0.07 | R3 | 0.20 ± 0.05 | 12 eyes (100%) | 0.014[b] |
| | | | R6 | 0.13 ± 0.04 | | 0.002[b] |
| | | | R12 | 0.10 ± 0.07 | | 0.000[b] |
| 8 months (S) n = 2 eyes | S0 | 0.51 ± 0.41 | S3 | 0.30 ± 0.30 | 2 eyes (100%) | 0.439 |
| | | | S6 | 0.20 ± 0.20 | | 0.317 |

[a]Functional or relative visual acuity improvement. [b]Significant, LogMAR: Log (minimum angle of resolution); P, Q, R, and S represent group of therapy based on duration of citicoline P0, Q0, R0, S0 = before therapy; P3, Q3, R3, S3 = at 3 months; Q6, R6, S6 = at 6 months; R12 = at 12 months.

In the severe amblyopia group there was no significant difference ($p>0.05$) (Table 4). This was because the number of eye samples used was too small to be analyzed statistically ($n = 2$). However, after further investigation, both eyes had clinical improvement in visual acuity. Both eyes have a change in BCVA over two Snellen chart lines (0.2 LogMAR units) and thus had improvement in functional visual acuity. This was also similar with the research of Hussein et al. [16] which showed that amblyopia patients with a BCVA of 20/200 or worse (BCVA>1.00 LogMAR units) had a high risk of functional therapeutic failure (final BCVA not anywhere near 6/9 to 6/12 nor normal eye BCVA 6/6). But, this study also showed patient with severe amblyopia who achieved clinically improvement of BCVA from LogMAR 0.92 to LogMAR 0.35.

Citicoline works by activating the biosynthetic structure of phospholipids in neuronal cell membranes. This activation causes an increase in brain metabolism and neotransmitter levels so that it has a neuroprotective effect [8]. It also enhances the visual retinal and postretinal pathways by stimulating the dopaminergic system. This stimulation can increase contrast sensitivity, visual acuity and visual response [7]. Because of this mechanism of action, citicoline is used for the treatment of glaucoma, amblyopia, nonarteritic ischemic optic neuropathy, brain stroke, dementia, Parkinson's disease, and other diseases [17].

The duration of oral administration of citicoline in children is up to one year and it is declared safe. But, the safety of this drug in the long term is still unknown [18]. However, in the research of Alvarez–Sabin and Roman [19] states that long-term use of citicoline therapy at an optimal dose is declared safe and effective by showing good tolerance.

Based on medical record data, four groups were classified according to the duration of citicoline administration. It varied from 3, 4, 6, or 8 months. Most of the patients received citicoline for three months (nine patients; 52.94%). The duration of administration of citicoline was varied depending on the patient's eye condition. Data on the duration of administration of citicoline in pediatric patients with refractive amblyopia can be seen in Table 3.

Based on Table 5, overall there was an improvement in visual acuity clinically in all groups at the end of therapy even though not all groups showed statistically significant improvement. Improvement of visual acuity clinically was defined as a decreased of LogMAR value from its initial value at the start of citicoline therapy. The results of visual acuity in the group of 3 and 6 months of drug administration showed a significant improvement at the 3rd month of observation (P3 and R3) ($p<0.05$). This was similar with the research conducted by Fresina et al. [6] who showed a significant improvement in visual acuity in the group with citicoline adjuvant therapy after observation at 90 days compared with the group that did not receive adjuvant therapy. In the group of 6 months administration of citicoline therapy with observation at 6th and 12th month (R6 and R12) also showed a significant improvement in visual acuity ($p<0.05$). This was also similar with Pawar et al. [8] who showed an improvement of visual acuity from the time of observation at the 5th month of therapy and the visual acuity remain good until the 12th month.

In the group of 4 and 8 months of drug administration, there was no statistically significant difference in the entire observation ($p>0.05$). This may be due to the small amount of data. However, the results of visual acuity in these groups showed improvement visual acuity clinically. In the group of 4 months administration of citicoline, this patient had moderate amblyopia in the right eye and mild amblyopia in the left eye. Both eyes achieved clinically improvement in amblyopia therapy within 6 months. This clinically improvement was seen through changes in visual acuity that exceeded 0.2 LogMAR units in the right eye and the end result of visual acuity that was closer to 6/9 in both eyes [14]. In terms of age, the patient was five years old. Patient was still in a critical period of vision development so this patient still have a high opportunity of effectiveness for early therapy [10, 11]. Based on the severity of amblyopia in the initial visual acuity, both eyes of the patient experienced relative and functional improvement. As previously described, the therapeutic efficacy of mild and moderate amblyopia in this study is relevant to other studies that have shown therapeutic success in patients with mild and moderate amblyopia [8, 15]. However, at the time of observation at the 3rd month, both eyes of this patient did not experience changes in visual acuity like the other eye samples. The absence of changes in visual acuity was probably due to non adherence factors for therapy in the first 3 months, either from primary therapy and/or citicoline adjuvant therapy. The lower the therapy adherence of the patient, the higher the risk of failure of the amblyopia therapy [16].

In the 8-month group, this patients had mild amblyopia in the right eye and severe amblyopia in the left eye. After further investigation, both eyes also experienced clinical success in amblyopia therapy within 4 months. The success of this therapy was evaluated from the final result of 6/6 visual acuity in the right eye and changes in visual acuity of more than 0.2 LogMAR units in the left eye [14]. In terms of age, this patient was six years old. At this age, the patient has a high chance of successful amblyopia therapy because the patient was still in a critical period of vision

development and thus early detection and therapy will promote further improvement [10, 11]. Based on the severity of the initial visual acuity, the patient's right eye experienced relative and functional improvement. Meanwhile, the left eye of the patient with severe amblyopia experienced relative improvement although there was no functional improvement. This was because the value of visual acuity at the start of the examination was too high [16].

## Conclusions

This study has shown that citicoline therapy resulted in a clinically and statistically significant improvement in visual acuity based on the severity of refractive amblyopia and the duration of citicoline therapy.

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Dewi Isadiartuti*, Noorma Rosita, Juni Ekowati, Achmad Syahrani, Toetik Ariyani and M. Ainur Rifqi

# The thermodynamic study of *p*-methoxycinnamic acid inclusion complex formation, using β-cyclodextrin and hydroxypropyl-β-cyclodextrin

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**Abstract**

**Objectives:** Cyclodextrin's ability to form an inclusion complex with a guest molecule is a function of two factors. The first is steric and depends on the relative size of cyclodextrin cavity to the guest molecule, while the second is the thermodynamic interaction between the different system components. This study therefore aims to determine the effect of β-cyclodextrin and hydroxypropyl-β-cyclodextrin as complex formers, on thermodynamic parameter values ($\Delta H$, $\Delta G$, and $\Delta S$) in the formation of inclusion complex with *p*-methoxycinnamic acid (*p*MCA).

**Methods:** The *p*MCA complex formation with β-cyclodextrin or hydroxypropyl-β-cyclodextrin was determined in 0.02 pH 4.0 M acetate buffer and 0.02 M pH 7.0 phosphate buffer, with a 0.2 μ value at 32, 37, and 42 ± 0.5 °C. This experiment was carried out in a waterbath shaker until a saturated solution was obtained. Subsequently, *p*MCA concentration was determined using UV spectrophotometer at the maximum *p*MCA wavelength, at each pH.

**Results:** The result showed *p*MCA formed inclusion complex with β-cyclodextrin or hydroxypropyl-β-cyclodextrin and exhibited increased solubility with increase in β-cyclodextrin or hydroxypropyl-β-cyclodextrin concentration. This temperature rise led to a decrease in the complex's constant stability ($K$). Furthermore, the interaction showed a negative enthalpy ($\Delta H<0$) and is a spontaneous processes ($\Delta G<0$). At pH 4.0, an increase in the system's entropy occurred ($\Delta S>0$), however, at pH 7.0, the system's entropy decreased ($\Delta S<0$).

**Conclusions:** The rise in *p*MCA solubility with increase in cyclodextrin solution concentration indicates an inclusion complex has been formed, and is supported by thermodynamic data.

**Keywords:** β-cyclodextrin; hydroxypropyl-β-cyclodextrin; inclusion complex; *p*-methoxycinnamic acid; solubility enhancement; thermodynamic.

## Introduction

The compound *p*-methoxycinnamic acid (*p*MCA) is an ethyl *p*-methoxycinnamic derivative obtained from sand ginger plant (*Kaempferia galanga* Linn.), and known to inhibit the enzymes cyclooxygenase 1 and 2 [1], thus, functioning as an analgesic. According to Ekowati and Dyah [2], *p*MCA present in sand ginger exhibits an even greater painkiller activity, compared to acetosal [2]. However, *p*MCA has a low solubility of 0.712 mg/mL in water, at 25 °C [3]. This causes low *p*MCA dissolution rate, thus, leading to incomplete absorption and low bioavailability [4]. The formation of inclusion complexes is therefore a suitable effort to improve *p*MCA solubility.

An inclusion complex is a system between drug molecules (guest) trapped in the cavity of a complex forming material (host) [5, 6]. Factors influencing an inclusion complex's formation are the accordance between the host and guest cavities' sizes and polarities, as well as thermodynamic parameters [7, 8]. Cyclodextrin is a constituent of inclusions with the ability to the ingredients' solubility in water.

In addition, cyclodextrin (CD) is a cyclic oligosaccharide compound with at least 6 D-(+)-glucopyranose units bound to α-1,4 glycoside bonds, a toroidal shape, and a cavity with a hydrophobic interior and hydrophilic exterior. These compounds are able to increase an ingredient's solubility by forming an inclusion complex with the guest molecules and stabilizing with several interactions, including hydrogen bonds, Van der Waals bonds, hydrophobic interactions and

***Corresponding author: Dewi Isadiartuti**, Faculty of Pharmacy, Department of Pharmaceutics, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 85100684948, E-mail: dewi-i@ff.unair.ac.id
**Noorma Rosita and M. Ainur Rifqi,** Faculty of Pharmacy, Department of Pharmaceutics, Universitas Airlangga, Surabaya, Indonesia
**Juni Ekowati and Achmad Syahrani,** Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Universitas Airlangga, Surabaya, Indonesia
**Toetik Ariyani,** Department of Clinical Pharmacy, Universitas Airlangga, Surabaya, Indonesia

electrostatic attraction. The cyclodextrin type most often used to form inclusion complexes is β-cyclodextrin, due to easy production, and relative inexpensiveness [8].

The cavity size of β-cyclodextrin and derivatives corresponds to the aromatic ring's size, thus the formation of inclusion complexes between these compounds and *p*MCA is highly likely [9].

Another commonly used cyclodextrin type for inclusion complex formation is hydroxypropyl-β-cyclodextrin (HPβCD). This is a derivative of β-cyclodextrin, with high solubility in water (above 50 g/100 mL, at 25 °C), due to the substitution of hydroxypropyl groups replacing the hydroxyl group in glucose. The compound is also less toxic and more environmentally friendly, compared to β-cyclodextrin [8, 10].

No previous studies have been conducted on the thermodynamics of inclusion complex formation between *p*MCA and cyclodextrin. This is a reversible reaction, and the complex compounds continuously formed undergo dissociation to produce free drugs and complex forming materials. However, the reaction is offset in a brief period of time, by the association of free drug molecules and complex forming materials. A molecule's ability to associate and dissociate to reach equilibrium is evident in the constant stability of complex formation ($K$) value [11]. This value is directly related to the complex formation free energy ($\Delta G$) value [12], and is influenced by pH, because the drug compound's ionized or unionized form (guest) influences the inclusion complex formation [8].

Therefore, this study aims to determine the effect of β-cyclodextrin and hydroxypropyl-β-cyclodextrin as complex formers, on thermodynamic parameter values ($\Delta H$, $\Delta G$, and $\Delta S$), in inclusion complex formation with *p*MCA, in a bid to better understand the interaction mechanism.

# Materials and methods

The materials used in this study include *p*-methoxycinnamic acid (Sigma-Aldrich), β-cyclodextrin (Sigma-Aldrich), hydroxypropyl-β-cyclodextrin (Sigma-Aldrich), distilled water, disodium hydrogen phosphate (Sigma-Aldrich), sodium dihydrogen phosphate (Sigma-Aldrich), citric acid (Sigma-Aldrich), sodium citrate (Sigma-Aldrich), 96% analytical grade ethanol, and 96% technical grade ethanol.

### Methods

#### Preparation buffer solutions

***0.02 M pH 4.0 citrate buffer solution:*** This was prepared by mixing 0.02 M sodium citrate solution with 0.02 M citric acid solution and adding sodium chloride, to obtain a 0.20 ionic strength.

***0.02 M pH 7.0 phosphate buffer solution:*** This was prepared by mixing 0.02 M disodium hydrogen phosphate solution with 0.02 M sodium dihydrogen phosphate solution, and adding sodium chloride, to obtain a 0.20 ionic strength.

#### Solubility testing

The *p*MCA's solubility in various β-cyclodextrin or hydroxypropyl-β-cyclodextrin concentrations ($0.10^{-3}$; $2.5 \times 10^{-3}$; $5.0 \times 10^{-3}$; $7.5 \times 10^{-3}$; and $10.0 \times 10^{-3}$ M) was tested in pH 4.0 and 7.0 solutions, with 0.20 ionic strengths, at 32, 37, and $42 \pm 0.5$ °C (305, 310, and 315 K). About 5.0 mL of β-cyclodextrin or hydroxypropyl-β-cyclodextrin solutions with certain pHs and concentrations were placed into a 15-mL vial. The vials were then inserted in the waterbath shaker and the temperature was adjusted as required. After the trial temperature was obtained, about 15 mg of *p*MCA was added and the mixture was shaken at a frequency of 140/min, until a saturated solution was formed. An optimized determination of *p*MCA saturated solubility in distilled water was indicated by the absence of a rise in *p*MCA concentration, after shaking for 5 h, under the solubility test's conditions. Subsequently, the solution was collected with an injection syringe, filtered with a 0.22 μm Millipore paper and the concentration was determined using UV spectrophotometer, at each pH's maximum *p*MCA $\lambda$. The maximum *p*MCA $\lambda$ were discovered to be 307.0 and 286.0 nm, pH 4.0 and 7.0, respectively.

# Results

## The solubility of *p*-methoxycinnamic acid in cyclodextrin solutions

Figures 1 and 2 show the *p*MCA solubility study in various β-cyclodextrin (βCD) and hydroxypropyl-β-cyclodextrin (HPβCD) solution concentrations, carried out at different pHs and temperatures.

## The complex stability constant and thermodynamic parameters

Table 1 shows the regression equation obtained from the curve of the relationship between the β-cyclodextrin (βCD) or hydroxypropyl-β-cyclodextrin (HPβCD) concentrations, and *p*MCA solubility.

The complex stability constant ($K$) is obtained using the formula below.

$$K_{(1:1)} = \frac{\text{slope}}{\text{intercept}\,(1 - \text{slope})} \quad (1)$$

Meanwhile, Table 2 shows the thermodynamic values of enthalpy ($\Delta H$), free energy ($\Delta G$), and entropy ($\Delta S$) values, calculated using the formula below. The slope value obtained from each regression equation in Table 1 was then used to calculate the enthalpy value ($\Delta H$ cal/mol) using Eq. (2), where $R$ represents the gas constant (1.987 calories/mol)

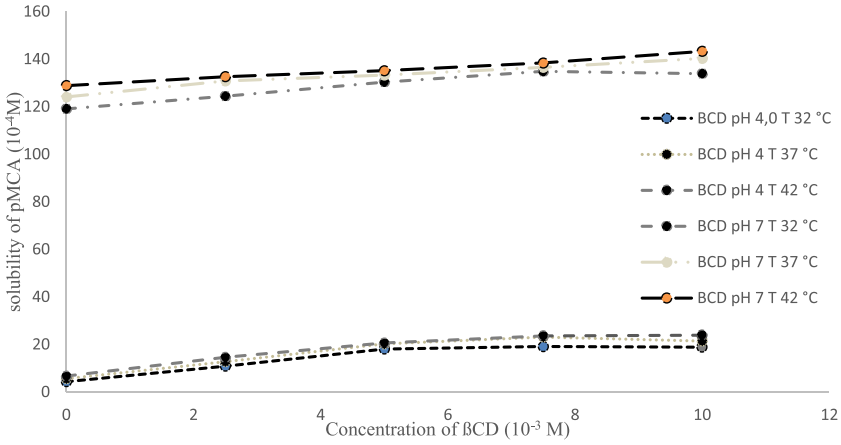


**Figure 1:** Solubility of *p*MCA in β-cyclodextrin (βCD) solutions at various pHs and temperatures.

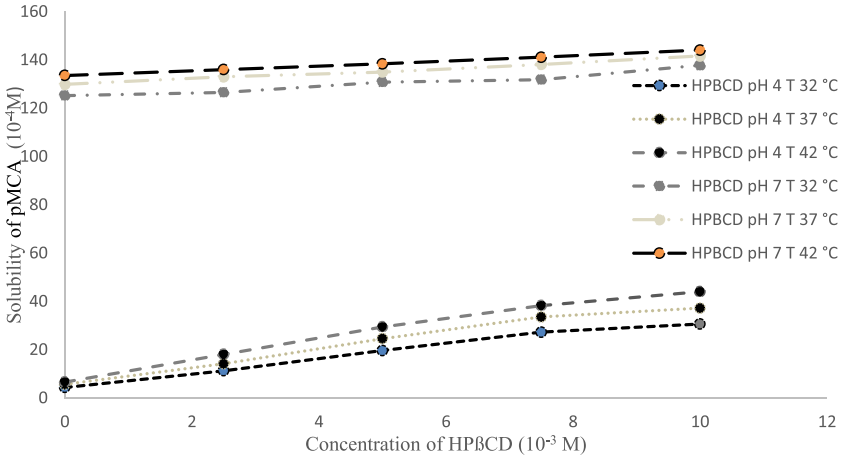


**Figure 2:** Solubility of *p*MCA in hydroxypropyl-β-cyclodextrin (HPβCD) solutions at various pHs and temperatures.

**Table 1:** Regression equation of the relationship between the pMCA solubility (M) and the concentration of cyclodextrin (M).

| Cyclodextrin | pH | Temperature (°K) | | |
|---|---|---|---|---|
| | | 305 | 310 | 315 |
| β-cyclodextrin | 4.0 | $y = 0.14864\,x + 6.8120.10^{-4}$<br>r = 0.9045 | $y = 0.16724\,x + 8.2880.10^{-4}$<br>r = 0.9072 | $y = 0.17372\,x + 9.1680.10^{-4}$<br>r = 0.9418 |
| | 7.0 | $y = 0.16040\,x + 120.50.10^{-4}$<br>r = 0.9491 | $y = 0.15248\,x + 125.40.10^{-4}$<br>r = 0.9849 | $y = 0.13824\,x + 128.73.10^{-4}$<br>r = 0.9938 |
| Hydroxypropyl-β-cyclodextrin | 4.0 | $y = 0.27440\,x + 4.8700.10^{-4}$<br>r = 0.9923 | $y = 0.33000\,x + 6.4753.10^{-4}$<br>r = 0.9899 | $y = 0.38040\,x + 8.2140.10^{-4}$<br>r = 0.9918 |
| | 7.0 | $y = 0.12140\,x + 124.33.10^{-4}$<br>r = 0.9699 | $y = 0.11444\,x + 129.74.10^{-4}$<br>r = 0.9962 | $y = 0.10452\,x + 133.36.10^{-4}$<br>r = 0.9949 |

$$\text{Slope} = -\Delta H\, 2,303\, R \tag{2}$$

Also, the free energy (Δ*G* cal/mol) was calculated using Eq. (3), where *R* represents the gas constant (1.987 calories/mol), *T* denotes absolute temperature (°K) and *K* indicates the complex stability constant value.

$$\Delta G = -2,303\, RT \log K \tag{3}$$

The value of entropy (Δ*S* cal/mol) was calculated using Eq. (4), by entering Δ*H* value as obtained from Eq. (2) and Δ*G* value as obtained from Eq. (3), where *T* denotes absolute temperature (°K).

$$\Delta G = \Delta H - T\Delta S \tag{4}$$

## Discussion

The solubility of *p*MCA in various β-cyclodextrin or hydroxypropyl-β-cyclodextrin concentrations ($0 \times 0.10^{-3}$

**Table 2:** The complex stability constant ($K$) and the thermodynamic parameters ($\Delta H$, $\Delta G$, and $\Delta S$) at various pHs and temperatures.

| $1/T$ (°K) | pH | β-Cyclodextrin | | | | Hydroxypropyl-β-cyclodextrin | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $K$ ($M^{-1}$) | $\Delta H$ (cal/mol) | $\Delta G$ (cal/mol) | $\Delta S$ (cal/mol) | $K$ ($M^{-1}$) | $\Delta H$ (cal/mol) | $\Delta G$ (cal/mol) | $\Delta S$ (cal/mol) |
| 305 | 4.0 | 256.30 | −2,200.40 | −3,361.89 | 3.81 | 776.53 | -0.879.21 | −4,033.57 | 10.34 |
| 310 | | 242.31 | −2,200.40 | −3,382.42 | 3.81 | 760.64 | -0.879.21 | −4,085.51 | 10.34 |
| 315 | | 229.32 | −2,200.40 | −3,402.48 | 3.82 | 747.44 | -0.879.21 | 4,136.99 | 10.34 |
| 305 | 7.0 | 15.85 | −4,391.34 | −1,674.84 | −8.91 | 11.11 | −4,833.31 | −1,465.48 | −11.04 |
| 310 | | 14.35 | −4,391.34 | −1,645.55 | −8.86 | 9.96 | −4,833.31 | −1,418.58 | −11.02 |
| 315 | | 12.46 | −4,391.34 | −1,585.61 | −8.91 | 8.75 | −4,833.31 | −1,354.97 | −11.04 |

to $10.0 \times 10^{-3}$ M) was tested in the medium of pH 4.0 and 7.0 buffer solutions, and the pH was selected based on the 4.04 *p*MCA pKa value. Based on the calculation using the Henderson–Hasselbalch equation, at pH 4.0 or pH equal to pKa, 50% of *p*MCA is in ionized form, while at pH 7.0 or 3 units above pKa, *p*MCA is about 99.9% ionized. The difference in the quantity of the guest molecule's unionized forms influences interaction with the hydrophobic cyclodextrin cavity.

The solubility study was conducted for a *p*MCA saturation time of 5 h. Figures 1 and 2 show the test results of *p*MCA solubility in β-cyclodextrin or hydroxypropyl-β-cyclodextrin. The *p*MCA solubility curve in β-cyclodextrin shows a BS type, meaning a rise in *p*MCA solubility occurred with increasing β-cyclodextrin concentration, but reached a plateau, and even tended to decrease at a certain point. This is because β-cyclodextrin's solubility in water is limited, thus, a complex deposition occurs at a certain concentration. Conversely, the *p*MCA solubility curve in hydroxypropyl-β-cyclodextrin shows an AL-type curve, meaning *p*MCA solubility rises with increasing hydroxypropyl-β-cyclodextrin concentration.

In the curves of *p*MCA solubility in β-cyclodextrin or hydroxypropyl-β-cyclodextrin, the calculated r value is greater, compared to the r table with a 95% confidence level of 0.754. This *p*MCA solubility curve's linearity shows the interactions occurring between guest molecules (*p*MCA) to form complexes with β-cyclodextrin or hydroxypropyl-β-cyclodextrin in a 1:1 M ratio. Table 1 shows the regression equation obtained from the *p*MCA solubility curve with β-cyclodextrin or hydroxypropyl-β-cyclodextrin concentration, while Table 2 shows the complex stability constant ($K$) value calculate using this equation.

According to Table 2, the increase in temperature causes a decrease in the complex stability constant ($K$). This shows the temperature rise causes the formed inclusion complex to become more unstable, meaning the interaction between *p*MCA and β-cyclodextrin or hydroxypropyl-β-cyclodextrin is released more easily. The cyclodextrin inclusion complex formation is a reversible reaction and temperature rise causes the guest *p*MCA molecule in the host cavity to dissociate out of the cyclodextrin cavity, as indicated by the lower $K$ value. In addition, a rise in pH reduces the complex stability constant ($K$), and the amount of ionized *p*MCA influences the compound's ability to enter the β-cyclodextrin or hydroxypropyl-β-cyclodextrin cavity. Ionized *p*MCA tends to come out of the hydrophobic cyclodextrin cavity, making the complex less stable.

At pH 4.0, hydroxypropyl-β-cyclodextrin's stability constant complex ($K$) is greater, compared to β-cyclodextrin, indicating the complex bonds formed in the hydroxypropyl-β-cyclodextrin are stronger. This is probably because hydroxypropyl-β-cyclodextrin has a hydroxypropyl group with the ability to provide steric barrier for *p*MCA to escape from the HPβCD cavity.

Based on Table 2, a curve of the relationship between log $K$ and $1/T$ is plotted, with β-cyclodextrin and hydroxypropyl-β-cyclodextrin inclusion complex formation at pH 4 as well as 7. Each curve provided a regression equation with a correlation coefficient value (r)>0.9, indicating a relationship between $1/T$ and log $K$. Subsequently, the slope obtained in each regression equation was used to calculate $\Delta H$ as in Eq. (2), as well as thermodynamic parameters ($\Delta G$ and $\Delta S$), as in Eqs. (3) and (4).

The results of β-cyclodextrin or hydroxypropyl-β-cyclodextrin at various pH both produced a negative enthalpy value ($\Delta H$), indicating an exothermic the dissolution process. Furthermore, the interaction between *p*MCA with β-cyclodextrin or hydroxypropyl-β-cyclodextrin is mainly hydrophobic. The free energy changes ($\Delta G$) also had a negative sign, indicating a spontaneous the complex formation, while the entropy change ($\Delta S$) in experiments with β-cyclodextrin or hydroxypropyl-β-cyclodextrin at pH 4.0 was positive, indicating an increase in system irregularities.

The regular water system surrounding the *p*MCA molecule becomes irregular because *p*MCA enters the cyclodextrin. This condition is not desirable because polar–nonpolar repulsion occurs between water molecules and the inner cyclodextrin cavity, thus, the condition is quickly replaced by the presence of guest molecules less polar than water molecules [13]. Meanwhile, in experiments with β-cyclodextrin or hydroxypropyl-β-cyclodextrin at pH 7.0, a negative value was obtained, indicating a lower system irregularity. This is possibly because the system irregularity caused by guest molecule entry and exit to and from the host molecule, no longer occurs. The cyclodextrin cavity is hydrophobic, thus, guest molecules in ionized form find it more difficult to enter.

## Conclusions

Based on the results, *p*MCA was concluded to form an inclusion complex with both β-cyclodextrin and hydroxypropyl-β-cyclodextrin. The interaction between *p*MCA and hydroxypropyl-β-cyclodextrin is stronger, compared to β-cyclodextrin, as indicated by the higher complex stability constant ($K$) value. In addition, the temperature rise led to a constant decrease in stability, and the interaction showed a negative enthalpy ($\Delta H<0$) as well as a spontaneous process ($\Delta G<0$). In pH 4.0 a rise in the system's entropy occurred ($\Delta S>0$), while in pH 7.0 a reduction in the system's entropy was observed ($\Delta S<0$)

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**Ethical approval:** Not applicable.

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Muhammad A. S. Rijal*, Hanah Masitah, Fanny Purvitasari and Retno Sari

# The effect of chitosan type and drug-chitosan ratio on physical characteristics and release profile of ketoprofen microparticles prepared by spray drying

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**Abstract**

**Objectives:** In order to minimize gastrointestinal irritation and to extend the absorption of ketoprofen, microparticles prepared with chitosan have been developed. In this study, chitosan type and drug-chitosan ratio were investigated to prepare microparticles of ketoprofen and evaluated for physical characteristics and drug release profiles.

**Methods:** Microparticles were prepared by using ionic gelation methods with chitosan, which has two different viscosities i.e., 19 and 50 cPs, cross-linked with tripolyphosphate, and dried by spray drying method. The microparticles were made with a drug-chitosan ratio of 5:15 and 6:15.

**Results:** The results showed that the microparticles had spherical shapes. Increasing the amount of ketoprofen improved the drug content and entrapment efficiency. Evaluation of drug release in simulated intestinal fluid (pH 6.8) showed that the microparticles prepared with chitosan 19 cPs had the slowest release rate than those of chitosan 50 cPs, while that of the microparticles prepared with chitosan 50 cPs with the ratio of drug/polymer 6:15 was the fastest, as shown by its slope value. The release rate of microparticles with chitosan 19 cPs was slower than those microparticles with chitosan 50 cPs.

**Conclusions:** It could be suggested that by increasing the amount of ketoprofen, it improved the entrapment efficiency and the release rate of microparticles.

**Keywords:** chitosan; ketoprofen; microparticle; spray drying; tripolyphosphate.

***Corresponding author: Muhammad A. S. Rijal**, Department of Pharmaceutics, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62315933150, E-mail: muh-a-s-r@ff.unair.ac.id
**Hanah Masitah, Fanny Purvitasari and Retno Sari,** Department of Pharmaceutics, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

## Introduction

Microparticles are spherical solid particles ranging in size from 1 to 1,000 μm that are made of polymers, wax, or other protective materials and intended for several purposes; which are to mask the taste and odor of the drug substance, to minimize the side effects of drugs, to improve drug's stability, to increase the solubility of drugs and to sustain the release of drugs [1].

Ketoprofen is nonsteroidal anti-inflammatory drugs that has been widely used for the treatment of arthritis rheumatoid disease. Ketoprofen is highly absorbed in the gastrointestinal tract (GIT) and has a short plasma half-life time of about 1.5–4 h. Therefore, it should be administered at a quite high frequency of use i.e., for 3–4 times a day. The severe side effects of ketoprofen are mostly found in the gastrointestinal tracts (GIT) that involve irritation, ulceration, bleeding, and perforation [2–4]. Microparticles are great tools to encapsulate ketoprofen thus they may be able for overcoming those problems.

Chitosan shows interesting polymeric characteristics for preparing microparticles. Chitosan is a natural linear biopolyaminosaccharide obtained from the alkaline deacetylation of chitin. Chitosan is a weak base with a pH of 6.3 and insoluble in water and organic solvents. However, chitosan is soluble in dilute acid solution at pH <6.5, in which a glucosamine unit changes into a form of $R\text{-}NH3^{+}$ that is water-soluble, so it can undergo cross-linking reaction with multivalent anions [5, 6]. In addition, chitosan provides antacid and antiulcer effects that can prevent irritation of the GIT [7].

Tripolyphosphate (TPP) is a polyanion compound that can interact with cationic compounds, such as chitosan, through electrostatic forces [8, 9]. In addition to TPP, glutaraldehyde is a cross connector that is often used in the preparation of microparticles. However, due to its high toxicity, glutaraldehyde is no longer used in pharmaceutical preparations [10].

There are some critical factors that affect the physicochemical characteristics of microparticles including the

molecular weight of the polymer, the ratio of drug-polymer, and the total mass of drug and polymer [1]. It has been known that the entrapment efficiency of the drug improves with the increasing concentration of initially fed drugs and the addition of a crosslinking agent. The drug release increases with the increase of drug loaded in microparticles [5, 11]. The ratio of drug-polymer has no significant effects on the particle shape of microparticles, but it affects the drug release from the microparticles [12].

For obtaining a small particle size, the spray drying method is often used to prepare microparticles [13]. It has several advantages such as a/the fast, simple, reliable, and reproducible process that can produce microparticles in nearly spherical form with a flat surface, the narrow or relatively homogenous particle size distribution, and less dependent on the solubility of the drug and polymer [14, 15]. However, the main disadvantage of this method is the loss of a large number of products, mainly due to the adhesion of the microparticles to the inner walls of the spray dryer [15]. Factors affecting the preparation of microparticles by spray drying method are the size of the nozzle, the inlet, pump rate, and flow rate [16]. The larger the size of the nozzle used, the larger the produced particle size is. Increasing the inlet temperature will fasten the drying process; however, it is likely that the damage to the drug's stability is also getting bigger [17]. The greater the flow rate and nozzle size is, the larger the size of the resulting microparticles is [14, 16].

Therefore, it is important to investigate the effect of the difference of chitosan types and ratio of chitosan-ketoprofen on physical characteristics and drug release profile of ketoprofen microparticles. Microparticles were prepared by spray drying method using chitosan with different types i.e., chitosan 19 and 50 cPs with the drug:chitosan ratio of 5:15 and 6:15. The concentration of chitosan solution was 0.25% w/v. Evaluation of the microparticles was performed to determine the physical characteristics, the drug content, and release of ketoprofen from the microparticles.

# Materials and methods

## Materials

Ketoprofen was obtained from Kimia Farma (Bandung, Indonesia); Chitosan was purchased from Biotech Surindo (Cirebon, Indonesia) and has a deacetylation degree of 86.63% and viscosity of 19 and 50 cPs. Sodium triphosphate pentabasic was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals and solvents used in this study were of the highest grade available.

## Methods

The composition of the microparticle formula can be seen in Table 1.

**Preparation of ketoprofen-chitosan microparticles:** Microparticles were prepared by adding chitosan solution into ketoprofen solution. The chitosan solution was prepared at a concentration of 0.25% w/v in 0.38% v/v acetic acid solution. About 0.25% w/v TPP solution was then made and added into ketoprofen-chitosan solution drop wisely while being stirred constantly for 3 h. After that, the mixture was allowed to stand overnight and dried using a spray dryer (tipe, SD – Basic Lab Plant UK Ltd, England) by setting the inlet temperature of 100 °C, pump speed of 3.5 scale, compressed air of 2 bars, and with a nozzle size of 1.0 mm.

**Fourier transform infrared (FT-IR) analysis of ketoprofen-chitosan microparticles:** The infrared (IR) spectrum of ketoprofen-chitosan microparticles was analyzed with Fourier-Transform Infrared (FTIR) spectrophotometer (Jasco FT-IR 5300, Easton MD, USA) and the sample was prepared using the KBr disc method. Ketoprofen and KBr were crushed until homogeneous, put into a vacuum dryer, and then pressed with a hydraulic press to obtain a transparent disc. The results were analyzed by comparing the IR spectrums with that of ketoprofen.

**Size distribution of ketoprofen-chitosan microparticles:** The size distribution of microparticles was determined using the microscopic method with an optical microscope (Olympus CHB Microscope, Olympus, Japan). The sample was a random sample of 300 particles.

**Physical morphology of ketoprofen-chitosan microparticles:** The morphology of microparticles that involves particle shape and surface texture was visually observed and the photomicrographs were then taken using an optical microscope (Olympus CHB Microscope, Olympus, Japan) with 1,000× magnification.

**Drug content and entrapment efficiency of ketoprofen in microparticles:** In order to quantify the amount of drug entrapped in microparticles, ketoprofen microparticles were weighed and added with ethanol to dissolve the particles. Phosphate buffer of pH 6.8 was then added to dilute the mixtures. The absorbance of ketoprofen was measured at 259 nm using spectrophotometer UV–Vis (Cary 50 Conc, Varian Inc., Australia). The analysis was carried out

**Table 1:** Formula of ketoprofen-chitosan microparticles.

| Materials | F1[a] | F2[b] | F3[a] | F4[b] |
|---|---|---|---|---|
| Ketoprofen, mg | 250 | 300 | 250 | 300 |
| Chitosan of 19 cPs (0.25% w/v), mg | 750 | 750 | – | – |
| Chitosan of 50 cPs (0.25% w/v), mg | – | – | 750 | 750 |
| Tripolyphosphate, TPP, mg | 300 | 300 | 300 | 300 |

[a]Ratio of drug/polymer=5:15, [b]ratio of drug/polymer=6:15.

in triplicates. Drug content and entrapment efficiency of ketoprofen in microparticles were calculated using the following formulas:

$$\text{Drug content} = \frac{\text{mass weight of ketoprofen entrapped in microparticles}}{\text{total mass weight of microparticles}} \times 100\%$$

$$\text{Entrapment efficiency} = \frac{\text{mass weight of ketoprofen entrapped in microparticles}}{\text{mass weight of ketoprofen added initially}} \times 100\%$$

***In vitro* ketoprofen released from ketoprofen-chitosan microparticles:** Evaluation of drug released from microparticles was performed using a basket method with a dissolution tester (Erweka DT-700, ERWEKA GmbH, Germany). Microparticles equivalent to 25.0 mg ketoprofen were weighed and put in the basket with a mesh size of 100 and the stirring stick was set at a speed of 50 rpm. The release media was a 900 mL of phosphate buffer pH 6.8 and heated at 37 ± 0.5 °C. During some interval times, about 5 mL of samples were taken and then filtered through 0.45 µm filter paper. At that sampling time, the dissolution medium was then replaced with 5 mL of phosphate buffer solution pH 6.8. Afterward, the absorption of each sample was observed using spectrophotometer UV–Vis (Cary 50 Conc, Varian Inc., Australia) at 259 nm. The sample was measured in triplicates. The results were then plotted into the curve of release profile with the square root of time as the axis and percent of dissolved ketoprofen as ordinate. The ketoprofen release rate was indicated by the value of $b$ (slope) of the linear equation of $y=bx + a$.

**Statistical analysis:** SPSS v18.0 statistical software was used for data analysis. The data of drug content, encapsulation efficiency, and release rate of ketoprofen-chitosan microparticles are expressed as the mean ± standard error of the mean (SD). A two-tailed Student's t-test was used for statistical analysis. $p<0.05$ was considered to indicate a statistically significant difference.

# Results

## The infrared spectrum of ketoprofen-chitosan microparticles

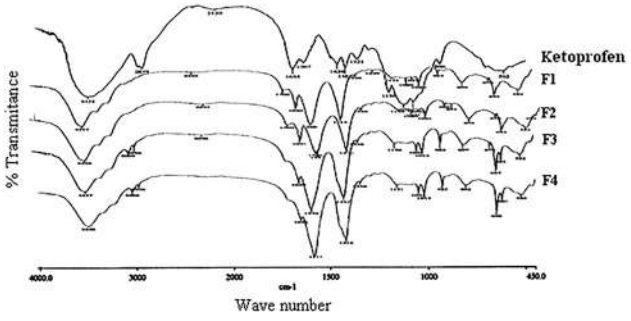


**Figure 1:** Infrared spectrum of pure ketoprofen, ketoprofen-chitosan microparticles from different formulae (F1, F2, F3, and F4).

## Morphology of ketoprofen-chitosan microparticles

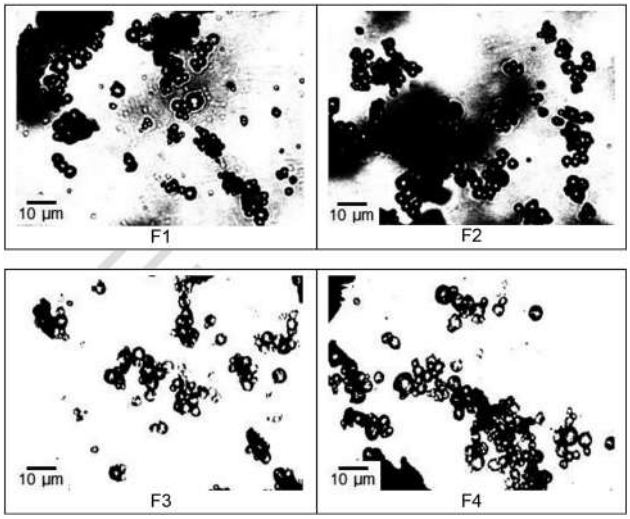


**Figure 2:** Morphology of F1, F2, F3, and F4 of ketoprofen-chitosan microparticles observed using an optical microscope (at a 1,000× magnification).

## Particle size distribution

**Table 2:** Size distribution of ketoprofen-chitosan microparticles.

| Particle size, µm | Percentage of microparticle number | | | |
|---|---|---|---|---|
| | F1 | F2 | F3 | F4 |
| 1.01 | 2.33 | 0.67 | 2.01 | 1.68 |
| 1.52 | 9.00 | 5.67 | 14.61 | 13.77 |
| 2.03 | 40.33 | 29.67 | 39.88 | 42.20 |
| 2.53 | 15.33 | 12.00 | 17.00 | 12.23 |
| 3.04 | 23.67 | 30.00 | 11.59 | 15.60 |
| 3.55 | 2.00 | 1.00 | 8.12 | 5.35 |
| 4.05 | 4.33 | 17.33 | 16.00 | 9.17 |
| 4.56 | 0.00 | 0.00 | 0.00 | 0.00 |
| 5.07 | 3.00 | 3.67 | 0.00 | 0.00 |
| Mean | 2.48 | 2.83 | 2.18 | 2.21 |

**Table 3:** Drug content, encapsulation efficiency (EE), and release rate of ketoprofen-chitosan microparticles.

| Formula | Drug content, % ± SD | EE, % ± SD | Release rate, %/minute$^{1/2}$ ± SD |
|---|---|---|---|
| Ketoprofen | – | – | 14.67 ± 0.29 |
| **F1** | 3.84 ± 0.04 | 45.69 ± 0.53 | 12.60 ± 0.44 |
| **F2** | 4.91 ± 0.04 | 49.40 ± 0.87 | 13.45 ± 0.58 |
| **F3** | 4.14 ± 0.02 | 55.22 ± 0.28 | 12.79 ± 0.19 |
| **F4** | 4.94 ± 0.04 | 57.09 ± 0.45 | 13.80 ± 0.58 |

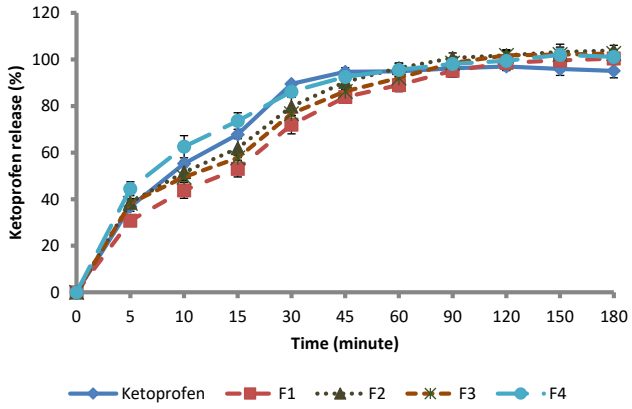


**Figure 3:** The profile of ketoprofen released from ketoprofen-chitosan microparticles in phosphate buffer pH 6.8.

## Discussion

On the evaluation of ketoprofen-chitosan microparticles using IR spectrophotometry (Figure 1), there was a peak PO4 $2^-$ a group of TPP at 1,090 $cm^{-1}$ region, which indicates an interaction between chitosan and TPP [18]. In addition, at the wavelength number region at 1,635 $cm^{-1}$, there was also a peak that shows a vibration deformation on NH in $NH3^+$ ions, indicating that there has been a bond in chitosan-TPP [19].

The morphology of microparticles was determined by an optical microscope. In general, the microparticles of F1, F2, F3, and F4 appear to be similar spherical forms and some particles formed agglomerates (Figure 2). It was because the concentration of chitosan solution used to prepare them was the same, which was 0.25% w/v so that the viscosity of the solutions prior to drying with a spray dryer was also similar. Therefore, no significant differences in the microparticles morphologies were observed.

The particle size distribution of ketoprofen-chitosan microparticles showed that microparticles of all formulas had particle sizes ranging from 1.00 to 5.00 μm with an average diameter less than 3.00 μm (Table 2). The average particle size was 2.48, 2.83, 2.18, and 2.21 μm for F1, F2, F3, and F4, respectively. These results are in accordance with another study that produced an average diameter of 2–3 μm [20]. Table 2 shows that majority of particles are in the fraction 2.03 μm. There was a slight shift to the 3.04 μm fraction at F2. This attributed to agglomerated particles, which shifted the mean particle size values to higher numbers. These results indicate that the ratio of drug-chitosan had no effects on the physical characteristics of ketoprofen-chitosan microparticles, but it affected the drug release from the microparticles.

Results of evaluation of ketoprofen content in microparticles showed that F1 contained ketoprofen of 3.84 ± 0.04%, F2 contained ketoprofen of 4.91 ± 0.04%, F3 contained ketoprofen of 4.14 ± 0.02%, and F4 contained ketoprofen of 4.94 ± 0.04% (Table 3). As shown in Table 3, the increase in drug content was more significant in increasing the ketoprofen: chitosan ratio than the chitosan type. The effect of ketoprofen-chitosan ratio on drug content was determined by performing statistical analysis on F1 with F2 and F3 with F4. The result in both analyzes shows a p-value=0.000 which indicates that increasing the amount of ketoprofen ratio from 5:15 to 6:15 will increase the drug content. This increase can be caused by the availability of space in the chitosan matrix to accommodate some drugs to be trapped in the matrix. Meanwhile, the encapsulation efficiency of F1, F2, F3, and F4 was 45.69 ± 0.53%, 49.40 ± 0.87%, 52.22 ± 0.28%, and 57.09 ± 0.45%, respectively. Statistical effect of ketoprofen ratio on encapsulation efficiency gave the same result as analysis of its effect on drug content

Results of release test and release profile of ketoprofen of microparticles can be seen in Figure 3. The release rate of microparticles was calculated based on the results of the microparticle release test by calculating the slope of the linear regression equation between the roots of the time and % of dissolved ketoprofen. The determination of the slope of each formula was calculated from the minute 0 until the 45 min. The release rate of ketoprofen, F1, F2, F3, and F4 was 14.668 ± 0.29; 12.590 ± 0.43; 13.448 ± 0.58 ($mg/min^{1/2}$); 12.790 ± 0.19; and 13.798 ± 0.58 ($\%/min^{1/2}$) respectively. From statistical analysis two-tailed Student's t-test (p=0.05), release rate between ketoprofen-chitosan 19 cPs and ketoprofen-chitosan 50 cPs microparticles was significantly different. However, the release of ketoprofen from different formulations showed high initial release (burst release) of the drug, and about 95–100% of the drug was released within 3 h. Increasing the cross-linking density can prevent the burst release [21].

## Conclusions

The development of ketoprofen-chitosan microparticles by spray drying technique producing spherical particles with uniform sizes. Increasing the drug-polymer ratio from 5:15 to 6:15 can improve drug content and encapsulation efficiency. Ketoprofen-chitosan 19 cPs type gave the slower release than those of ketoprofen-chitosan 50 cPs type. However, the release of ketoprofen from different formulas showed a burst release in the first hour. From these results, it is necessary to carry out further studies on increasing the crosslink density to prevent the drug burst release

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Anisyah Achmad*, Tika Yasmin Rahmayanti and Bagus Putu Putra Suryana

# The maximum dose and duration in the therapy single use methotrexate to achieve remission by rheumatoid arthritis patients through disease activity score 28 (DAS28)

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**Abstract**

**Objectives:** One of the treatments for rheumatoid arthritis (RA) was methotrexate which a disease modifying antirheumatic drug therapy. The use of methotrexate required the right dose and length of therapy to achieve remission. The effectivity of methotrexate could be accounted by disease activity score 28 (DAS28) as a tool has been used clinically with a combination number of tender joints, swollen joints, erythrocyte sedimentation rate, and global clinical assessment by the patient. The aim of this study was to determine the effective dose and length of therapy methotrexate was measured by DAS28 score.

**Methods:** This research was a cross-sectional study and data was collected from patient medical records in Saiful Anwar Hospital, Malang, from February to July 2018. The research has been given ethical clearance. The inclusion criteria for the 88 subjects were men and women, over 20 years of age, usage of only methotrexate for at least three months, an erythrocyte sedimentation rate score, uncomplicated inflammatory bowel disease, cancer, and systemic lupus erythematosus. All data obtained was entered in formula DAS28. The Statistic analysis used both Pearson and Spearman's rank correlation.

**Results:** Only 16 patients achieved remission. There were not significant correlation in statistical analysis between DAS score and cumulative dose (r=−0.091; p=0.400), average dose (r=0.043; p = 0.692), maximum dose (r=0.074; p=0.492), and length of therapy (r=−0.075; p = 0.489). The initial dose of therapy methotrexate was different and the length of therapy was adjusted to the patient's health condition.

**Conclusions:** The maximum dose and length of therapy methotrexate was required to achieve remission in RA.

**Keywords:** DAS28; methotrexate; rheumatoid arthritis.

## Introduction

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune disease that causes inflammation, leading to joint deformity and disability [1]. RA affects 0.5–1.0% of the population with the number of female patients being higher than that of male patients [2], RA patients will experience a decline in the quality of social and economic.

Methotrexate is the first choice of disease modifying antirheumatic drugs (DMARDs) for therapy of RA. It can be used as monotherapy or combination with leflunomide, hydroxylchloroquine, sulfasalazine, adalimumab, etanercept, and infliximab. Regiment therapy using methotrexate can achieve the remission clinic condition through maintained tapering dose of methotrexate if higher dose were clinically administered [3]. The methotrexate is always chosen as the first choice in treatment and has been given to 70% of patients who suffer from this disease [4]. The reason for choosing methotrexate is based on a comparison of the risks and benefits of equal use, guaranteed safety, and affordable prices [5]. Methotrexate can also be used monotherapy or in combination, each of which can be easily monitored for clinical recurrence. If the combination is carried out with DMARD, more intensive monitoring of the disease is needed [6]. Another reason for choosing methotrexate, it does not cause dependence on steroids when the use of methotrexate is stopped [1].

Improvement and effectiveness of therapy of RA can be measured through instruments such as DAS28. Its score is low for therapy effectivity. There are two types of DAS checking. The namely are DAS28-ESR and DAS28-CRP. There are 28 joints measured when they experienced pain

***Corresponding author: Anisyah Achmad**, Department of Clinical Pharmacy, Major of Pharmacy, Medical Faculty, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia, Phone: +62 81255636910, E-mail: achmadanisyah@gmail.com
**Tika Yasmin Rahmayanti,** Medicine Faculty, Department of Pharmacy, Brawijaya University, Malang, Indonesia
**Bagus Putu Putra Suryana,** Department of Rheumatology, Internal Medicine, Saiful Anwar Hospital, Malang, Indonesia

and swelling during the examination for the patient RA. It has been widely used clinically with a combination number of the tender joints, the swollen joints, erythrocyte sedimentation rate (ESR), or C-reactive protein (CRP), and global clinical assessment by the patient [7]. ESR result shows manifestation as the chronic phase in RA disease and usually does not have different significant results from previous examinations. ESR is more interpretive of disease activity in the last weeks when blood samples are drawn whereas CRP can change its value in a quicker and more sensitive time so that it can change the interpretation of the value of the patient's disease activity. DAS28-ESR has been considered by EULAR (European Alliance of Associations for Rheumatology) and its calculations have been validated. Disease activity in RA is more represented using DAS28-ESR than DAS28-CRP [7].

Long-term therapy with adjusted dose of methotrexate is needed in order to achieve the remission condition for RA. Dose of methotrexate and length of therapy has been proven as a contributing factor to therapy efficacy, with regiment therapy of methotrexate with starting dose of 7.5–12.5 mg and usage interval of once weekly [3]. Different patient conditions become one of the considerations for decisions on methotrexate dose optimization and length of therapy. The previous research conducted by Yamanaka et al. said that there is a positive correlation in statistically between an average dose of methotrexate >8 mg per week and DAS28 score <3.2 [8].

The results of this study can be used by clinicians and pharmacists for evaluating the dose and length of therapy methotrexate in RA patient. The efficacy of therapy can be monitored by DAS28-ESR score.

## Materials and methods

The research was done at the Rheumatology Polyclinic of Saiful Anwar Hospital (RSSA), Malang, from February to July 2018 and had been given ethical clearance number 400/32/K.3/302018 by the ethics commission of RSSA. The research was a cross-sectional study, with quantitative analysis for the research method for acknowledging the correlation between methotrexate dose as well as length of therapy and RA disease activity as measured by DAS28. The subjects who became involved in the study were calculated using the purposive sampling method and the subjects agreed to sign a consent form. In this study, the DAS28 calculation used ESR data.

The inclusion subjects for this research were men and women >20 years old who have been diagnosed with RA, have used only methotrexate for at least three months, possess laboratory results as differential diagnosis, and were proven to have RA with ESR. Patients did not possess other disease complications (such as IBD, SLE, or cancer). The exclusion was patients who smoke and/or drink alcohol. There were three different kinds of dose in this study which are cumulative dose, average dose, and maximum dose, found through observation of medical records. Cumulative dose was defined as the product of methotrexate dose and length of therapy, average dose was defined as the sum of methotrexate dose variations during therapy, and maximum dose was defined as the highest dose of methotrexate received by a patient. All doses were calculated in milligrams per week (mg/week). The length of therapy was counted from patient have used oral methotrexate for at least three months until May 2018. Calculation of scoring results from DAS28 was performed using mathematical equations such as the following [3, 9, 10].

$$\text{DAS28} = 0.56\sqrt{(\text{TJC28})} + 0.28\sqrt{(\text{SJC28})} + 0.70\ln(\text{ESR}) + 0.014\text{xGH}$$

The following was an explanation for the formula above: TJC = tenderness in 28 joints, SJC = swelling in 28 joints, ESR = erythrocyte sedimentation rate in the first 1 h, GH = patient's Assessment of General Health measured by VAS (visual analog score).

Indonesia Rheumatology Association [3] divides RA disease activity into the four severity degrees of remission, low activity, medium activity, and high activity, for which the classification was detailed in Table 1.

**Table 1:** Interpretation of DAS28-ESR [3].

| Disease activity | DAS28-ESR score |
|---|---|
| Remission | ≤2.6 |
| Low activity | >2.6 to ≤3.2 |
| Medium activity | >3.2 to ≤5.1 |
| High activity | >5.1 |

In this study, a statistical analysis of maximum dose, average dose, accumulated dose (mg/week), and length of therapy (week) will be carried out of DAS28 score. The statistical analysis used both Pearson and Spearman's rank correlation ($p<0.05$).

## Result

Result of demographic data between age and gender were in Table 2. The average age of subjects was approximately 46–55 years old with 30 subjects in total and more suffered by women as many as 74 of 88 patients.

Methotrexate was the DMARDs group and the first choice drug for RA therapy [7]. Methotrexate dose and length of therapy became influence factors for the improvement of RA patient therapy. The purpose of grouping dose and length of therapy was to simplify identification of the distribution for the group that has the highest number of patients. Based on empirical Sturges' rule, methotrexate dose were divided into seven classes with the interval for each class being 893 mg for cumulative dose and 1.8 mg for average dose and maximum dose. Meanwhile, length of therapy was divided into seven classes with the interval for each class being 87 weeks. The results showed that the highest numbers of patients are 61

**Table 2:** Demographic data of RA patients.

| Demographic data | | |
|---|---|---|
| **Age group, years** | **Total** | **Percentage** |
| **Age** | | |
| 17–25 | 7 | 8% |
| 26–35 | 2 | 2% |
| 36–45 | 11 | 12% |
| 46–55 | 30 | 34% |
| 56–65 | 26 | 30% |
| >65 | 12 | 14% |
| Total | 88 | 100% |
| **Gender** | | |
| Male | 14 | 16% |
| Female | 74 | 84% |
| Total | 88 | 100% |

patients in the cumulative dose class of 75–968 mg, 31 patients in the average dose class of 6.9–8.7 mg, 27 patients in the maximum dose class of 8.8–10.6 mg, and 58 patients in the length of therapy class of 14–101 weeks. Result of this study for dose and length methotrexate can be seen in Table 3.

After observing and measuring RA patients, the results were as shown in Table 4. The results showed that there were 16 patients who achieved clinical remission while 54 patients achieved medium activity. This indicates that the methotrexate therapy had not been effective. Based on the results of correlation analysis, there was not significant correlation of cumulative dose, average dose, maximum dose and length of therapy methotrexate with DAS-ESR28 score (Table 5).

**Table 3:** Methotrexate dose and length of therapy distribution.

| Dose group | Total | Percentage |
|---|---|---|
| **Cumulative dose, mg** | | |
| 75–968 | 61 | 69% |
| 969–1862 | 10 | 11% |
| 1863–2,756 | 11 | 12% |
| 2,757–3,650 | 4 | 5% |
| 3,651–4,544 | – | – |
| 4,545–5,438 | – | – |
| 5,439–6,332 | 2 | 3% |
| Total | 88 | 100% |
| **Average dose, mg** | | |
| 5–6.8 | 6 | 7% |
| 6.9–8.7 | 31 | 35% |
| 8.8.-10.6 | 25 | 27% |
| 10.7–12.5 | 20 | 23% |
| 12.6–14.4 | 2 | 3% |
| 14.5–16.3 | 3 | 4% |
| 16.4–18.2 | 1 | 1% |
| Total | 88 | 100% |
| **Maximum dose, mg** | | |
| 5–6.8 | 2 | 3% |
| 6.9–8.7 | 23 | 25% |
| 8.8–10.6 | 27 | 30% |
| 10.7–12.5 | 21 | 24% |
| 12.6–14.4 | – | – |
| 14.5–16.3 | 14 | 16% |
| 16.4–18.2 | 1 | 2% |
| Total | 88 | 100% |
| **Length of therapy, weeks** | | |
| 14–101 | 58 | 66% |
| 102–189 | 14 | 16% |
| 190–277 | 9 | 11% |
| 278–365 | 3 | 3% |
| 366–453 | 1 | 1% |
| 454–541 | 1 | 1% |
| 542–629 | 2 | 2% |
| Total | 88 | 100% |

# Discussion

The results of the study for the largest age were at 46–55 years by 30 patients (34%) and the smallest at 17–25 years by seven patients (8%). For the category of the largest gender were women as many as 74 patients (84%) and men 14 patients (16%). RA can happen not only caused by immune system. Increasing age towards geriatrics will thin the protective layer of the joint and synovial fluid begins to thicken so that movement in the joint or bone will increase the risk of RA. The women usually have smaller muscle types than men. The muscles can affect the strength in holding a mechanical load. Besides it, the biological factors of women and men can differ in providing therapeutic effects on treatment.

When the spread of RA in this study is compared with other studies, it shows the same results that the incidence of RA is estimated to be 4–13 in every 100,000 population for men and 13–36 in every 100,000 population for adult women [11]. The presence of the hormone estrogen can reduce the immune response and secretion of

**Table 4:** DAS28-ESR score distribution.

| Disease activity score | DAS28-ESR score | Total patients | Percentage |
|---|---|---|---|
| Remission | ≤2.6 | 16 | 18% |
| Low activity | >2.6 to ≤3.2 | 15 | 17% |
| Medium activity | >3.2 to ≤5.1 | 54 | 62% |
| High activity | >5.1 | 3 | 3% |
| Total | | 88 | 100 |

**Table 5:** Statistical correlation analysis.

| Tested variable | DAS28-ESR | | |
|---|---|---|---|
| | R (correlation) | p (significant) | n (size of patient) |
| Cumulative dose | −0.091 | 0.400 | 88 |
| Average dose | 0.043 | 0.692 | |
| Maximum dose | 0.074 | 0.492 | |
| Length of therapy | −0.075 | 0.489 | |

proinflammatory cytokines and can inhibit the formation of osteoclasts and synovial pannus so that it can inhibit the progression of RA[12]. The incidence of RA in women is influenced by the hormone estrogen which can reduce the immune response and secretion of proinflammatory cytokines [13]. Estrogen can inhibit the formation of osteoclasts and synovial pannus so RA progression can be inhibited [14]. The conditions post menopause will show differences in the development of RA [15, 16]. The incidence of RA in this study increased at age >45 years. At that age many menopausal conditions occur. The level of androgen is inversely proportional to the severity of RA while testosterone has a mechanism of inhibiting the humoral and cellular immune system [17, 18]. This can affect RA which is an autoimmune disease and requires immunosuppressant compounds. This can explain why the incidence of RA in men is lower. The higher incidence of RA in women can also be explained because the duration of RA therapy in women requires a longer time than men. This is also related to therapeutic adherence.

## Correlation of methotrexate dose with DAS28-ESR score

Based on the results of the study, the maximum dose of methotrexate was 8.8–10.6 mg/week for 27 patients (30%), the average dose was 6.9–8.7 mg/week for 31 patients (35%), and the cumulative dose. 75–968 mg/week for 61 patients (69%). Some references wrote that the maximum methotrexate dose is 7.5–25 mg/week [18]. The methotrexate is given to patients differ based on disease conditions and tolerability. The target of RA therapy is remission with a DAS28 score ≤2.6 [19]. The results of this study showed only 16 patients achieved remission.

If we pay attention to the use of methotrexate in this study, patients has not reached the maximum dose of 25 mg/week. The maximum dose only for one patient is 15 mg/week. It can be recommended for clinicians to provide therapeutic dose up to 25 mg in RA patients who have not yet reached remission. Increasing the dose can be done if after 4 weeks have not reached remission. Likewise with the initial dose of MTX can be recommended to increase the dose of 2.5–5 mg every 2–6 weeks depending on the severity of the disease, until reaching a maximum dose of 25 mg/week [20]. Doctors may avoid side effects such as nausea, vomiting, gastritis, and headaches when using large and uncontrolled dose [21]. Methotrexate usually use for the treatment of RA with NSAIDs as an analgesic. The use of methotrexate and NSAIDs at the same time can increase side effects [22].

Poor prognostic factors, such as citrullinated protein antibodies, rheumatoid, disease severity, joint erosion, and no response to conventional therapy, have proven to contribute to the improvement of RA therapy. The decision take a combination therapy using other conventional DMARDs or biological agents, will be applied to the patient therapy regimen if the condition of the patient worsens [18].

## Correlation of length of therapy with DAS28-ESR score

Out of 16 of 88 patients who achieved the remission condition (DAS28 <2.6) and have been receiving methotrexate for 120–240 weeks and the longest length of therapy was 624 weeks. The shortest length of therapy out of 88 patients was 12 weeks. Although statistically there is no significant relationship, the length of therapy with MTX can reduce the DAS28 score. This shows the effectiveness of MTX therapy. Methotrexate is absorbed rapidly and efficiently in the proximal part of the small intestine after oral administration. Its bioavailability (BA) is relatively high in the range of 64–90%; plasma concentration reaches the maximum concentration within 1–2 h and then declines and becomes undetectable after 24 h [1]. Maximum absorption can occur in low oral dose of methotrexate [22]. Half-life ($t^1/_2$) of methotrexate has different based on dosage. Half-life ($t^1/_2$) of methotrexate on low dose is 3–10 h, while on higher dose of 10–15 h. The metabolic process of methotrexate produces active metabolites namely which hydroxymethotrexate [23]. This metabolite has a half-life ($t^1/_2$) of 12 h. Half-life ($t$ ½) of methotrexate affects the length of time influence in the body and also the effectiveness in regulating the dose and interval of giving therapy.

Methotrexate therapy must be monitored every week. Monitoring of therapy methotrexate is done every 2–4 weeks, 8–12 weeks and more than 12 weeks. The treatment of methotrexate by oral should be done a

maximum of three months or 12 weeks to avoid toxicity and organ disorders [24]. Methotrexate therapy should monitor organ function through complete laboratory blood count data, liver transaminase levels, and serum creatinine [25].

The success of methotrexate therapy not only depends on the dose and length of therapy, but adherence to treatment therapy also plays an important role. Methotrexate also requires steady state time to be able to provide optimal efficacy. Methotrexate of therapy reached a steady state after 6–8 week [26, 27]. Methotrexate can be administered at night in order to optimize therapeutic effects and minimize recurrent symptoms of RA such as morning stiffness and swollen and tender joints [3], which usually occurs in the morning due to the increase in pro inflammation cytokines.

In the study, some limitations should also be recognized. It should be noted that RA is a relapsing remitting disease and some changes in the DAS28 over the six months may have arisen due to natural variation rather than as a result of the MTX intervention. Smaller differences may have been missed. While small differences are less likely to be clinically useful individually. Overall, the results of this study can be used as a basis for RA therapy in practice to improve the quality of life for RA patients.

The use of methotrexate for RA should be monitored for the effectiveness of treatment, especially at the dose and length of therapy. It is necessary to increase the methotrexate dose by 25 mg/week to get more remission patients and reduce the length of therapy, which is a maximum of 12 weeks. In this study, the patient is in remission after 120–240 weeks. The patient of adherence may be one of the factors that can increase the effectiveness of treatment.

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Shah Faisal, Junaidi Khotib and Elida Zairina*

# Knowledge, attitudes, and practices (KAP) towards COVID-19 among university students in Pakistan: a cross-sectional study

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**Abstract**

**Objectives:** Pakistan has taken unprecedented measures to control the spread of COVID-19. Complete lockdown followed by smart lockdown and quarantine centres was established. Their awareness and attitude towards COVID-19 had an impact on the individual behaviour of the precautionary measures. The current study examined the knowledge, attitudes and practices of university students in Pakistan.

**Methods:** An online cross-sectional study was conducted among university students in Pakistan. A questionnaire containing demographic and KAP information related to COVID-19 has been created.

**Results:** A total of 358 students responded to the survey, and 353 participants completed the study. Among the respondents, 61.5% were male, 76.8% were single, and 58.4% enrolled in a bachelor's degree. The results showed that most of the respondents (68%) had good knowledge about COVID-19, while the overall knowledge score was 8.78 ± 1.63 (range 1–10). The majority of the respondents (90.9%) were aware of COVID-19, 95.8% knew the sign and symptoms, and 83% of them knew about its transmission. We found a significant difference in knowledge scores across education and area of study $p<0.05$. More than half (53.5%) of the respondents were satisfied with the facilities provided by the government of Pakistan. The average practices score among the students was 5.08 ± 1.312. A significant difference was found among practice score and area of study $p<0.05$.

**Conclusions:** Most of the students have an adequate level of knowledge and are doing better preventive measures against COVID-19. Health education initiatives are required to ensure best practice among the high-risk groups.

**Keywords:** attitudes; COVID-19; knowledge; Pakistan; practices; university students.

## Introduction

An outbreak of enigmatic pneumonia, characterized by fever, dry cough, fatigue, and gastrointestinal symptoms, was reported in China in December 2019 [1]. Later, following the local health authority's announcement of an epidemiological warning on 1 January 2020, the market was closed [2]. Fever, cough mostly dry, fatigue, muscle aches, and difficulty breathing are the main clinical manifestations of this profoundly infectious disease, while the advanced stage of the disease is described as respiratory discomfort, septic shock, and coagulation problems [3, 4]. The World Health Organization (WHO) announced a global public health emergency on 30 January 2020 [5].

The Government of Pakistan and the Ministry of Health have taken unprecedented steps to restrict the spread of the virus in the country. These include a complete lockdown at the start of the pandemic, followed by a smart lockdown in major cities and educational institutions' closure [6]. The Ministry of National Health Services Regulation and Coordination (MoNHSRC) report identified 295,636 confirmed cases by 30 August 2020. The total number of cases in Sindh provinces was 129,268 among all the Pakistan regions, with 2,398 reported deaths. Punjab has 96,741 confirmed cases and 2,196 deaths, and Khyber Pakhtunkhwa has 36,017 confirmed cases and 1,250 deaths, Balochistan has 12,842 confirmed cases and 141 reported deaths, Gilgit Baltistan has 2,863 confirmed cases and 67 deaths, Azad Jammu Kashmir has 2,294 confirmed cases and 61 deaths, while the Islamabad Capital Territory has 15,611 confirmed cases and 175 deaths [7].

***Corresponding author: Elida Zairina**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; Center for Patient Safety Research, Universitas Airlangga, Surabaya, Indonesia; and Innovative Pharmacy Practice and Integrated Outcomes Research (INACORE) Group, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 031 5933150, E-mail: elida-z@ff.unair.ac.id.
https://orcid.org/0000-0003-0845-4640
**Shah Faisal,** Magister Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Junaidi Khotib,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

The pandemic has put the education system in a difficult situation, especially for university students who were at their time free but with limited experiences. Their behaviours and attitudes were assumed to be affected by the outbreak. The outbreak of SARS in 2003 suggested that public knowledge and attitudes towards the pandemic were linked to panic emotions, which in turn had an impact on measures to control the pandemic. As a result, the assessment of knowledge and attitudes about COVID-19 insights, into preventive practices could be gained. During the pandemic, the government of Pakistan closed down educational institutions. Knowing about the 'student's' knowledge (K), attitudes (A), and practices (P) against COVID-19 will help governments and policymakers to control the pandemic by targeting vulnerable populations. The study aimed to evaluate the student's knowledge (K), attitudes (A), and practices (P) against COVID-19.

## Materials and methods

An online cross-sectional survey was conducted between 15 June and 20 July 2020 at various universities in Pakistan. The data was collected using a random sampling method. The questionnaire was based on information provided on the WHO and the Government of Pakistan websites for COVID-19 [8, 9]. The questionnaire was not validated and was used to collect information from students about awareness of COVID-19. The online link to the questionnaire was shared with university students through social media (WhatsApp and Facebook messenger). The questionnaire included the informed consent of the participants prior to the completion of the questionnaire. The researcher inserted a brief introduction to the background, purpose, procedure, voluntary participation, confidentiality, participation notes and completion of the questionnaire. The first part of the questionnaire concerned the students' demographic characteristics—the second part of the questionnaire (K1–K10) dealt with the knowledge and transmission of COVID-19. Every question was answered as yes/no and not sure. The score of 1 was given for each correct answer and 0 for each incorrect/not sure answer. A total score of ≥8 was considered good knowledge; a score of 7 was deemed to be fair, while a score of ≤6 was regarded as poor knowledge. The third part of the study consisted of two questions (A1–A2) about attitudes towards COVID-19 with a yes/no answer choice. The fourth part comprised six questions to the practices toward COVID-19, which were answered as yes/no. A mean score ≥5, indicated good practices against COVID-19, a score of 4 as fair practices, while three or less than 3 showed poor practices among the university students.

## Statistical analysis

The data were analysed using the statistical package for social science (SPSS) version 25.0. A descriptive analysis was used to measure the study characteristics, the level of knowledge, attitudes, and practices. Standard deviation and mean were used to compare each variable based on demographic classification. In addition, the Mann Whitney test and ANOVA test with a 95% confidence level were carried out to examine the difference between variables.

## Results

A total of 358 respondents responded to the survey. Five respondents were not willing to participate, and 353 of the respondents completed the survey. The demographic characteristics of the respondents were shown in Table 1. The study showed that the majority of the respondents (68%) had good knowledge of COVID-19. The mean knowledge score for COVID-19 was 8.78 ± 1.63 (range 1–10). The high knowledge score was in the 28–38 age group, with a mean score of 8.94 ± 1.81 SD. Ph.D. students have a high score of knowledge compared to master and bachelor students. Respondents in Azad Jammu Kashmir territory have higher knowledge score (9.36 ± 0.92) as compared to students in other regions. There was a significant difference in knowledge score across education and study province ($p<0.05$), as shown in Table 1.

while the percentage of respondents' level of practice is shown in Table 3.

Assessing the respondents' attitudes, 53.5% stated that they were satisfied with the facilities provided by the government of Pakistan. Responses to statements of attitudes can be seen in Figure 1.

The students' mean practice score was 5.08 ± 1.312 SD (Range 1–6), which showed that the students do better practices against the pandemic. The students from Azad Jammu Kashmir do better practices 5.45 ± 1.21 as compared to students in other provinces of the study. We found a significant difference in practice scores between the study area ($p<0.05$), as shown in Table 1. The results of the student responses to COVID-19 practice statements were shown in Table 4, while the students' level of practice is shown in Table 3.

## Discussion

Since the outbreak of COVID-19, it has promptly become a menace to public health and has led to massive socioeconomic damages worldwide. Strenuous measurements have been enforced, including lockdowns, social distancing, closing educational institutions, banning public gatherings, and establishing quarantine centres in Pakistan to mitigate the outbreak effectively.

**Table 1:** Respondents demographics and scores of knowledge, attitudes, and practices toward COVID-19.

| Demography | Frequency (n=353) | Percentage | Knowledge score | | Attitude score | | Practice score | |
|---|---|---|---|---|---|---|---|---|
| | | | Mean ± SD | p-Value | Mean ± SD | p-Value | Mean ± SD | p-Value |
| Gender | | | | | | | | |
| Female | 136 | 38.5 | 8.76 ± 1.82 | 0.369 | 1.04 ± 0.87 | 0.576 | 5.15 ± 1,17 | 0.630 |
| Male | 217 | 61.5 | 8.79 ± 1.50 | | 0.99 ± 0.87 | | 5.03 ± 1.392 | |
| Age group, years | | | | | | | | |
| 17–27 | 285 | 80.5 | 8.74 ± 1.59 | 0.688 | 1.01 ± 0.87 | 0.073 | 5.07 ± 1.36 | 1.69 |
| 28–38 | 62 | 17.6 | 8.94 ± 1.81 | | 0.91 ± 0.83 | | 5.03 ± 1.10 | |
| Above 39 | 7 | 2 | 8.86 ± 1.57 | | 1.71 ± 0.75 | | 6.00 ± 0.00 | |
| Marital status | | | | | | | | |
| Single | 271 | 76.8 | 8.74 ± 1.58 | 0.612 | 0.99 ± 0.86 | 0.469 | 5.06 ± 1.36 | 0.625 |
| Married | 81 | 22.9 | 8.88 ± 1.81 | | 1.04 ± 0.89 | | 5.15 ± 1.13 | |
| Divorced | 1 | 0.4 | 10.00 ± 00 | | 2.00 ± 0.00 | | 4.00 ± 0.00 | |
| Education | | | | | | | | |
| Bachelor | 206 | 58.4 | 8.64 ± 1.76 | 0.025 | 1.05 ± 0.88 | 0.187 | 5.04 ± 1.36 | 0.442 |
| Master | 126 | 35.7 | 8.86 ± 1.45 | | 0.90 ± 0.86 | | 5.08 ± 1.27 | |
| PhD | 21 | 5.9 | 9.62 ± 1.63 | | 1.19 ± 0.81 | | 5.43 ± 0.97 | |
| Province | | | | | | | | |
| Azad Jammu Kashmir | 11 | 3.1 | 9.36 ± 0.92 | 0.034 | 1.00 ± 1.00 | 0.589 | 5.45 ± 1.21 | 0.001 |
| Baluchistan | 22 | 6.2 | 7.86 ± 1.93 | | 1.18 ± 0.85 | | 4.14 ± 1.45 | |
| Gilgit Baltistan | 6 | 1.7 | 7.83 ± 2.40 | | 1.16 ± 0.98 | | 4.00 ± 1.78 | |
| Khyber-Pakhtunkhwa | 184 | 52.1 | 8.92 ± 1.55 | | 0.96 ± 0.90 | | 5.16 ± 1.31 | |
| Punjab | 110 | 31.2 | 8.75 ± 1.61 | | 0.99 ± 0.82 | | 5.25 ± 1.11 | |
| Sindh | 20 | 5.7 | 8.78 ± 1.63 | | 1.30 ± 0.73 | | 4.60 ± 1.50 | |
| University | | | | | | | | |
| Private university | 120 | 34 | 8.67 ± 1.57 | 0.367 | 1.05 ± 0.84 | 0.468 | 5.06 ± 1.32 | 0.830 |
| Public university | 233 | 66 | 8.83 ± 1.66 | | 0.98 ± 0.88 | | 5.09 ± 1.30 | |

The respondents' responses to the knowledge statements were shown in Table 2, while the percentage of respondents' level of practice is shown in Table 3. Assessing the respondents' attitudes, 53.5% stated that they were satisfied with the facilities provided by the government of Pakistan. Responses to statements of attitudes can be seen in Figure 1.

In this study, the students have good knowledge of COVID-19 as the mean knowledge score was 8.78 ± 1.63 (range 1–10). The overall correct student knowledge was 87.75%. This study is in range with previous research conducted in Vietnam and China, where participants' correct knowledge score was high [10, 11]. We found a significant association in knowledge score between educational level and study area ($p<0.05$). The high study level was associated with high knowledge score of respondents. These results of this study are consistent with a study conducted in China [12]. Most of the respondents in this study (95.8%) were aware of the disease's clinical signs and symptoms, and 89.5% were aware that there was no specific treatment as of the date of the survey. The results of this study are consistent with prior research conducted in Saudi Arabia [13].

The question from K6–K10 was about the mode of transmission where the overall score of respondents was 83%. The majority of respondents (94.3%) indicated that isolation and quarantine are common strategies that can help control the spread of diseases, while the correct score for human-to-human transmission by droplet and contaminated hand was 94.1%. These results are supported by previous studies in Malaysia and Ethiopia [14, 15]. The correct scores of the respondents for the virus cannot be transmitted through mosquitos were 72.2%. Our study result is in line with the study conducted in Nigeria [16]. The respondents' attitude towards the government approach to the pandemic was satisfactory. The responses to attitudes are different from the study conducted in China and Saudi Arabia, where most of the respondents were optimistic about the 'government's' approach to the pandemic [11, 13]. Students have shown better preventive practices to control the spread of the pandemic. The mean student preventive practice score was 5.08 ± 1.312 SD. The majority of respondents (90.9%) indicated that they often wash their hands with soap for 20 s, while 76.5% use sanitizers consistent with previous research [14]. The student's practices of avoiding crowded places were also high, as 88.1 % reported avoiding crowded places, which is in line with the Malaysia study [14]. The majority of respondents (93.2%) indicated that they avoided contact with

**Table 2:** Knowledge of the students towards COVID-19.

| Questions | Frequency, n | Percentage, % |
|---|---|---|
| K1. COVID-19 is an infectious disease caused by a newly discovered coronavirus. | | |
| Yes | 321 | 90.9 |
| No | 11 | 3.1 |
| Not sure | 21 | 5.9 |
| K2. The main symptoms of coronavirus include coughing, shortness of breath, hot fever, and sore throat. | | |
| Yes | 338 | 95.8 |
| No | 3 | 0.8 |
| Not sure | 12 | 3.4 |
| K3. Presently, there are no vaccines that protect against the disease. | | |
| Yes | 319 | 90.4 |
| No | 11 | 3.1 |
| Not sure | 23 | 6.5 |
| K4. There is no specific treatment. A supportive treatment use to reduce the symptoms. | | |
| Yes | 316 | 89.5 |
| No | 17 | 4.8 |
| Not sure | 20 | 5.7 |
| K5. People with chronic lung disease or moderate to severe asthma are at high risk for severe illness from COVID-19. | | |
| Yes | 320 | 90.7 |
| No | 7 | 2 |
| Not sure | 26 | 7.4 |
| K6. People with weak immune system with diseases like cancer, smoking, organ transplantation, poorly controlled HIV, and prolonged use of corticosteroids had more risk for severe problems from COVID-19. | | |
| Yes | 312 | 88.4 |
| No | 8 | 2.3 |
| Not sure | 33 | 9.3 |
| K7. It's likely that the virus is originated in an animal, and then spread to humans. | | |
| Yes | 245 | 69.4 |
| No | 52 | 14.7 |
| Not sure | 56 | 15.9 |
| K8. COVID-19 cannot be transmitted through mosquitos. | | |
| Yes | 262 | 74.2 |
| No | 27 | 7.6 |
| Not sure | 64 | 18.1 |
| K9. COVID-19 spreads through contaminated droplets from infected persons (through coughing or sneezing) or contaminated hands. | | |
| Yes | 332 | 94.1 |
| No | 6 | 1.7 |
| Not sure | 15 | 4.2 |
| K10. Isolation and quarantine are common strategies that can help to limit the spread of the virus. | | |
| Yes | 333 | 94.3 |
| No | 11 | 3.1 |
| Not sure | 9 | 2.5 |

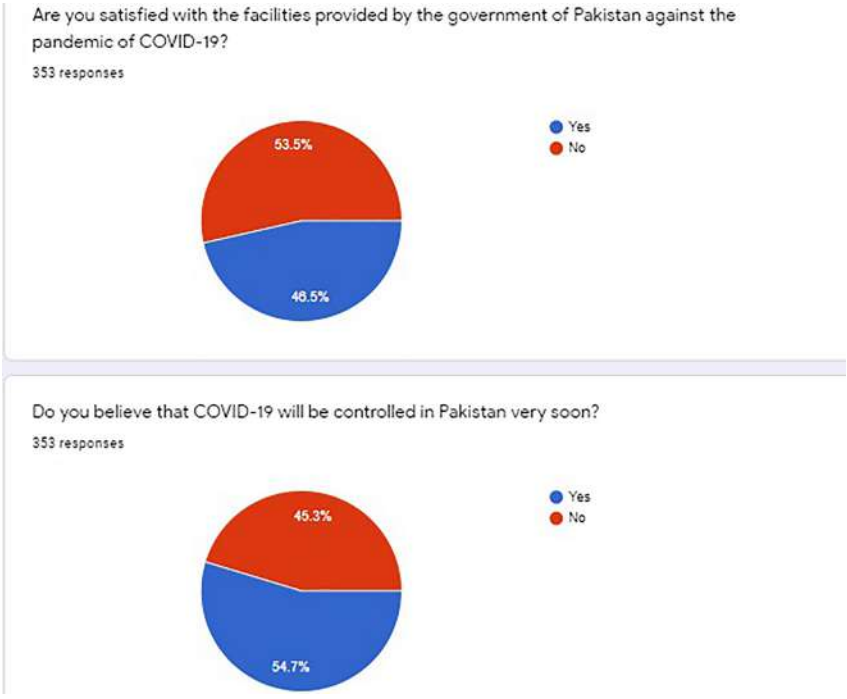


**Figure 1:** Attitudes towards COVID-19 by the students

**Table 3:** Percentage of level of knowledge, and practices of the respondents.

| Variable | Level | Frequency, n | Percentage, % |
|---|---|---|---|
| Knowledge | Poor knowledge | 36 | 10.2 |
| | Fair knowledge | 77 | 21.8 |
| | Good knowledge | 240 | 68 |
| Practices | Poor practice | 43 | 12.2 |
| | Fair practice | 39 | 11 |
| | Good practice | 271 | 76.8 |

people who respiratory illness, symptoms, and covered their nose and mouth while sneezing which is in line with the study conducted in South Korea [17].

## Limitation of the study

In this study, most of the respondents were from two provinces Khyber-Pakhtunkhwa, and Punjab, which is

**Table 4:** Respondents practices against COVID-19.

| Questions | Frequency | Percentage |
|---|---|---|
| I wash my hands often with soap for 20 s. | | |
| Yes | 321 | 90.9 |
| No | 32 | 9.1 |
| I use sanitizer when soap and water are not available. | | |
| Yes | 270 | 76.5 |
| No | | |
| I avoid crowded places (social distancing). | | |
| Yes | 311 | 88.1 |
| No | 42 | 11.9 |
| I avoid close contact with anyone showing symptoms of respiratory illness. | | |
| Yes | 329 | 93.2 |
| No | 24 | 6.8 |
| I do not share eating. | | |
| Yes | 239 | 67.7 |
| No | 114 | 32.3 |
| I cover my mouth and nose with a tissue (not by hands) while coughing or sneezing | | |
| Yes | 323 | 91.5 |
| No | 30 | 8.5 |

well-developed and has easy access to the Internet. The number of respondents from the least developed areas such as Baluchistan and Gilgit Baltistan was low due to limited Internet access, which may be considered as the study's limitation. Further studies are needed in these areas to evaluate the knowledge, attitudes and practices of students. Another shortcoming of the study may be the small number of respondents who participated in the study may be another shortcoming of this study.

## Conclusions

The study showed a comprehensive assessment of the student's awareness of COVID-19 when all the institutions were closed. The results showed that most respondents have good knowledge of COVID-19 and follow precautionary practices to combat the rise of COVID-19. This study's results may be useful for policymakers and healthcare authorities on further health interventions, awareness campaigns toward COVID-19, and health education programs. Health education programs aimed at mobilizing and improving information related to COVID-19 are required, particularly for vulnerable groups, to increase their preventive practices.

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**Competing interests:** Authors state no conflict of interest.
**Informed consent:** Informed consent was obtained from all individuals included in this study.
**Ethical approval:** The local Institutional Review Board deemed the study exempt from review since it is for the purpose of internal validation of the questionnaire for organization to get more data about international student awareness regarding COVID-19.

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Aniek Setiya Budiatin*, Maria Apriliani Gani, Chrismawan Ardianto, Samirah, Sahrati Yudiaprijah Daeng Pattah, Fitroh Mubarokah and Junaidi Khotib

# The impact of glutaraldehyde on the characteristics of bovine hydroxyapatite-gelatin based bone scaffold as gentamicin delivery system

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**Abstract**

**Objectives:** Biomaterials are widely used as drug delivery systems targeting bone tissue, such as to treat bone infectious disease. However, the addition of drugs to biomaterials weakens their mechanical properties. Crosslinkers are compounds that improve the mechanical properties of biomaterials. This study aims to determine the effect of glutaraldehyde (GTA) as a crosslinker on the characteristics of bovine hydroxyapatite-gelatin-based bone scaffold with gentamicin as antibiotics (BHA-GEL-GEN-GTA).

**Methods:** BHA-GEL-GEN-GTA scaffold with GTA solid content ranging from 0.1 to 1.4 wt% was made by direct compression. The compressive strength test was carried out using autograph. Scaffold degradation test was carried out by dissolving the scaffolds in PBS. Scaffold toxicity was performed by MTT assay using BHK-21 fibroblast cells.

**Results:** There was a significant difference in the scaffolds' compressive strength due to differences in GTA volume. Scaffold crosslinked using GTA with solid content 0.1 and 0.2 wt% in 2 mL solution had higher compressive strength than those in 1 mL solution. Furthermore, GTA with solid content 0.6, 1, 1.2, and 1.4 wt% showed higher compressive strength than those without GTA. Degradation test results showed that GTA increased the percentage of weight loss and swelling of the scaffold. The scaffold exhibited a nontoxic profile in MTT assay.

**Conclusions:** GTA with optimum solid content shows great compressive strength, stable swelling profile with low percentage of scaffold's weight loss, and is considered as nontoxic.

**Keywords:** biomaterials; bovine hydroxyapatite; compressive strength test; degradation test; glutaraldehyde; infectious disease.

## Introduction

Bone defect is a serious condition mostly caused by local trauma. Every year, more than 2.2 million people need surgical procedures to deal with bone defects [1]. Osteomyelitis is the most common complication due to bone defects. Drug delivery to the bone for osteomyelitis therapy is very challenging in the pharmaceutical and orthopedic fields. This is because the oral route is unable to produce sufficient MIC to the bone tissue. On the other hand, the systemic route is potentially toxic to other organs when antibiotic concentration is too high [2, 3]. Thus, the development of biomaterials as an antibiotic delivery system with direct targets on bone tissue is increasing [3].

Bovine hydroxyapatite (BHA) is one of the biomaterials composing bone scaffold with chemical formula and physical properties similar to human bone hydroxyapatite. BHA has a carbonate substitution group on the apatite that distinguishes it from synthetic hydroxyapatite [4]. The carbonate group is known to increase osteoblast proliferation, thus accelerating the synthesis of new bone matrix [5]. Gelatin (GEL) is a polymer similar to bone organic minerals and is useful for supporting apatite crystal formation in the synthesis of new bone matrix [6, 7]. This makes BHA and GEL widely used as scaffold components for bone tissue engineering. Moreover, the BHA-GEL complex is a potential matrix for antibiotics delivery in the treatment of osteomyelitis [3]. Based on the study of Budiatin et al., matrix containing BHA-GEL is known to sustainably release gentamicin (GEN) to inhibit the growth of *Staphylococcus aureus* in a concentration-dependent manner [3].

***Corresponding author: Aniek Setiya Budiatin**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62 818 597 732, E-mail: anieksb@yahoo.co.id
**Maria Apriliani Gani, Chrismawan Ardianto, Samirah, Sahrati Yudiaprijah Daeng Pattah, Fitroh Mubarokah and Junaidi Khotib,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia

However, the addition of antibiotics to the biomaterial weakens the scaffold's mechanical properties causing premature degradation [8]. Therefore, to improve scaffold mechanical properties, a crosslinker such as glutaraldehyde (GTA) is needed to induce chemical bonds in the biomaterial polymer chain [3, 9]. GTA is a dialdehyde compound which has a very reactive aldehydic group. GTA causes covalent bonds with amine or hydroxyl groups on the polymer biomaterial, which increases the scaffold's mechanical strength [3, 9, 10]. In contrast, there is no enough evidence about the impact of GTA on the characteristics of the BHA-GEL-GEN-GTA scaffold. Thus, this study aimed to determine the effect of GTA concentration in BHA-GEL-GEN-GTA scaffolds on compressive strength, degradation (weight loss and swelling profile), and scaffold's toxicity.

# Materials and methods

### Fabrication of BHA-GEL-GEN-GTA scaffold

Eight grams of BHA (Airlangga University, Indonesia) with 1 g of GEN (Yantai Justaware Pharmaceutical, China) were stirred in the mortar until homogeneous. Then, 20 wt% GEL (Cartino, Thailand) was added in the mixture. After that, GTA (Merck, USA) with solid content of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 1.2, and 1.4 wt% from GTA 25 wt% was added on the BHA-GEL-GEN by spraying on the mixture while stirring until homogeneous. After forming a dense mass, the BHA-GEL-GEN-GTA mixture was sifted with a mesh size of 1.0 mm. The granules obtained were then dried at 37 °C for 24 h. After that, as much as 100 mg of granules were weighed and pressed into scaffolds.

### Compressive strength test

Before the compressive strength was conducted, the scaffold body's diameter and length are measured using a micrometer to calculate the scaffold's surface area. The compressive strength was measured using a calibrated autograph which previously calibrated (Shiamdzu AG-10 TE, Japan). Compressive strength is calculated based on the formula $\sigma = \frac{4F}{(\pi d^2)}$ [11].

### Degradation test

The initial scaffold weight (mi) was weighed before the degradation test was conducted. The PBS solution was prepared by mixing 0.7075 g of $Na_2HPO_4$, 0.118 g of $KH_2PO_4$, and 0.9 g of NaCl into 100 mL of distilled water. Furthermore, the pH of the solution was checked using a pH meter (PBS solution has a pH of 7.4 ± 0.2). After that, 2 mL of PBS solution was inserted, each into the 5 mL venoject. The scaffold was then inserted into a venoject containing PBS and incubated (37 ± 1 °C). After 1, 2, 3, 6, and 12 h, the scaffolds were taken and dried with filter paper until the PBS fluid on the scaffolds did not remain. After the scaffolds dry, the scaffolds are weighed (m). Next, the scaffold was returned to the venoject containing the new PBS solution and placed in an incubator at 50 °C for four days. After four days, the final scaffold was weighed (md). The percentage of scaffold weight loss and swelling are calculated based on [12]:

$$\text{Weight loss}\,(\%) = \left(\frac{\text{md} - \text{mi}}{\text{mi}}\right) \times 100,\ \text{Swelling}\,(\%) = \left(\frac{\text{m} - \text{md}}{\text{md}}\right) \times 100 \quad [12].$$

Here m is the weight of degraded scaffold measured at t time, mi is the initial weight of the scaffold, and md is the final weight of the scaffold (degraded scaffold after drying at 50 °C for four days) [12].

### MTT assay

The cell that used for MTT test was BHK-21 fibroblasts. The BHK 21 suspension was placed into a 96-well (50 µL/well) plate and added with DMEM media as much as 100 µL/well. The 96-well plates filled with cells were then incubated in a $CO_2$ incubator (temperature 37 °C for 24 h). After 24 h, scaffolds that previously smoothed were inserted into each well (2 mg/well). The 96-well plate that already contained BHK 21 cell and scaffold powder then incubated for 24 h. After 24 h, the remaining powder in the well was removed, and the well washed with PBS. Furthermore, DMEM media was added to the well, followed by MTT (µL/well), cells then were incubated for 3 h. After that, DMSO was added to the well (50 µL/well) and incubated for another 5 min. Furthermore, the absorbance (abs) was read by an ELISA reader (Thermo Scientific, USA) at a wavelength of 620 nm. The cell viability was calculated based on [13]:

$$\text{Cell viability}\,(\%) = \frac{\text{Abs treatment} - \text{Abs media}}{\text{Abs control} - \text{Abs media}} \times 100 \quad [13].$$

Material is non-toxic when the cell viability is more than 50% [14].

# Results

A study about GTA concentration on the compression strength and degradation of BHA-GEL-GEN-GTA scaffolds was done. Before the compressive strength test was carried out, a test was conducted to optimize GTA solution volume. There was a significant difference in the scaffolds' compressive strength due to differences in the crosslinker solution volume. The scaffolds that were crosslinked using GTA with solid content of 0.1 wt% in 2 mL solution showed a higher compressive strength than those with the same solid content in 1 mL solution (One Way ANOVA, $p<0.001$; Figure 1A). Similarly, scaffold crosslinked using GTA with solid content 0.2 wt% in 2 mL solution showed a higher compressive strength than those in 1 mL of solution (One Way ANOVA, $p<0.05$; Figure 1A). Thus, the volume of 2 mL GTA solution was used for the next experiment. Moreover, GTA with solid content of 0.6, 1, 1.2, and 1.4 wt% increases the compressive strength of scaffolds (one way ANOVA, $p<0.01$ vs. BHA-GEL-GEN; Figure 1B).

Furthermore, degradation test was carried out to see the effect of GTA concentration on scaffold degradation.

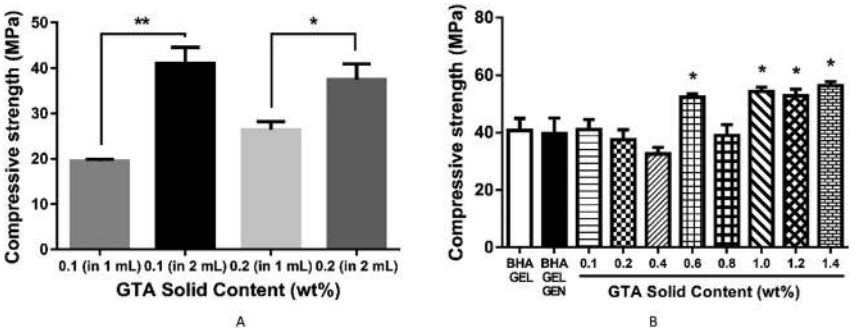


**Figure 1:** Optimation of the volume of GTA solution on the scaffold's compressive strength. Solid content is the weight of GTA (wt%) to the total weight of the scaffold.
**p<0.001, *p<0.05 (A). The compressive strength test results of scaffolds with different solid content of GTA (all in 2 mL) (B). Each bar represents the mean ± SEM compressive strength of the five scaffolds, *p<0.01 compared to BHA-GEL-GEN. The statistical test used in both figures was ANOVA one way.

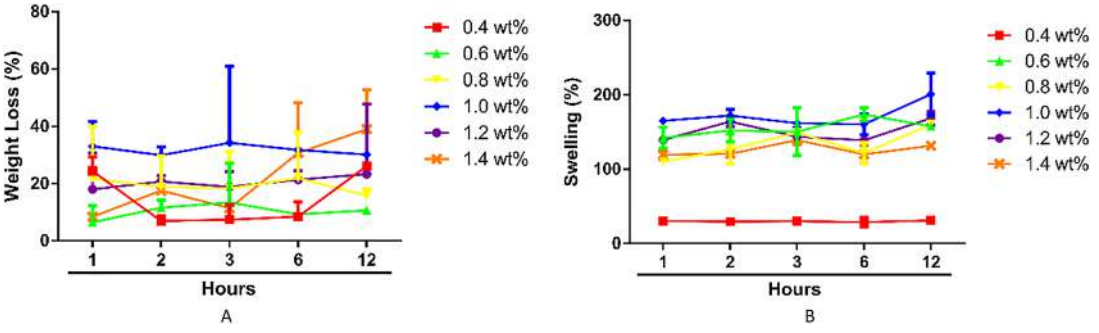


**Figure 2:** The results of the BHA-GEL-GEN-GTA scaffold degradation test.
Percentage of scaffolds' weight loss (A), percentage of scaffolds swelling (B). Each point shows the mean weight loss or swelling ± SEM of the two scaffolds.

The degradation test results, namely percentage of weight loss and swelling, are shown in Figure 2. Based on the weight loss percentage profile (Figure 2A), the increase in GTA concentration causes an increase in the percentage of weight loss from scaffolds. The scaffold swelling profile shows that the increase in GTA concentration also increases the scaffolds' swelling (Figure 2B).

Considering that GTA is a toxic compound, MTT assay was carried out to examine the effect of GTA concentration on the viability of BHK-21 fibroblast cells. Based on the results, the viability of fibroblast cells in each group was 103.383 ± 7.504, 138.307 ± 8.810, 124.089 ± 11.314, 86.510 ± 2.793, and 96.011% ± 5.588 respectively fot BHA-GEL and BHA-GEL-GEN-GTA with GTA solid content of 0.0, 0.1 and 0.4 wt% (Figure 3).

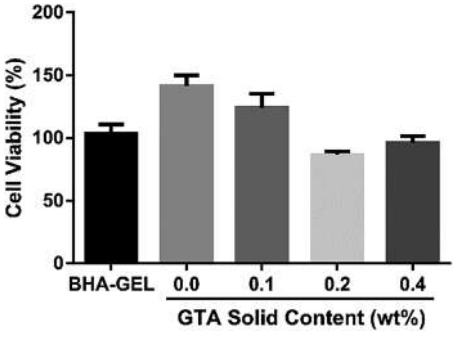


**Figure 3:** The results of the BHA-GEL-GEN-GTA scaffold toxicity test with different solid content of GTA. All groups tested had cell viability above 50%.

## Discussion

GTA is a dialdehyde compound with a very reactive aldehydic group and form covalent bonds with functional groups such as amines and thiols phenols, hydroxyl, and imidazoles [10]. Because of this, GTA is widely used as a crosslinker to increase the mechanical strength of biomaterials [9, 10]. The present study showed that the volume of GTA solution affects the compressive strength of scaffolds, even with the same solid content of GTA. This is possibly because an increase in the volume of solution

increases contact area between the scaffold and GTA, which may speed up the chemical reactions and increase the formation of crosslink. Thus the present data indicates that an increase in GTA solid co[ntent increases the crosslink formed.

The compressive strength test results showed that GTA with solid content of 0.6, 1, 1.2, and 1.4 wt% had higher compressive strength value compared with the group without GTA. This high value in compressive strength may increase the physical performance for clinical use. This is in line with the research conducted by Pinto et al., which states that increasing the concentration of GTA causes an increase in the strength of chitosan-based scaffold [15]. GTA is a compound that highly soluble in water [16]. Therefore, even the compressive strength increase due to the increase in the GTA solid content, does not guarantee the decrease in degradation of the scaffolds in water. Thus, the scaffold degradation test was performed. The test results indicated that an increase in the solid content of GTA led to an increase in the percentage weight loss of scaffolds. Furthermore, the swelling test showed that an increase in GTA's solid content also led to an increase in the percentage of scaffold swelling. This is because an increase in GTA's solid content may increase the volume of water drawn by the scaffold, thereby increasing the percentage of scaffold swelling and weight loss [16]. The swelling capacity of a scaffold is one factor that supports nutrient diffusion and cell adhesion to the biomaterial. However, excessive swelling diminish the scaffold's mechanical integrity, which causes premature degradation before the completion of the new bone matrix synthesis [17]. This may eliminate the scaffold function in bone remodeling. In addition, premature degradation may also be toxic to the bone tissue microenvironment because GTA and the delivered drugs are highly released [9]. In this study, scaffolds that are crosslinked using GTA with solid content of 0.4 wt% had a stable swelling profile with a low percentage of weight loss. Present finding indicates that the scaffold has an excellent capacity to absorb fluid and may not experience premature degradation *in vivo*.

To examine the toxicity of the scaffold, the MTT assay was carried out. Based on the results, the group with 0.0 wt% GTA showed the highest cell viability. This was because there was no GTA present on the scaffold, so the metabolic activity was not disturbed by GTA, thus making cell proliferation run well [10]. Furthermore, the addition of GTA demonstrates cell viability above 50% in the solid content of GTA up to 0.4 wt%. The present data indicates that the addition of GTA to the scaffold may not disturb the activity of cells involved in the bone tissue regeneration. This is in line with the study conducted by Bharatham et al. that the use of 2% GTA as a crosslinker was considered as nontoxic based on MTT assay [18].

## Conclusions

The present study found that the increase in GTA solution, the better the efficiency of the crosslink formed. Furthermore, the increase in GTA's solid content leads to an increase in the scaffolds' compressive strength. GTA with a lower solid content showed stable swelling profile with low percentage of scaffold's weight loss. Based on the toxicity test, scaffold with the optimum solid content of GTA is considered as non-toxic. Therefore, BHA-GEL-GEN-GTA scaffold with optimum GTA solid content is potentially investigated in further *in vivo* study with bone infection animal model to test its effectiveness in delivering GEN.

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**Informed consent:** Not applicable.
**Ethical approval:** Not applicable.

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Lisa Narulita, Suharjono*, Kuntaman and Mohammad Akram

# Analysis of the use of antibiotics profile and factors of surgical site infections study on digestive and oncology surgeries

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**Abstract**

**Objectives:** The incision method operation with a high risk of infection in a clean and clean-contaminated operation requires the use of prophylactic antibiotics to minimize the risk of infection. This study was designed to analyze the effectiveness of prophylactic antibiotics in patients with digestive and oncology surgeries.

**Methods:** The statistical method used was chi-square to determine the risk factors for infection at surgical site infections (SSI) in patients with digestive and oncology surgeries. This study had received ethical approval from the Ethics Committee of Dr. H. Slamet Martodirdjo Hospital, Pamekasan.

**Results:** There were 67 patients consisted of 48 digestive surgeries (71.6%) and 19 oncology surgeries (28.4%). The criteria of observation on day 30 showed that as 1 (1.5%) SSI patient experienced purulence, inflammation, and erythema around the surgical wound so an analysis of $p>0.05$ was carried out so that there was no association with the incidence of SSI during hospitalization, but other factors originating from the patient, such as a lack of personal hygiene at home and lack of nutritious food intake was measured in temperature, pulse, respiration, and white blood cells examination before surgery and 24 h after surgery, all within normal ranges. The qualitative analysis of prophylactic antibiotics using the Gyssen method showed that 31 (46.3%) rationales needed an improvement process.

**Conclusions:** The widely used prophylactic antibiotics, namely cefazolin and cefuroxime are recommended antibiotics used in incision surgery and rationale used.

**Keywords:** Gyssens; prophylactic antibiotics; surgical.

## Introduction

The process of preventing surgical site infections (SSI) is by giving the appropriate antibiotics. Antibiotics are a class of antibacterial drugs that are widely used in hospitalized patients either alone or in combination [1]. This is evidenced by the use of antibiotics in developed countries, which is around 13–37%, while in developing countries it is 30–80% [2]. The use of antibiotics is divided into three, namely as prophylaxis, empiric therapy, and definitive therapy. These divisions are based on the patient's condition when the antibiotics are given. Prophylactic antibiotics are antibiotics given to patients who have not had an infection or have not had the disease. However, it is thought to provide a great chance of getting infected or if the patients are infected. Prophylactic antibiotic administration must be accompanied by correct consideration [3].

The choice of antibiotics for prophylaxis is based on considerations to achieve effective concentrations in the tissue before the surgical procedure achieve therapeutic levels during the operation process and after the closed incision with the aim and means, minimize the development of bacterial resistance processes, pay attention to economic factors, and choose low toxicity and the injection dosage form that is selected on the basis of safety and process in the operating procedure [4, 5]. In several journals and guidelines for the use of antibiotics, the first-line therapy recommended for prophylaxis in surgical patients is cefazolin or cefuroxime. It aims of give prophylactic antibiotics to suppress the colonization of microorganisms that are present during surgical procedures [6].

The factors that contribute to the development of infection in surgery can be divided into several variables, including those related to the patients and procedures in surgery involving the indication for surgery, the length of

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 812 1733 877, E-mail: suharjono@ffunair.ac.id
**Lisa Narulita,** Master of Clinical Pharmacy Program, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Kuntaman,** Department of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Mohammad Akram,** Department of Surgery, Dr. H. Slamet Martodirdjo Hospital, Pamekasan, Indonesia

surgery performed, the operating room in each hospital and the pathogens of microorganisms that are one of the causes of SSI, reported by World Health Organization (WHO), namely *Staphylococcus aureus* (with 30.4%), followed by Gram-negative *staphylococci* (11.7%), *Escherichia coli* (9.4%) and *Enterococcus faecalis* (5.9%). Inflammation is a local response to an infection or injury. The function of inflammation is to attack or deactivate antigens and is a stage of tissue repair [7].

The impact of SSI can cause morbidity and mortality of patients after surgery, in which it occurs in surgical patients worldwide. SSI as defined by The United States Centers for Disease Control and Prevention (CDC) is defined as an infection associated with a surgical procedure occurring at or near a surgical incision (incision or organ/space) after surgery within 7 days, 30 days of the procedure or within a time of 90 days if the prosthetic material is implanted at the surgery [8]. The WHO shows the overall prevalence of SSI is around 11.2 per 100 patients across incident studies and for a large-scale study of 850,000 on noninsurance surgery from across the United States puts the incidence of SSI to be around 1.9% and in Southeast Asia, it is estimated at around 7.8%. This condition encourages the need for countries in Southeast Asia to pay attention to specific risk factors and develop effective prevention strategies which will certainly have a more cost-effective impact [9, 10].

Infection in the area of operation has an impact on the length of patient care with higher medical costs and can reduce the quality of health services. SSI is the most expensive case with an estimated cost of $3.3 billion per year and associated with an additional value of nearly one million rupiah per day for inpatients each year [11, 12]. Inflammation in the surgical process can be observed visually in the area around the wound with characteristics of heat, erythema, pain, and swelling, or redness, burning sensation in the incision area, pain, swelling, and decreased function. Meanwhile, the signs of SSI are based on physiological parameters and the characteristics, such as the presence of systemic inflammatory response syndrome (SIRS): Hyperthermia (>37.5 °C) or hypothermia (<36.5 °C), tachycardia (heart rate >90$x$/min), tachypnea (respiratory rate >20), leucocytosis (white blood cell count >12,000/mm$^3$) or leucopenia (white blood cell count <4,000/mm$^3$), elevated C-reactive plasma protein or procalcitonin. To prevent SSI from occurring, it is necessary to have antibiotic prophylaxis recently recommended for use in the preoperative and postoperative periods to reduce SSI levels [5, 13–16].

The administration of surgical prophylactic antibiotics at each clean-contamination surgery has been shown to be effective in reducing the incidence of surgical wound infection. The effectiveness of surgical prophylactic antibiotics is highly dependent on the antibiotic concentration in the incision area, where at least the inhibitory concentration is minimal. Therefore, the choice of antibiotics, the antibiotic dose, the time and duration of administration, and the method of administration of antibiotics are the determining factors for the success of the prophylactic action [17].

Therefore, prophylactic antibiotics are usually given parenterally. To reach high enough tissue levels at the time of surgery, prophylactic antibiotics should be given 30–60 min preoperatively. Provision of prophylactic antibiotics that continued longer does not reduce the risk of further infection in the area of the operation [1,18]. Digestive and oncology surgeries are ones of the major surgeries are ones of the abdomen in the clean-contaminated category so that it has a high risk of infection and 4.46 times of the risk of experiencing SSI compared to other types of surgeries [19, 20].

## Materials and methods

This research was an analytical and descriptive observational study. The data were taken prospectively which analyzed the use of prophylactic antibiotics in patients with digestive surgery and oncology surgeries and analyzed the risk factors for infection in the surgical area at Dr. H. Slamet Martodirdjo Hospital, Pamekasan Regency.

This study aims to determine the pattern of prophylactic antibiotics and the incidence factors of SSI in digestive surgery and oncology surgery patients. In this study, in surgical patients with clean and clean-contaminated surgery criteria who received prophylactic antibiotics by the inclusion criteria, there were 67 out of 382 patients at medicine staff surgery room in April–June 2020. Clinical parameters included measurements of temperature changes, pulse, Respiratory and laboratory examinations in the form of WBC values before surgery, 24 h after surgery, and on 3–7 days after surgery as well as monitoring the condition of the surgical wound on day 30. The statistical method used was chi-square to determine the risk factors for infection in the operating area (SSI) in digestive surgery and oncological surgery patients. This study had received ethical approval from the Ethics Committee of Dr. H. Slamet Martodirdjo Hospital, Pamekasan Regency.

## Results

Patients with digestive and oncology surgeries (N=67) were measured their clinical conditions before and after surgery in the form of body temperature, respiration, pulse, laboratory tests, and prophylactic antibiotics, then the physical condition of the surgical wounds was examined on days

3 and 7. After surgery (when wound care was performed), patients were then followed for up to 30 days, especially their wound after surgery to find out if there was an infection in the area of operation (SSI). This study had been approved by the Ethics Committee of Hospital Dr. H. Slamet Martodirdjo, Pamekasan Regency with a certificate of passing the ethical review number: 070/252/432.603/KEPK/2020.

The total number of 67 patients consisted of 48 digestive surgeries (71.6%) and 19 oncology surgeries (28.4%). The research sample categories were classified descriptively based on gender, age, and guarantor type. Of the total 67 patients, there were male, 40 (59.7%) patients, and 27 (40.3%) female patients, with ages between 18 and 45 years as many as 37 patients (55.2%) and the patient insurance status. There were 3 (4.5%) more patients who did not use insurance and those who used national insurance coverage as many as 64 (95.5%) in digestive surgery and oncology surgery, Moreover 45 samples of digestive surgery consisted of 32 (66.7%) male patients and 16 (33.3%) female patients with 25 patients aged 18–45 years (52.1%) and the insurance status used was National Insurance Coverage as many as 46 (95.8%) patients. Classification of patient demographic relationships in detail can be seen in Table 1.

The results of the Gyssens analysis for the use of antibiotics based on the type of surgery are shown in Table 2, slowing that the clean and clean operations contained almost the same amount, with only a different one point. Even though the dominant oncology surgery was clean, there were only 19 out of 67 samples. Operation category 0–IV p-value>0.005 implied no difference involving the use of antibiotics in clean-contaminated and clean surgeries. From the data analysis, it was found that clean-contaminated category 0, appropriate and rational antibiotics were only 9 (27.2%) compared to clean surgery 20 (58.8%) which was higher. Category I was the inappropriate timing of giving antibiotics for two patients because it was more than 30 min, Category II was good for clean-contaminated surgery and clean statement of inaccurate doses because the time given to surgery was more than 3 h, so that in these patients there needs to be an additional dose. In category III, the duration was too long from the drug injected until the operation was complete because there is a delay in operating hours. Whereas in category IV there was a more effective/cheaper/narrow spectrum as much as 37.5% in prophylactic use. So there needs to be a control or evaluation of uniform exercises in contaminated clean and clean surgeries.

Based on the analysis of risk factors for infection with the incidence of infection in the area of operation (SSI), which stated that 64% of the prophylactic antibiotic cefazolin did not occur SSI, while ceftriaxone although only one patient had an incidence of SSI of 1.5%. Digestive surgical prophylactic antibiotics requested by WHO were cephalosporin antibiotics generation I and created II as prophylaxis to prevent multiresistant pathogens, superinfection, and infection of *Staphylococcus* spp. In choosing antibiotics, you must pay attention to the pattern of germs and antibacterial sensitivity in the hospital. In the period of study, the germ patterns and antibacterial sensitivity in the hospital were not yet available. The choice of prophylactic antibiotics was also based on the type of surgery and its medicinal properties. Therefore, prophylactic antibiotics must be nontoxic and, bactericides, which were available in the elderly form, could achieve therapeutic levels in a short tissue time, as well as a long half-life. The prophylactic antibiotic used in digestive surgery at Dr. H. Slamet Martordirdjo Hospital Pamekasan was a cephalosporin class of antibiotics creating the first, namely cephalosporin, cefuroxime created the second and ceftriaxone which was the third generation and there was also metronidazole and ciprofloxacin injection.

**Table 1:** Demographic characteristics of patients with digestive and oncology surgeries.

| No | | Characteristics of research subjects | Types of surgery | | | | Total samples (n=67) | |
|---|---|---|---|---|---|---|---|---|
| | | | Digestive surgery (n=48) | | Oncology surgery (n=19) | | | |
| | | | n, % | | n, % | | n, % | |
| 1 | Gender | Male | 32 | 66.7 | 8 | 42.1 | 40 | 59.7 |
| | | Female | 16 | 33.3 | 11 | 57.9 | 27 | 40.3 |
| 2 | Age, years | 18–45 | 25 | 52.1 | 12 | 63.1 | 37 | 55.2 |
| | | 46–65 | 21 | 43.7 | 5 | 26.3 | 26 | 38.8 |
| | | 66–80 | 2 | 4,2 | 2 | 10.6 | 4 | 6 |
| 3 | Guarantee status | Noninsurance | 2 | 4.2 | 1 | 5.3 | 3 | 4.5 |
| | | National insurance coverage | 46 | 95.8 | 18 | 94.7 | 64 | 95.5 |

**Table 2:** The results of the analysis of the quality of the use of prophylactic antibiotics by type of surgery.

| Category | Type of surgery | | | | | | p-Value |
|---|---|---|---|---|---|---|---|
| | Clean-contaminated (n=33) | | Clean (n=34) | | Total Usage (n=67) | | |
| | n, % | | n, % | | n, % | | |
| Category IV | 13 | 39.4 | 12 | 35.3 | 25 | 37.3 | 0.15 |
| Category III | 4 | 12.1 | 1 | 2.9 | 5 | 7.5 | 0.35 |
| Category II | 5 | 15.2 | 1 | 2.9 | 6 | 9.0 | 0.42 |
| Category I | 2 | 6.1 | 0 | 0 | 2 | 3.0 | 0.23 |
| Category 0 | 9 | 27.3 | 20 | 58.8 | 29 | 43.3 | 0.3 |
| Total | 33 | 100 | 34 | 100 | 67 | 100 | |

Category 0, Appropriate and rational use of antibiotic; Category I, Not on time for antibiotics; Category II, Incorrect dose/interval/route; Category III, Duration too long/too short; Category IV, Some are more effective/less toxic/less expensive/narrow spectrum.

The time of administration that occurred SSI occurred in the range of 31–40 min, and the patient went home on the third day of hospitalization in room surgery then observed on the seventh day, There was no infection in the operating area after the 30th day and it was found that there was an infection in the operating area in patients with appendicitis with elective surgery and the type of clean surgery contaminated with an operation time of 35 min to 1 h and waiting time on days 1 and 2, it is by the existing initial stages.

## Discussion

Some things need to be considered after the actions taken (SSIs) which are also a common cause of health problems related to infection [21, 22]. In this study, after being observed on the third and seventh days of the patient during hospitalization in the operating room, there were no signs of SSI. However, after the patient went home, it was observed that 30 patients there was 1 (1.5%) patient whose wounds were less than the 67 samples in this study, in which there were purulent, inflammatory, and erythema around the surgical wounds which are shown in Table 4. As a result, we can visually observe the SSI around the wound with the characteristics of heat, pain, fluid, and swelling [13, 23, 24]. The WHO's Global Guidelines for the Prevention of Surgical Site Infection states that the incidence of SSI/SSI for developing countries for 100 surgical patients, is approximately (ranging from 1.2 to 23.6%) and many factors are identified as contributing to SSI risk [25].

The choice of prophylactic antibiotics for surgical patients which is the first line is cefazolin or cefuroxime. To present prophylactic antibiotics to stop and reduce colonization of microorganisms present during operative operations by selecting the narrowest spectrum for limiting occurring bacterial resistance, low toxicity, economic factors, injection dosage forms for effective conditions, and the ability to access tissue before surgical procedures and achieve therapeutic levels during surgery and several hours after the incision is closed [26].

Also, prophylactic antibiotics must be sensitive to infection-causing bacteria. If a germ map is needed in the operating room, then the existing recommendations, namely cefuroxime with alternative use of cefuroxime or metronidazole plus gentamicin are selected [27–29]. Cefazolin and cefuroxime which are the most prophylactic antibiotic used in this study in Table 3 are beta-lactam antibiotics for the first generation of cephalosporins, including broad-spectrum antibiotics with greater effectiveness against Gram-positive bacteria, which are the most common SSI germs at the age of 24–48 h postsurgery and, high lipophilic so that it is easily penetrated by the network. Streptococcus is the bacteria that causes many infections, including cellulitis. Streptococcal cellulitis is an acute inflammation that occurs in the skin and subcutaneous tissue in the event of a fire, wound, or surgical wound or after minor trauma [30].

Cefazolin is a generation I cephalosporin that is dominant in Gram-positive, in terms of a cheaper price than cefuroxime and can suppress the growth of bacteria such as *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, and *Klebsiella* spp. On the other hand cefuroxime is a second-generation cephalosporin that tends to be Gram-negative, and the price is more expensive than cefuroxime and ceftriaxone in the 2020 e-catalog. The prophylactic antibiotic dosage regimen given based on the recommendation is 2 g of cefazolin, for all patients weighing under 120 kg. Ceftriaxone is not recommended because it has greater activity against Gram-negative bacteria and less activity against Staphylococcus which is a common cause

**Table 3:** Analysis of risk factors for infection with the incidence of infection in the area of operation (SSI).

| No | | Risk factors | Infection area of operation | | | | Total sample (n=67) | |
|---|---|---|---|---|---|---|---|---|
| | | | Not SSI (n=66) | | SSI (n=1) | | | |
| | | | n, % | | n, % | | n | p-Value |
| 1 | Types of antibiotics used | Cefuroxime | 9 | 13.4 | 0 | 0 | 9 | 0.963 |
| | | Cefazolin | 43 | 64.2 | 0 | 0 | 43 | |
| | | Ceftriaxone | 9 | 13.4 | 1 | 1.5 | 10 | |
| | | Ciprofloxacin | 3 | 4.5 | 0 | 0 | 3 | |
| | | Ciprofloxacin and metronidazole | 2 | 2.9 | 0 | 0 | 2 | |
| 2 | Time to offer antibiotics (minutes) | 20–30 | 15 | 22.4 | 0 | 0 | 15 | 0.831 |
| | | 31–40 | 44 | 65.6 | 1 | 1.5 | 45 | |
| | | 41–50 | 7 | 10.4 | 0 | 0 | 7 | |
| 3 | Observation after prophylaxis (days) | 1 | 28 | 41.8 | 0 | 0 | 28 | 0.783 |
| | | 3 | 28 | 41.8 | 1 | 1.5 | 29 | |
| | | 7 | 10 | 14.9 | 0 | 0 | 10 | |
| 4 | Based on diagnosis | Appendicitis | 20 | 2.8 | 1 | 1.5 | 21 | 0.788 |
| | | Hernia | 12 | 17.9 | 0 | 0 | 12 | |
| | | Obstruction | 17 | 2.3 | 0 | 0 | 17 | |
| | | Tumor | 8 | 11.9 | 0 | 0 | 8 | |
| | | Rust | 9 | 13.4 | 0 | 0 | 9 | |
| 5 | Nature of operations | Urgent (Cito) | 6 | 8.95 | 0 | 0 | 6 | 1.000 |
| | | Elective | 60 | 89.5 | 1 | 1.5 | 61 | |
| 6 | Type of operation | Clean | 33 | 49.2 | 0 | 0 | 33 | 0.313 |
| | | Clean-contaminated | 30 | 44.7 | 1 | 1.5 | 31 | |
| 7 | Length of operation (minutes) | 0–30 | 26 | 38.8 | 0 | 0 | 26 | 0.801 |
| | | 35–60 | 27 | 40.2 | 1 | 1.5 | 28 | |
| | | 65–90 | 10 | 14.9 | 0 | 0 | 10 | |
| | | 95–120 | 3 | 4.5 | 0 | 0 | 3 | |
| 8 | Long wait before surgery(days) | 1–2 | 62 | 92.5 | 1 | 1.5 | 63 | 0.812 |
| | | 3–4 | 2 | 2.9 | 0 | 0 | 2 | |
| | | 5–6 | 2 | 2.9 | 0 | 0 | 2 | |

of postoperative wound infection [31]. The effectiveness of antibiotic use in this study was carried out by comparing the five parameters listed in Tables 3 and 4 in the form of measuring clinical parameters (temperature, pulse, and respiration) plus monitoring the condition of the operation wound and checking laboratory parameters. There are no SIRS criteria as marked by hyperthermia (>37.5 °C) or hypothermia (<36.5 °C), tachycardia (heart rate >90*x*/min), and tachypnea (respiratory rate >20) in 67 sample in the operating room [32].

**Table 4:** Value of measurement of clinical and laboratory data before and after prophylactic antibiotics.

| Type | Time of examination | Range of clinical | Mean ± SD |
|---|---|---|---|
| Temperature, °C | Pre-op | 36–38 | 36.5 ± 0.4 |
| | 24 h post-op | 34–37 | 35.8 ± 4.0 |
| | Days 3–7 post-op | 35–37 | 36.5 ± 0.6 |
| Heart Rate (HR), *x*/min | Pre-op | 55–105 | 83.2 ± 10.3 |
| | 24 h post-op | 60–90 | 83.3 ± 10.6 |
| | Days 3–7 post-op | 60–95 | 51.7 ± 11.8 |
| Respiratory (RR), *x*/min | Pre-op | 16–20 | 18.4 ± 1.2 |
| | 24 h post-op | 18–20 | 18.5 ± 1.0 |
| | Days 3–7 post-op | 16–20 | 11.5 ± 0.9 |
| WBC, $\times 10^3/\mu L$ | Pre-op | 5.38–25.05 | 48.71 ± 20.7 |

The use of prophylactic antibiotics in Table 4 shows the results patient's clinical and laboratory conditions measurements in the form of temperature, pulse, and respiration, both before surgery (pre-op) and, 24 h after surgery in columns 4 and 5. For the results of measuring clinical conditions, measurements can be made at all patients and all results were still in the normal category with normal parameters according to the SIRS criteria. The importance of wound care was carried out to support the healing process of surgical wounds. In the study, there was no rapid infection after surgery on days 1, 3, and 7 but on day 30 there were surgical wounds shown in the figure in appendix 6 and the patients were at home where there was erythema, inflammation, fluid around the incision wound

and conditions improved after 42 days after control to the clinic on day 32 who received wound care and additional drugs.

Wound monitoring was performed in all patients on day 3 and 7 postoperatively at the inpatient medical staff surgical. Meanwhile, on the 30th day, when the control patients went to the clinic or were hospitalized at their home by telephone, the health care workers were asked about the patient's condition because it was not possible for the patient's condition to go to the clinic during the pandemic because there was a priority scale for the poly patient. On days 3 and 7 according to the study, there were no signs of SSI in the patient so that this study was continued to the 30th day. It turned out that one patient had inflammation according to the SSI criteria (1.5%). This condition could be caused by various factors including patient factors from cleanliness and supporting factors such as less balanced nutritional intake of patients while at home. This was evidenced by questions to patients about habits while at home, both regarding hygiene and diet. The patients admitted that they did not take a bath and the food they consumed had less protein value, only because they were worried that the stitches would not dry out.

Analysis of the effectiveness of prophylactic antibiotics in clean and clean-contaminated surgeries can be done by measuring clinical and laboratory signs as well as by monitoring the wound condition. As mentioned, surgery carries the risk of various complications including SSI. Prophylactic antibiotics with the right type, amount, duration, method, and time of administration are expected to reduce the risk of postoperative complications. In this study, the overall of the five clinical and laboratory parameters and surgical scar conditions were normal. The optimal time for prophylactic antibiotics was 15–30 min or 60 min before the incision in Table 3 which means that the administration time in the normal range was 20–50 min. This needs to be considered because this administration is related to the time it takes to reach the maximum level in the tissue so that it can inhibit infection due to various contaminations [33].

Time provides the duration of the operation so that the correct dose is given. There were two patients (2.9%) who did not administer antibiotics on time, either because they were given too long before the incision were too close or given after the incision was made. It must be paid attention that the administration that takes too long time before the incision is carried out is not effective because the maximum concentration ($C_{max}$) in the blood has decreased. Then, the t½ of cefazolin is between 90 and 150 min.

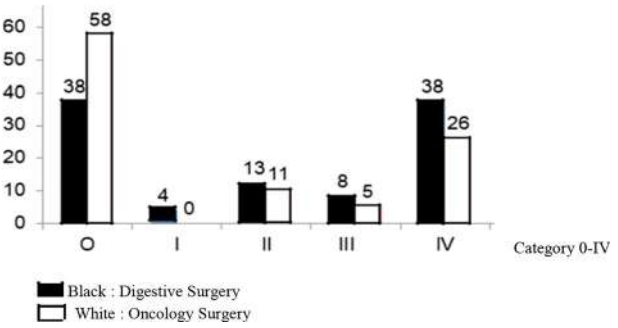


**Figure 1:** Qualitative analysis of use of prophylactic antibiotics.

Qualitative analysis of this prophylactic antibiotic is shown in (Table 2).

A qualitative analysis of the use of antibiotics was also carried out in this study using the Gyssens method analysis [34]. The analysis was carried out on the use of prophylactic antibiotics in contaminated clean surgery and clean surgery in Table 2 where the qualitative analysis results showed that the correct and rational=31 of category 0 (46.26%), category I 2 (2.9%); Category II 6 (8.9%); Category III 5 (7.4%) and Category IV 23 (34.3%). In Category IV, because the cefuroxime used was a patient preparation, the price was more expensive than the catalog price, and there were antibiotics such as Ciprofloxacin and injection metronidazole because there were antibiotics that were more effective than these antibiotics (Figure 1).

Actions that should be taken are to improve communication between health care workers with the provision of antibiotics as well as guidelines and regular procedures for rational use of antibiotics such as the Guidelines for the Use of Antibiotics which that refer to the Clinical Practice Guidelines, Standard Operational Procedure, National Formulary and periodic evaluations to improve the antimicrobial resistance control program at Dr. H. Slamet Martodirdjo Hospital to increase knowledge, discipline, and uniformity for all professionals.

## Conclusions

Analysis prophylactic antibiotics have been performed by doctor and pharmacist. In 31 patients, there are the use of cefazolin (64.2%) and the rational use of antibiotics (46.26%). Evaluation in the administration of prophylactic antibiotics is needed. The widely used prophylactic antibiotics, such as cefazolin and cefuroxime are recommended antibiotics to be used in incision surgery and rationale used.

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Nunuk Dyah Retno Lastuti*, Nur Rusdiana and Poedji Hastutiek

# Second internal transcribed spacer (ITS-2) as genetic marker for molecular characterization of *Sarcoptes scabiei* in rabbits from several areas of East Java, Indonesia

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**Abstract**

**Objectives:** The purpose of this study is to use the second internal transcribed spacer (ITS-2) to determine the molecular characteristics of *Sarcoptes scabiei* in rabbits from several areas of East Java.
**Methods:** Collecting *S. scabiei* mites from rabbits with clinical signs of scabies; DNA extraction with minikit QIAamp DNA; polymerase chain reaction amplification; nucleotide sequence analysis; homology and phylogenetic tree using the Neighbor-Joining method in the program molecular evolutionary genetics analysis-7 (MEGA-7).
**Results:** Sequence analysis of ITS-2 *S. scabiei* from five regions in East Java showed an identity >91.23% with isolates from China (KX695125.1). The phylogenetic analysis of ITS-2 *S. scabiei* from Mojokerto rabbits has a close relationship with AB82977.1; Surabaya and Nganjuk rabbits are closely related to KX695125.1; while Sidoarjo and Pasuruan rabbits are closely related to EF514469.2. and AB369384.1.
**Conclusions:** The homology analysis of all samples showed identity of more than 91.23% with isolate China (KX695125.1). The sequences of ITS-2 gen of *S. scabiei* from rabbits in several areas were relatively close to *S. scabiei* obtain various hosts from National Centre for Biotechnology Information (NCBI) data.

**Keywords:** ITS-2; rabbit; *Sarcoptes scabiei*.

***Corresponding author: Nunuk Dyah Retno Lastuti**, Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, E-mail: nunukdyah53@gmail.com
**Nur Rusdiana,** Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia
**Poedji Hastutiek,** Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

## Introduction

Scabies is a highly contagious skin disease caused by *Sarcoptes scabiei* (*S. scabiei*) is one of the most important human and animal diseases. It has been reported that around 100 million people in the world are infected with *S. scabiei* with a prevalence between 0.2 and 71.4% [1]. Currently, scabies is an emerging or re-emerging parasitic skin disease and could threaten the health of humans and animals in the world [2, 3]. Scabies in humans is a public health problem, characterized by intense itching, inflammation, manifests as skin allergies (hypersensitivity type IV), and associated with the mites burrowing into the stratum granulosum of the epidermis [4]. Scabies diagnosis was carried out by using the skin scraping method but it was difficult in mild infections. The development of serological diagnosis and vaccine sub-units in humans and animals is still needed through molecular research with various gene loci [2, 5]. For the molecular typing and molecular characterization of *S. scabiei* were used various genetic markers such as ITS-2, COX-1, 12S rRNA, and 16S rRNA [3, 6, 7, 8]. The ITS-2 gene locus has advantages compared to other molecular regions, it has a high level of sensitivity, 100 genome replication, and also the ITS-2 has a high rate of evolution so it can be used as a genetic marker for detection of genetic mutations due to differences in geographic location [9]. The purpose of this study is to use the second internal transcribed spacer (ITS-2) to determine the molecular characteristics/identity of *S. scabiei* in rabbits from several areas of East Java. This research is a preliminary study that can be further developed for the study of serological diagnostic kits and subunit vaccines in animals, and in humans using a sample of *S. scabiei* var. *hominis*.

## Materials and methods

This research according to standard operating procedures and approved by the Ethic committee of Veterinary Medicine Faculty, Airlangga University, certificate No. 630-KE. In this study, skin

scrapings of 22 rabbits with clinical symptoms of scabies were collected from rabbit farms in Sidoarjo, Pasuruan, Mojokerto, Nganjuk, and Surabaya. The character of the selection of the five-area samples is as follows: (1). the largest number of rabbit farms in the area, (2) high cases of scabies and a severe degree of infection, (3) dirty cage conditions, especially the dry season. The scraped mites are collected for molecular examination processing [8, 10].

### DNA extraction and polymerase chain reaction (PCR) assay

DNA extraction was carried out using a QIAamp DNA Minikit (Qiagen, Hilden, Germany) according to the factory protocol. Amplification of the 304 bp ITS-2 *S. scabiei* fragment was carried out using the forward primer (5′ CGG TTT CGT CAC ACT TCG ATG 3') and reverse (5′ CGG GTA TTC TCG CTT GAT CTG 3′). Subsequently, the PCR test of the ITS-2 encoding gene segment thermocycling in an automatic thermocycler (Biorad) with an initial denaturation at 94 °C for 5 min; followed by 35 cycles of template denaturation, 94 °C for 30 s, primer annealing at 54 °C for 30 s, DNA extension at 72 °C for 30 s, and final extension at 72 °C for 5 min (Qiagen, Hilden, Germany). Analysis of the PCR product was carried out by electrophoresis using 2% agarose gel [7, 11]. PCR products were purified using the QIAquick, PCR purification kit (Qiagen).

### DNA sequencing and phylogenetic tree analysis

DNA sequencing was carried out in an automatic DNA sequencer (ABI 3730XL, Solgent Co. Ltd., South Korea). The nucleotide sequence was read using molecular evolutionary genetics analysis-7 (MEGA-7) software and the DNA sequence analysis was carried out using the Basic Local Alignment Search Tool (BLAST) on the Gene Bank ("http://www.ncbi.nlm.nih.gov/BLAST"). The phylogenetic tree was analyzed using the MEGA-7 software with the Construct/Test Neighbor-joining tree and Bootstrap method and multiscale Bootstrap analyses with 1,000 Replications were conducted.

## Results

The Results of the PCR test were read on 2% agarose gel electrophoresis showing that the PCR product with high specifications was a single band at the 304 bp position in accordance with the amplification target. The DNA sequencing analysis results of *S. scabiei* from five regions in East Java showed an identity 91.23–98.68% for *S. scabiei* Chinese isolates (Accession number on Gene Bank: KX695125.1).

### Sequencing result of *S. scabiei* nucleotide

Multiple alignment results from the nucleotide sequences showed differences in nucleotide arrangements of deletion and substitution mutations from the ITS-2 *S. scabiei* encoding gene that infected rabbits from the Surabaya, Sidoarjo, Mojokerto, Pasuruan, and Nganjuk isolates if aligned with Chinese isolate (KX695125.1) (Figure 1).

## Result of phylogenetic tree analysis

The phylogenetic tree analysis results of *S. scabiei* from several regions of East Java isolates with data on the Gene Bank showed that *S. scabiei* from rabbit isolates of Mojokerto and *S. scabiei* on *Capricornus scispus* Japan isolate with accession number AB82977.1 have a close relationship, *S. scabiei* Surabaya and Nganjuk isolate have a close relationship with the reference sequence that used for primer design of Chinese isolate with accession number KX695125.1, and *S. scabiei* Sidoarjo and Pasuruan isolate have a close relationship with Chinese isolate (accession number EF514469.2) and *S. scabiei* of *feral raccoon* isolate from Japan with accession number AB369384.1. *S.scabiei* isolates from Surabaya, Sidoarjo, Pasuruan, Nganjuk, and Mojokerto also showed close kinship with *S.scabiei* from several hosts in several countries with a pairwise distance showing at 0.000 (Figure 2).

## Discussion

The nucleotide composition change that occurred in *S. scabiei* isolates from Surabaya, Nganjuk, Pasuruan, Sidoarjo, and Mojokerto cities showed deletions and substitutions (Figure 1). The only changes in the partial nucleotide sequence of *S. scabiei* DNA were silent mutations because genetic mutations that occurred have a percentage of less than 9%, and the identity level was more than 91.23%. In this study, *S. scabiei* isolates came from five regions in East Java with different geographical locations. The degree of polymorphism can be influenced by the diversity of the host and geographic location and by different genetic markers [4, 5, 12]. Deletion and substitution of a nucleotide may be caused by the mite's activity to adapt of host cells including made of tunnels in stratum granulosum during their life cycle to obtained nutrients from host cells [13, 14]. Phylogenetic tree analysis showed that *S. scabiei* isolate from Mojokerto was in the same branch as the Japanese isolate *C. crispus* (AB820977.1). The Surabaya and Nganjuk isolates are in the same branch as the Chinese rabbit isolates (KX695125.1) and these isolates are also used as reference sequences on primer design to PCR tests. Sidoarjo and Pasuruan isolates are in the same branch as

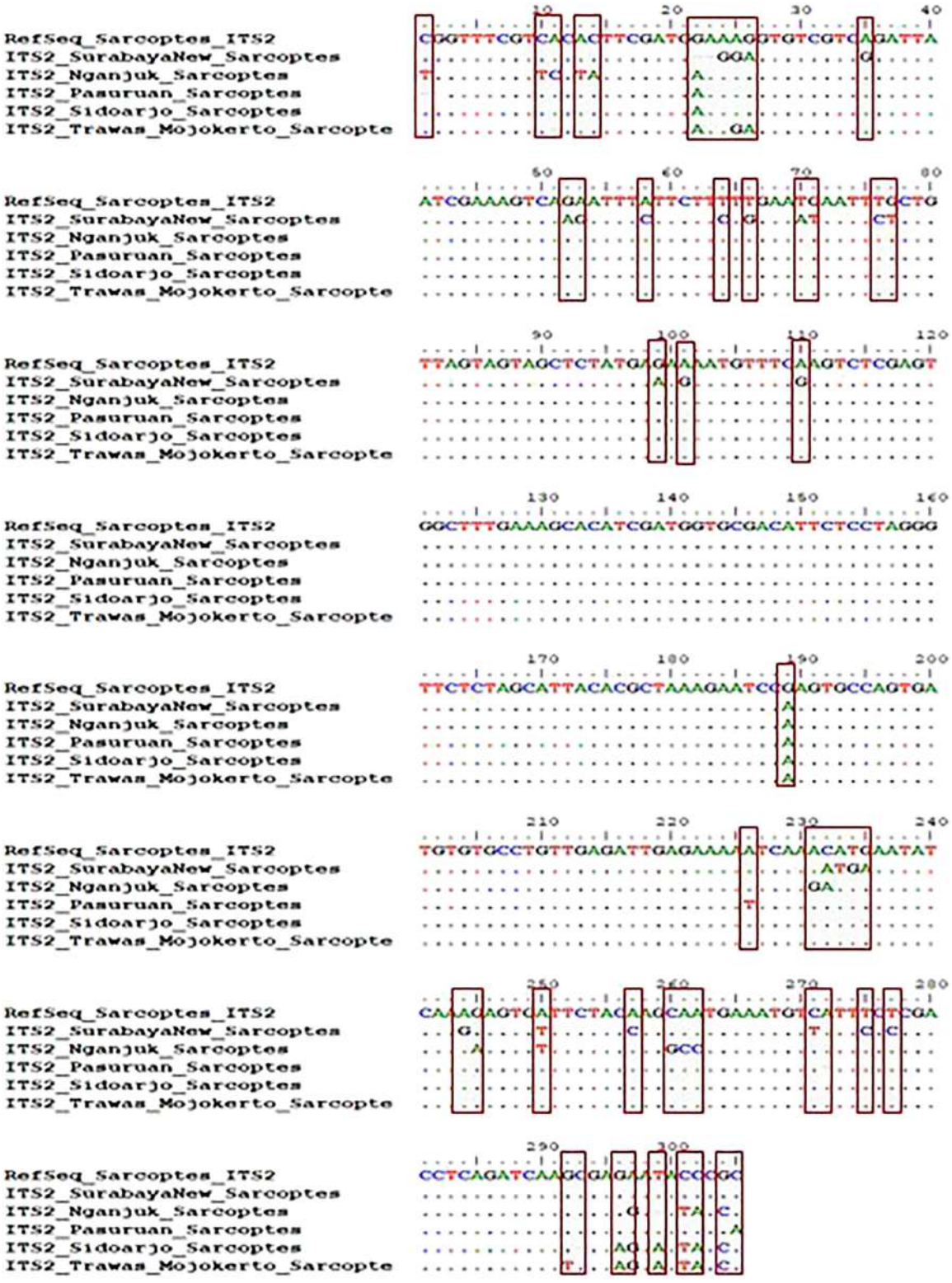


**Figure 1:** Multiple alignments of *S. scabiei* nucleotide sequences isolate from Surabaya, Nganjuk, Pasuruan, Sidoarjo, and Mojokerto with Chinese isolate (KX695125.1).

the Chinese isolate from rabbits (EF514469.2) as seen in Figure 2. The kinship that occurs can be caused by *S. scabiei* migration through the host of rabbits or other animals and humans [2, 5].

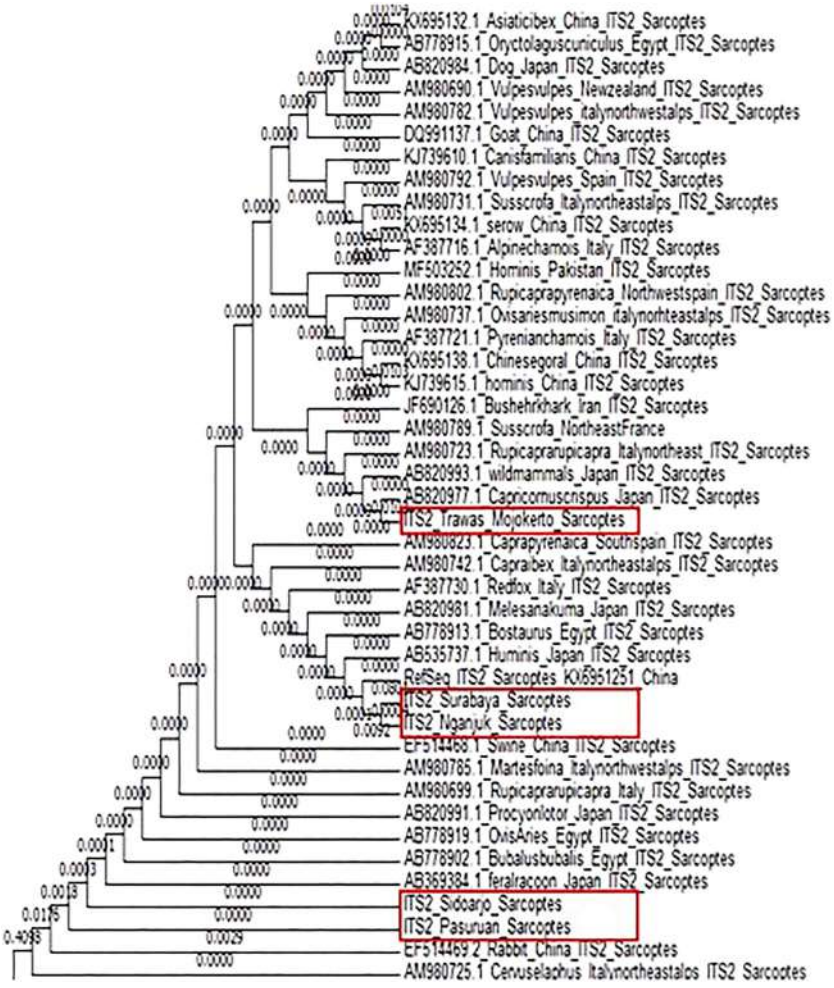


**Figure 2:** Phylogenetic tree analysis of the ITS-2 encoding gene of *S. scabiei* from Surabaya, Nganjuk, Pasuruan, Sidoarjo, and Mojokerto rabbit isolates on Gene Bank *S. scabiei* data.

## Conclusions

The homology analysis of all samples showed an identity of more than 91.23% with isolate China (KX695125.1). The sequences of the ITS-2 region of *S. scabiei* from rabbits in several areas were relatively close to *S. scabiei* obtain various hosts from National Centre for Biotechnology Information (NCBI) data.

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Nuzul W. Diyah*, Isnaeni, Shabrina W. Hidayati, Bambang T. Purwanto and Siswandono

# Design of gossypetin derivatives based on naturally occurring flavonoid in *Hibiscus sabdariffa* and the molecular docking as antibacterial agents

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**Abstract**

**Objectives:** This study was purposed to design gossypetin derivatives which have higher activity than the parent compound found in *Hibiscus sabdariffa* and to find the most potent compound as the antibacterial agent.
**Methods:** Twenty-five gossypetin derivatives were designed by conjugation the molecular structure of gossypetin with acyl group from some natural phenolic acids. The antibacterial activity was predicted by docking simulation on *Escherischia coli* DNA gyrase (PDB. 1KZN) which was performed by Molegro Virtual Docker. Potency as an antibacterial agent was evaluated based on binding affinity, hydrogen bond, and similarity of binding pattern with reference ligand Clorobiocin.
**Results:** Almost all derivatives showed higher binding affinity than gossypetin (docking score –113.43 kcal/mol). The most active compound was 3G19 with docking score –167.42 kcal/mol which was comparable to clorobiocin (docking score –167.75 kcal/mol). The compounds displaying higher activity than gossypetin were belonged to 7,4′-dimethyl and 3,7,4′-trimethylgossypetin of coumaric acid, caffeic acid, and also ferulic acid. The compounds showed similar binding mode with clorobiocin especially in interaction with Asn46.
**Conclusions:** Gossypetin derivatives designed by conjugating the gossypetin with phenolic acyl increased *in silico* antibacterial activity of the parent compound. The 3,7,4′-trimethylgossypetin of coumaric acid was selected as the most potent compound for antibacterial agents.

**Keywords:** conjugation; DNA gyrase; docking; gossypetin; *Hibiscus sabdariffa*.

## Introduction

Microbial resistance to well-known antibiotics is still a global health challenge today. Various strategies have been attempted to solve this problem including development of new antimicrobial drugs with novel mechanism of action. There is growing interest in the metabolite of plant towards discovery of more potent bioactive agents. Flavonoids are a large class of natural compounds which have been extensively studied for their antibacterial activity [1]. Some synthetic flavonoids also exhibited remarkable antibacterial activities which are more potent than the standard drug against multidrug-resistant (MDR) Gram-negative and Gram-positive bacteria [2].

Flavonoids are widely distributed in various plants including *Hibiscus sabdariffa* (Roselle) which has been used as traditional herbal medicine for a long time. Aqueous extract of roselle flower and calyx showed varying degree of antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* [3, 4]. *E. coli* is a Gram-negative bacteria that dominates the cause of Urinary Tract Infection (UTI) and are generally developed as MDR bacteria, but are still sensitive to some antibiotics such as aminocoumarins and fluoroquinolones if used in controlled dosage regimen [5]. The *H. sabdariffa* (HS) contained polyphenols of flavonoid in simple or polymerized form. The chemical constituents found in the flower of HS include anthocyanin, gossypetin, gossypitrin (gossypetin 3-glucoside), hibiscetin, hibiscitrin

***Corresponding author: Nuzul W. Diyah**, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Kampus C, UNAIR. Jl. Mulyerejo, Surabaya, 60115, Indonesia, E-mail: nuzul-w-d@ff.unair.ac.id
**Isnaeni, Bambang T. Purwanto and Siswandono,** Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, E-mail: isnaeni@ff.unair.ac.id (Isnaeni), bambang-t-p@ff.unair.ac.id (B.T. Purwanto), prof.siswandono@yahoo.com (Siswandono)
**Shabrina W. Hidayati,** Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia,
E-mail: 1930912320093@mhs.ulm.ac.id

(hibiscetin-3-glucoside), sabdaretin, sabdaritrin, and other gossypetin glucosides [6]. The gossypetin isolated from the whole flower including calyx of HS exhibited antibacterial activity which may be due to its polyphenolic nature [7].

So far, there still no reports of any attempts to enhance the activity of gossypetin through molecular design or synthesis of the gossypetin derivatives. This study was focus on designing of new gossypetin derivatives in an attempt to discover more specific bioactive agents than the isolate of HS extract. To increase the antibacterial activity, gossypetin derivatives were designed by conjugating the hydroxyl group on gossypetin structure with certain acyl group of simple carboxylic acids and natural phenolic acids. Selection of functional groups that will be conjugated with parent structure is a critical point in designing new derivatives of active constituent from medicinal plants [8]. The acyl functionality of naturally occurring phenolic acids was considered to give synergistic biological activity for gossypetin. The phenolic acids which will be combined with gossypetin also exhibited an antibacterial activity, such as cinnamic acid [9], coumaric acid [10], ferulic acid [11], and caffeic acid [12].

According to some derivatives of gossypetin already existed, molecular modification could be achieved by attachment of methyl substituent (methylation) to hydroxyl group at position 3; position 3,7; position 3,7,4′ [13]. Natural glucoside of gossypetin bound the monosaccharide at the position 3 but glycosylation also took place at position 7 or 8. The attachment of acyl substituent to hydroxyl group at position 8 to obtain gossypetin ester of the biologically active phenolic acids was predicted to increase the antibacterial activity of derivatives compared to the parent compound. The position 8 of the flavonoid core (Table 1) is the substituent's position which is the closest to the oxygen of lactone ring, which was considered as pharmacophore that could not be replaced. The oxygen in lactone ring as pharmacophores are also found in aminocumarin antibiotics which have polycyclic ring that are similar to flavonoids [14].

This study aimed to find the gossypetin derivatives which have stronger potency as antibacterial agent based on in silico molecular interaction with targeted protein in microbial cells. It was reported that some flavonoids inhibited DNA gyrase B in *E.coli* [15]. DNA gyrase is a type II DNA topoisomerase that introduces negative supercoils into DNA and it is exists only in prokaryotes making it an attractive target of antibacterial drugs [16]. Clorobiocin, one of aminocoumarin antibiotics, was an inhibitor of DNA gyrase which could eliminated some *E. coli* plasmids such as ampicillin-resistant R28K plasmids [17]. To explore the molecular interactions and selected the most potent compound, the *E.coli* DNA gyrase (PDB ID:1KZN) was selected as targeted enzyme. Evaluation of absorption, distribution, metabolism, elimination, and the toxicity (ADMET) profile of drugs candidate is an important step in drug design so that the gossypetin derivatives which highly potential as antibacterial substances were evaluated for their in silico ADMET prediction using online tools.

## Materials and methods

### Materials

The Computer ASUS VivoBook series with an Intel Core i5-8250U CPU and Windows 10 Ultimate operating system was used as hardware. The software used is ChemDraw Pro 16.0 from Cambridge Soft, Molegro Virtual Docker (MVD) ver 6.0 program from CLC bio, and Discovery Studio Visualizer (DSV) v.19.1.0.18287 from BIOVIA. Crystal structure in three-dimensions (3D) of targeted protein obtained from the Protein Data Banks (http://rcsb.org).

### Ligand preparation

The 25 molecules studied include gossypetin as the parent compound, three compounds were methylgossypetin, 12 were methylgossypetin ester of phenolic acids, and nine were methyl gossypetin ester of carboxylic acids (Table 1). The two-dimensional (2D) structure was drawn using ChemDraw Pro 16.0 and the 3D geometries were optimized to minimal energy using MMFF94. The optimized structure then saved in Sybyl2 format (*.mol2) prior to use for *in silico* molecular docking.

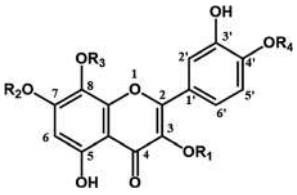
### Protein preparation

The crystal structure of *E. coli* DNA gyrase as targeted molecule was available from protein data bank site (www.rcsb.org). The DNA gyrase (PDB ID: 1KZN) contains co-crystallized ligand Clorobiocin (PDB: CBN_1), an aminocoumarin antibiotic similar to novobiocin and coumermycin A1 [18]. The protein in the *.pdb format was downloaded into workspace of MVD, all the bound water molecules, ligands, and unused cofactors were removed (preprocessed) from the protein. The binding site used in docking simulation was inspected by detecting cavity containing CBN_1 which served as reference ligand. The docking protocol was validated by redocking the reference ligand extracted from targeted protein [19].

### Molecular docking

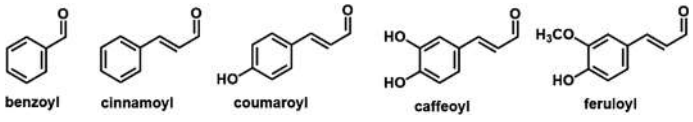
The main objective of the molecular docking is to identify the energetically favorable binding modes of test ligands in the targeted protein's binding site [20]. Docking simulation for all compounds were performed by MVD using same method as the reference ligand. The DNA gyrase

**Table 1:** The molecular structure of gossypetin derivatives designed for molecular docking.




| No | Code | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Energy, kcal/mol |
|---|---|---|---|---|---|---|
| 1 | Goss | H | H | H | H | −67.682 |
| 2 | Gm1 | $CH_3$ | H | H | H | −67.81 |
| 3 | Gm2 | $CH_3$ | $CH_3$ | H | H | −86.927 |
| 4 | Gm3 | $CH_3$ | $CH_3$ | $CH_3$ | H | −101.33 |
| 5 | 1G1 | H | $CH_3$ | $CH_3C{=}O$ | H | −99.615 |
| 6 | 1G2 | H | $CH_3$ | $(CH_3)_2C{=}CH{-}CH_2$ | H | −117.089 |
| 7 | 1G3 | H | $CH_3$ | benzoyl | H | −143.289 |
| 8 | 1G4 | H | $CH_3$ | cinnamoyl | H | −125.255 |
| 9 | 1G5 | H | $CH_3$ | coumaroyl | H | −113.785 |
| 10 | 1G6 | H | $CH_3$ | caffeoyl | H | −112.342 |
| 11 | 1G7 | H | $CH_3$ | feruloyl | H | −126.164 |
| 12 | 2G8 | H | $CH_3$ | $CH_3C{=}O$ | $CH_3$ | −114.326 |
| 13 | 2G9 | H | $CH_3$ | $(CH_3)_2C{=}CH{-}CH_2$ | $CH_3$ | −131.467 |
| 14 | 2G10 | H | $CH_3$ | benzoyl | $CH_3$ | −158.484 |
| 15 | 2G11 | H | $CH_3$ | cinnamoyl | $CH_3$ | −139.966 |
| 16 | 2G12 | H | $CH_3$ | coumaroyl | $CH_3$ | −128.615 |
| 17 | 2G13 | H | $CH_3$ | caffeoyl | $CH_3$ | −127.174 |
| 18 | 2G14 | H | $CH_3$ | feruloyl | $CH_3$ | −141.331 |
| 19 | 3G15 | $CH_3$ | $CH_3$ | $CH_3C{=}O$ | $CH_3$ | −115.15 |
| 20 | 3G16 | $CH_3$ | $CH_3$ | $(CH_3)_2C{=}CH{-}CH_2$ | $CH_3$ | −133.081 |
| 21 | 3G17 | $CH_3$ | $CH_3$ | benzoyl | $CH_3$ | −159.866 |
| 22 | 3G18 | $CH_3$ | $CH_3$ | cinnamoyl | $CH_3$ | −141.376 |
| 23 | 3G19 | $CH_3$ | $CH_3$ | coumaroyl | $CH_3$ | −129.371 |
| 24 | 3G20 | $CH_3$ | $CH_3$ | caffeoyl | $CH_3$ | −128.125 |
| 25 | 3G21 | $CH_3$ | $CH_3$ | feruloyl | $CH_3$ | −141.879 |

The acyl functional group for $R_3$




were defined as the receptors and the gossypetin derivatives were defined as the ligands. Scoring function used was MolDock score [21], and ligand was docked into binding site origin of cavity-1 with volume 1.52 Å and surface 168.96 Å. The docking parameter used for run was set as default. The data extracted from docking process were the free energy of binding (ΔG) represented by docking score (MolDock score = MDS), the number of hydrogen bonds, and type of amino acid residues. ΔG described binding affinity of ligand-receptor complex after docking. The more negative binding energy indicated higher ligand affinity against the binding site of receptor. Scores of all test ligand were compared with reference ligand to evaluate the potency of ligands as inhibitor [22]. To assess the similarity in binding mode between test ligand and reference ligand, the amino acid residues interacting with both ligands were compared. The 2D-visualization of ligand-receptor interactions was inspected using DSV program.

### ADME prediction

The ADMET prediction was performed using the online pkCSM tool [23]. The 3D-structure of selected compounds drawn using Chem3D 16.0 program were saved as *.sdf files, then translated into the SMILES format using Online SMILES Translator (https://cactus.nci.nih.gov/translate/). The SMILES format of compounds were processed using the online pkCSM tool to predict ADMET parameters [24].

## Results

The molecular structure of gossypetin derivatives and their 3D molecular energy was listed in Table 1. The lower

molecular energy of gossypetin derivatives compare with the gossypetin indicated that substitution of hydroxyl group with substituent larger than methyl decreased the energies which means that the geometry of substituted-gossypetin molecules were more stable energetically.

## Molecular docking

The molecular docking identified the ligands that bound in similar orientation with Clorobiocin (PDB: CBN_1). Validation results of ligand CBN_1 were provided root-mean-square deviation (RMSD) score 1.807 Å (<2 Å), indicated that the targeted protein used was valid for docking assessment [25]. Most of the ligands were in good docking poses and showed similar binding conformations. Figure 1 display docked conformation of selected ligands in binding site of DNA gyrase and Table 2 shows docking scores as representation of ligand-protein complex's binding energy. It is notably from Figure 1 that the binding site of these compounds and reference ligand was similar.

All gossypetin derivatives excluding Gm3 have MDS value lower than gossypetin which indicated that designed compounds exhibited higher binding affinity than gossypetin in the binding site of DNA gyrase. Particularly 3G19 have binding energy value of 167.42 kcal/mol, comparable to the redocked ligand Clorobiocin (167.75 kcal/mol). Binding site analysis of the compounds as ligand for DNA gyrase revealed that the molecules had well fitted into binding pocket of the protein, and the ligand molecules can interact by hydrogen bonding, van der Waals, and steric interactions with amino acid residues in the cavity-1. Type of amino acid residues which built hydrogen bond with ligands was also displayed in Table 2. Most of the compounds performed H-bond interactions with the enzyme (Table 2) except the 3,7,4′-trimethylgossypetin ester of carboxylate series (3G15, 3G16, 3G18, 3G19, 3G21). It was found that gossypetin formed hydrogen bonds with amino acid Asn46 and Val71 which were important in the interaction for reference ligand CBN_1. The hydroxyl group at 3,7, and 4′-position of gossypetin were vital to form hydrogen bonds with Asn46, Pro79, and Val71 respectively, so that methylation in this position eliminated the hydrogen bonds according to data for Gm1, Gm2, Gm3. Surprisingly, 3G19 still have higher binding affinity than its 7,4′ dimethyl analogs (2G12) even though there was no hydrogen bond. The type of amino acids and binding interactions of all ligands in their docked conformations were visually inspected using MVD program. The ligand exhibiting lowest docking scores were selected to compare its binding interaction with gossypetin and the picture was taken using DSV program (Figure 2).

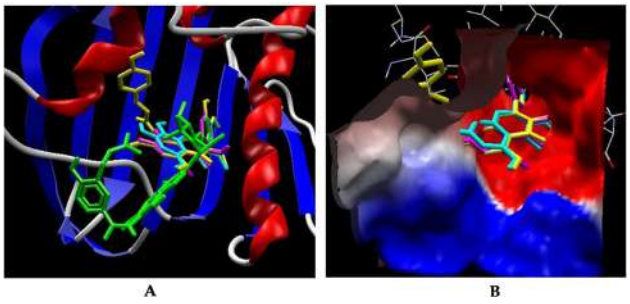



**Figure 1:** Docked conformation of gossypetin and its derivatives in ATP-binding site of *E. coli* DNA gyrase (PDB. 1KZN). Purple color is gossypetin, blue is 3-methylgossypetin (Gm1), yellow is 3G19, and green is reference ligand CBN_1.
(A) test ligands in same position with CBN_1. (B) test ligands in binding pocket of cavity-1 where the flavonoid core are deep inside.

## ADMET profiles

The predicted ADMET parameters resulting from pkCSM online (Table 3) were the profile of five selected compounds including the gossypetin, the most potent compound 3G19, second-highest potential compounds (2G13, 2G14), and a compound with low antibacterial potency, Gm1.

According to data on Table 3, substituted-gossypetin is considered to have a better absorption than gossypetin because intestinal absorption is good if the value >80% while absorption is considered as poor if it is <30%. All compounds have a good skin permeability as skin permeability is low if log Kp >−2.5. The higher distribution volume (VDss) value indicated the more drugs are distributed in the tissue rather than in blood plasma. The distribution of a compound was considered as low if log VDss value <−0.15. Based on data in Table 3, the highly potent of gossypetin derivatives (3G19, 2G13, 2G14) were distributed in plasma more higher than in tissue. All compounds showed poor ability to penetrate the blood–brain barrier and CNS because their log BB <−1 and log PS value <−3. The gossypetin and derivatives would not be metabolized by CYP2D6 and CYP3A4 but 3G19, 2G13, and 2G14 could acted as inhibitor of CYP3A4. Meanwhile, gossypetin and Gm1 (3-methyl derivatives) might inhibit CYP1A2. Inhibitors of the CYP450's can alter the pharmacokinetics of drugs which are metabolized by these enzymes.

Organic Cation Transporter 2 (OCT2) plays a key role in the disposition and renal clearance of mostly cationic drugs and endogenous compounds, such as metformin and cisplatin which are important clinical substrates. Gossypetin and derivatives are not OCT2 substrate so they don't have potential adverse interactions with codirected

**Table 2:** Binding energy and interaction of gossypetin derivatives with amino acids in *E.coli* DNA gyrase.

| No | Code | MolDock score[a] (kcal/mol) | Σ[b] H-bonds | Amino acids that interact with ligands | |
|---|---|---|---|---|---|
| | | | | Hydrogen bonds | Van der Waals interaction |
| 1 | Goss | −113.43 | 2 | Asn46, Pro79, Val71 | Ala47, Asp73, Asn46, Glu50, Ile78, Pro79, Val43, Val71 |
| 2 | Gm1 | −119.72 | 1 | Val71 | Ala47, Asn46, Asp73, Glu50, Ile78, Val43, Val71 |
| 3 | Gm2 | −117.82 | 1 | Val71 | Ala47, Asn46, Asp73, Glu50, Ile78, Ile90, Pro79, Val43, Val71 |
| 4 | Gm3 | −109.12 | 1 | Asn46 | Ala47, Asn46, Asp73, Glu50, Ile78, Val43, Val71 |
| 5 | 1G1 | −121.20 | 3 | Asn46, Val71 | Ala47, Asn46, Asp73, Ile78, Pro79, Val43, Val71 |
| 6 | 1G2 | −145.93 | 3 | Asn46, Val71 | Ala47, Asn46, Asp73, Ile78, Ile90, Ile134, Pro79, Val43, Val71 |
| 7 | 1G3 | −143.14 | 7 | Asn46, Arg136, Ile78,Thr165, Val71 | Ala47, Asp73, Gly77, Ile78, Ile134, Pro79, Thr165, Val43, Val71 |
| 8 | 1G4 | −141.69 | 3 | Asn46, Thr165, Val71, | Ala47, Ala86, Ala87, Asn46, Asp73, Ile78, Ile90, Ile134, Pro79, Thr165, Val43, Val89, Val71 |
| 9 | 1G5 | −152.72 | 6 | Asn46, Ala86, Ile78, Ile90, Thr165, Val71 | Ala47, Ala86, Ala87, Asn46, Asp73, Gly77, Ile78, Ile90, Ile134, Pro79, Thr165, Val43, Val89,Val71 |
| 10 | 1G6 | −156.77 | 7 | Asn46, Val71, Ala86, Ile78, Ile90, Thr165 | Ala47, Ala86, Ala87, Asn46, Asp73, Gly77, Ile78, Ile90, Ile134, Met91, Pro79, Thr165, Val43, Val89,Val71 |
| 11 | 1G7 | −151.54 | 7 | Asn46, Val71, Ala86, Ile78, Ile90, Thr165 | Ala47, Ala86, Ala87, Asn46, Asp73, Gly77, Ile78, Ile90, Ile134, Met91, Pro79, Thr165, Val43, Val89,Val71 |
| 12 | 2G8 | −120.28 | 1 | Asn46 | Ala47, Asn46, Asp73, Glu50, Ile78, Ile90, Pro79, Val43, Val71 |
| 13 | 2G9 | −146.58 | 2 | Asn46, Glu50 | Ala47, Asp73, Glu50, Ile78, Ile90, Pro79, Val43, Val71 |
| 14 | 2G10 | −140.60 | 5 | Asn46, Arg136, Ile78, Thr165 | Ala47, Ala87, Arg136, Asn46, Asp73, Glu50, Gly77, Ile78, Ile134, Pro79, Thr165, Val43, Val71 |
| 15 | 2G11 | −157.12 | 1 | Asn46 | Ala47, Ala86, Ala87, Asn46, Asp73, Ile78, Ile90, Ile134, Pro79, Val43, Val71, Val89 |
| 16 | 2G12 | −157.16 | 2 | Asn46, Ile90 | Ala47, Ala86, Ala87, Asn46, Asp73, Ile78, Ile90, Ile134, Pro79, Val43, Val71, Val89 |
| 17 | 2G13 | −162.56 | 2 | Asn46, Ile90 | Ala47, Ala86, Ala87, Asn46, Asp73, Ile78, Ile90, Ile134, Pro79, Val43, Val71, Val89 |
| 18 | 2G14 | −162.39 | 2 | Asn46, Ile90 | Ala47, Ala86, Ala87, Asp73, Glu50, Ile78, Ile90, Ile134, Pro79, Val43, Val71, Val89 |
| 19 | 3G15 | −119.14 | 0 | – | Ala47, Asp73, Asn46, Glu50, Ile78, Ile90, Met91, Pro79, Val43, Val71 |
| 20 | 3G16 | −147.37 | 0 | – | Ala47, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71 |
| 21 | 3G17 | −140.22 | 1 | Glu50 | Ala47, Ala86, Ala87, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71 |
| 22 | 3G18 | −155.18 | 0 | – | Ala47, Ala86, Ala87, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71, Val89 |
| 23 | 3G19 | −167.42 | 0 | – | Ala47, Ala86, Ala87, Ala96, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71, Val89 |
| 24 | 3G20 | −160.37 | 1 | Ile90 | Ala47, Ala86, Ala87, Ala96, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71, Val89 |
| 25 | 3G21 | −151.08 | 0 | – | Ala47, Ala86, Ala87, Ala96, Asp73, Asn46, Glu50, Ile78, Ile90, Pro79, Val43, Val71, Val89 |
| 26 | CBN_1[c] | −167.68 | 5 | Asn46, Arg136, Val71 | Asp73, Thr165, Val71 |

[a]MolDock score as representation of binding energy; [b]number of H-bonds; [c]reference ligand (clorobiocin).

OCT2 substrates or inhibitors. The higher the total clearance (CL total) of the compound, the faster the excretion process. It can be seen in Table 3 that the log CLtotal of 3G19 was similar with gossypetin and Gm1 which have higher total clearance than 2G13 and 2G14. Toxicity prediction indicated that the gossypetin derivatives were not mutagenic (no AMES toxicity) and also were not hepatotoxic. The $LD_{50}$ acute toxicity of the compounds were similar [23].

# Discussion

The docking results showed that all gossypetin derivative, except Gm3 (gossypetin 3,7,4′-trimethyl ether), have a higher binding affinity than parent gossypetin, so that they have the potential to be developed as antibacterial agents. The most potent compound 3G19 possessed the lowest interaction energy (low MDS) even though it didn't

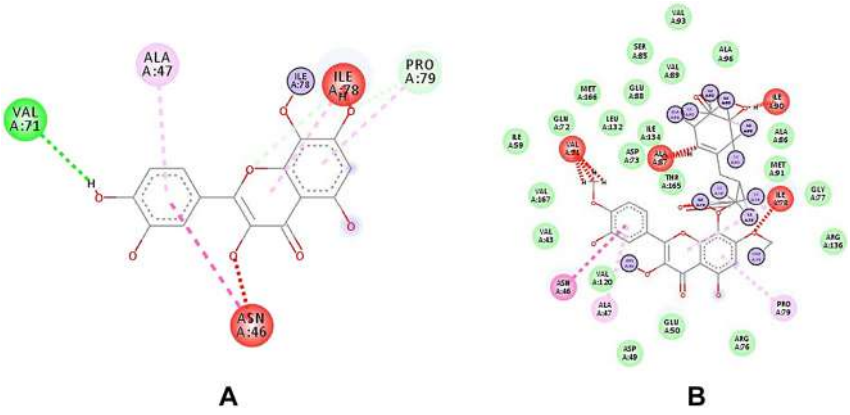



**Figure 2:** Comparison of molecular interactions with DNA gyrase. (A) gossypetin. (B) 3G19. The 3G19 forms covalent interaction (purple) with more amino acids and displays more van der Waals interactions (green) than gossypetin. Both ligands show steric interactions (pink).

**Table 3:** ADMET profile of highly potent gossypetin derivatives compare to unsubstituted-gossypetin.

| ADMET parameter | Gossypetin | Gm1[a] | 3G19[b] | 2G13[c] | 2G14[c] |
|---|---|---|---|---|---|
| **Absorption** | | | | | |
| Intestinal absorption, % | 53.599 | 73.086 | 76.588 | 86.937 | 90.557 |
| Skin permeability (log Kp, cm/h) | −2.735 | −2.735 | −2.735 | −2.735 | −2.735 |
| **Distribution** | | | | | |
| VDss (log, L/kg) | 0.48 | 0.201 | −0.573 | −0.944 | −0.6 |
| BBB permeability (log BB) | −1.756 | −1.303 | −1.588 | −2.021 | −1.765 |
| CNS permeability (log PS) | −3.602 | −3.558 | −3.769 | −3.346 | −3.848 |
| **Metabolism** | | | | | |
| CYP2D6 and CYP3A4 substrate | No | No | No | No | No |
| CYP1A2 inhibitor | Yes | Yes | No | No | No |
| CYP2C19 and CYP2D6 inhibitor | No | No | No | No | No |
| CYP3A4 inhibitor | No | No | Yes | Yes | Yes |
| **Excretion** | | | | | |
| Renal OCT2 substrate | No | No | No | No | No |
| Total clearance (log, mL/min/kg) | 0.522 | 0.455 | 0.431 | −0.013 | 0.226 |
| **Toxicity** | | | | | |
| AMES toxicity | No | No | No | No | No |
| Hepatotoxicity | No | No | No | No | No |
| $LD_{50}$ Oral Rat Acute Toxicity, mol/kg | 2.53 | 2.702 | 2.442 | 2.671 | 2.464 |

[a]Lowest potent derivative. [b]The most potent derivative. [c]The second-highest potent derivatives; BBB, Blood–brain barrier; CNS, Central nervous system; OCT2, organic cation transporter; VDss, Steady state of volume distribution.

form hydrogen bonds (H-bond). Apparently, its high binding affinity was due to the formation of covalent bonds with the amino acids in the binding site (Figure 2B). The MolDock scoring function (MolDock Score) used by MVD is based on a piecewise linear potential (PLP) [22], $E_{MolDock\ Score} = E_{inter} + E_{intra}$, where $E_{inter}$ represents the ligand-protein interaction energy. The lower interaction energy indicated the more stabil interaction. A covalent bond is formed by equal sharing of electron pairs between atoms. The sharing of electrons allows each atom to achieve a stable electronic configuration and attain a lower potential energy when atoms are bonded than when they are separated [26]. Base on this theory, the formation of covalent bond between some functional groups in the compound 3G19 with amino acids in protein target could decrease the interaction energy which was showed as low MDS. Low docking score means high affinity against the corresponding protein. This study did not intentionally design a covalent inhibitor because the design of selective covalent inhibitors is conceptually very attractive but in practice hard to achieve. There were many desirable features of a covalent inhibitor, including increased biochemical

efficiency of target disruption, lower sensitivity toward pharmacokinetic parameters and increased duration of action of the compound [27].

Based on analysis of the structure and *in silico* activity (Table 2), the hydroxyl group at 4′-position was important for binding affinity of gossypetin. Methylation of hydroxyl group at this position removed the hydrogen bond with Val71 and decreased binding affinity. H-bond was still retained in the 3-methyl and 3,7-dimethyl gossypetin. The methylation of hydroxyl group at position 3 increased *in silico* activity of gossypetin, but the subsequent methylation resulted in 3,7,4′-trimethyl gossypetin derivatives decreased the activity. However, additional substitution of 8-hydroxyl by acyl of aromatic acids could increase the binding interaction of methylgossypetin derivatives (1G, 2G, and 3G series) but simple acetyl group could not increase the binding affinity. This result supports our prediction that phenolic acyl substitution on 8-hydroxyl could increase *in silico* interaction with targeted protein.

Prenylation also reduced the binding energy, even its capability was higher than benzoyl from aromatic acid. Apparently, the double bond in the prenyl group connected with the conjugated electrons in the flavonoid ring, thereby lowering the energy. It had been reported that prenylated flavonoids possess antimicrobial activity. The addition of prenyl groups to the flavonoid backbone usually assumed to facilitate attachment to cell membranes. Prenylation may increase the potential bioactivity of its original flavonoid [28]. The influence of the vinylic double bond on increasing in silico activity was shown by the lower docking score of 2G11 and 3G18 which contained cinnamoyl group, compare with 2G10 and 3G17 which contained benzoyl group without aliphatic double bonds. These results were similar with some cinnamic acid derivatives which have higher antimicrobial activity than the benzoic acid derivatives [29].

The phenolic group contributed to reduce binding energy as well; methyl gossypetins containing phenolic acyl (coumaroyl, caffeoyl, feruloyl) showed lower scores than methyl gossypetins containing benzoyl and cinnamoyl. The additional phenolic group in caffeoyl was also lowering binding energy of 1G6 and 2G13 compare with 1G5, 1G7, 2G12, 3G14 which contained coumaryl and feruloyl. This was seen in 3-methyl, 3,7-dimethyl and 3,7,4′-trimethyl derivatives with the exception of gossypetin 3,7,4′-trimethyl ether 8-coumarate (3G19). These results were consistent with study which reported that some coumaric esters had higher antibacterial activity than esters of cinnamic, caffeic, and ferulic acid against *E. coli* [9]. It was suggested that aromatic hydroxyl group and vinylic double bond, in coumaric and other phenolic acyl, contributed to electronic stabilization in flavonoid ring which have bound methyl groups. It has been mentioned in the early of discussion that high affinity of 3G19 was supported by the formation of covalent bond with amino acids in DNA gyrase. The atoms in the hydroxyphenyl and vinyl groups were involved in covalent bonds with amino acids, and the presence of these groups augmented the covalent bonds of the flavonoid backbone with amino acids. Based on docking study, it can be concluded that the ligand affinity was not only determined by the formation of hydrogen bonds but also supported by the electronic stabilization of the phenolic acyl.

The docking result recommended compound 3G19 as the most the most potent compound followed by 2G13 and 2G14 as the second-best docked compound. ADMET profile of 3G19 showed that the compound had a good profile as a drug candidate. It had a good intestinal absorption so it could be used as orally administered agent. All compounds had a good skin permeability which indicated that they would be advantageous to be developed as topical antibacterial drugs. That the compounds did not crossed BBB and also unable to penetrate CNS can be considered beneficial in terms of reducing side effects and toxicities because the BBB secures the brain from exogenous compounds. Other supporting properties are that the compound is predicted to be immediately excreted, is not expected to be nontoxic to the liver and is not mutagenic. Based on all the results, it can be concluded that 3G19, 2G13, and 3G14 that have high in silico activity against DNA gyrase also have pharmacokinetic advantages and is relatively nontoxic.

## Conclusions

This research has successfully designed the most potent the gossypetin 3,7,4′-trimethyl ether 8-coumarate (3G19) as antibacterial agent which targeted *E. coli* DNA gyrase. Generally, all designed phenolic acyl of methyl gossypetins showed high binding affinity to the gyrase and also acted as potential antibacterial candidate. Binding site analysis concluded that 3G19 and the other methylgossypetin ester of phenolic acid which presented novel covalent interaction with the amino acid provided an opportunity for the development of gossypetin derivatives as new gyrase inhibitors. The designed gossypetin derivatives were proper to be synthesized and then evaluated for their *in vitro* antibacterial activity.

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**Ethical approval:** Not applicable.

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Muhammad Amirul Asyraf Noh, Siti Sarah Fazalul Rahiman, Habibah A Wahab* and Amirah Mohd Gazzali*

# Discovery of new targeting agents against GAPDH receptor for antituberculosis drug delivery

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**Abstract**

**Objectives:** Tuberculosis (TB) remains a public health concern due to the emergence and evolution of multidrug-resistant strains. To overcome this issue, reinforcing the effectiveness of first line antituberculosis agents using targeted drug delivery approach is an option. Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH), a common virulence factor found in the pathogenic microorganisms has recently been discovered on the cell-surface of *Mycobacterium tuberculosis*, allowing it to be used as a drug target for TB. This study aims to discover active small molecule(s) that target GAPDH and eventually enhance the delivery of antituberculosis drugs.

**Methods:** Ten ligands with reported *in vitro* and/or *in vivo* activities against GAPDH were evaluated for their binding interactions through molecular docking studies using AutoDock 4.2 program. The ligand with the best binding energy was then modified to produce 10 derivatives, which were redocked against GAPDH using previous protocols. BIOVIA Discovery Studio Visualizer 2019 was used to explore the ligand-receptor interactions between the derivatives and GAPDH.

**Results:** Among the 10 ligands, curcumin, koningic acid and folic acid showed the best binding energies. Further analysis on the docking of two folic acid derivatives, F7 (γ-{[tert-butyl-N-(6-aminohexyl)]carbamate}folic acid) and F8 (folic acid N-hydroxysuccinimide ester) showed that the addition of a bulky substituent at the carboxyl group of the glutamic acid subcomponent resulted in improved binding energy.

**Conclusions:** Folic acid and the two derivatives F7 and F8 have huge potentials to be developed as targeting agents against the GAPDH receptor. Further study is currently on-going to evaluate the effectiveness of these molecules *in vitro*.

**Keywords:** antituberculosis; Autodock; drug delivery; folic acid; GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

## Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (M. TB) that mainly affects the lungs. It is spread through air droplets expelled by people inflicted with TB. Despite the discovery of BCG (Bacillus Calmette–Guérin) vaccine 100 years ago, TB remains as one of the leading causes of death in the world [1]. In 2018, 1.2 million people worldwide died from tuberculosis [1].

The treatment approach of TB could be divided into first- and second-line treatment options. First line treatment includes drugs such as isoniazid, rifampicin, ethambutol and pyrazinamide [2]. Albeit old, these drugs are still effective to kill M. TB. However, in the recent years, the increasing number of MDR-TB (Multiple Drug Resistance-Tuberculosis) and XDR-TB (Extensively Drug Resistance-Tuberculosis) cases has rendered these drugs to be ineffective in advanced cases. As an alternative, second-line agents such as kanamycin, cycloserine and ethionamide are used despite their relatively high cost, limited availability and significant side effect profiles [2]. It is therefore essential to prevent or minimize the occurrence of resistance cases and concurrently expand the currently available first-line treatments.

With the advancement in technology, targeted drug delivery is an attractive approach as compared to the conventional drug delivery system in order to improve drug therapy by increasing the drug concentration, prolonging and localizing the drug effects at the target sites and concurrently decrease drug uptake at the nontarget sites [3]. This in turn, will improve the efficacy of the drug while

***Corresponding authors: Amirah Mohd Gazzali and Habibah A Wahab**, Discipline of Pharmaceutical Technology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, 11800, Penang, Malaysia, Phone: +604 6533888,
E-mail: amirahmg@usm.my (A.M. Gazzali), habibahw@usm.my (H.A. Wahab)
**Muhammad Amirul Asyraf Noh,** Discipline of Pharmaceutical Technology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, Penang, Malaysia. https://orcid.org/0000-0003-1525-4233
**Siti Sarah Fazalul Rahiman,** Discipline of Physiology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, Penang, Malaysia

reducing the associated side effects [4]. There are two types of targeted drug delivery: active and passive drug targeting. Active targeting utilizes specific ligands conjugated to the drug, which will bind to the appropriate receptors expressed by the cells at the targeted site. Passive targeting on the other hand includes targeting by nanoparticles, which exploits the environment of the targeted cells such as leakier environment at the cell junctions [5].

To date, there are limited work reported on M. TB cell-targeting in the literature. Majority of studies on targeted drug delivery towards TB focuses on passive targeting using nanoparticles [6]. However, a recent paper by Malhotra et al. [7] described a cell surface receptor called Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) discovered on the surface of M. TB. GADPH, a common virulence factor found in pathogenic microorganisms, was reported to interact and internalize lactoferrin and transferrin into the cytoplasm in order to increase the intake of iron into M. TB [8]. This highlights the potential of GAPDH in the internalization of anti-TB drug conjugates into the M. TB cells and thus, this receptor could be a useful target for active drug targeting.

This research aims to discover the active small molecule (s) that target GAPDH and eventually enhance the delivery of antituberculosis drugs directly against M. TB using molecular docking studies.

## Materials and methods

Autodock 4.2 was employed to predict the resulting conformations of the ligand after docking with the receptor [9]. The receptor was treated as a rigid structure during the docking, but the ligands were allowed to rotate freely.

GAPDH macromolecule used as the receptor throughout this study was obtained from the Protein Databank (PDB id: 6IEP). By using AutoDockTools (ADT), a graphical user interface available in the Autodock 4.2 software, the GAPDH receptor was loaded in PDB format and any unrelated ions, waters and ligands were removed. Subsequently, polar hydrogens and Kollman charges were added to the receptor, before saving the file in PDBQT format.

Selection of the ligands was conducted in the following way. Relevant studies of GAPDH inhibitors and common targeting agents were explored in the available literature by using specific keywords; GAPDH inhibitors or ligands and targeting agents. Based on the findings, suitable molecule(s) were selected as ligands for this study based on their *in vitro* and/or *in vivo* activities against GAPDH and the molecules' relative molecular weight. Docking of small ligands (less than 900 Da) using AutoDock® give a more reliable results as compared to the large ligands [10]. Their chemical structures were obtained from the chemical databases such as PubChem, ChemSpider or constructed from scratch if the ligands were not available in these databases using PerkinElmer ChemDraw 17.0. After the chemical structure of the ligands was obtained, energy minimization was carried out on the 3D structure of the ligands using PerkinElmer Chem3D 17.0. The minimized 3D structures were then saved in PDB format in Chem3D. Next, the ligands were prepared in ADT by adding Gasteiger charges, specifying suitable torsion centers as well as the number of rotations. Later, these ligands were saved in PDBQT format in the ADT.

Following that, a grid box of dimensions $60 \times 60 \times 60$ units along $x$, $y$ and $z$ axis was set to cover the active site of the receptor, which was determined by the location of the cocrystalized NAD ligand. NAD is a native ligand of GAPDH that plays an important role in the latter's glycolytic function [11]. The docking was then executed using Lamarckian Genetic Algorithm as follows: 150 search runs, population size of 150 and 2,500,000 maximum number of energy evaluations. After completion of the docking for each ligand, the resultant conformations were ranked according to their lowest binding energy and clustered together if their RSMD values were within 0.5 Å from one another. The lowest binding energy, inhibition constant and number of hydrogen bonds for each ligand were tabulated. The ligands with the lowest binding energy were then chosen to be visualized using Biovia Discovery Studio 2019 to identify their interactions with the amino acid residues of the receptor.

After the initial docking studies were completed, the ligand with the lowest binding energy was modified to produce derivatives. Different strategies including the variation of substituents and extension of structures were employed to maximize the interactions of the ligands with their binding sites in order to improve their binding affinity. These derivatives were redocked against GAPDH using previously described protocols and the lowest binding energy, inhibition constant and numbers of hydrogen bonds for all ligands involved were reported.

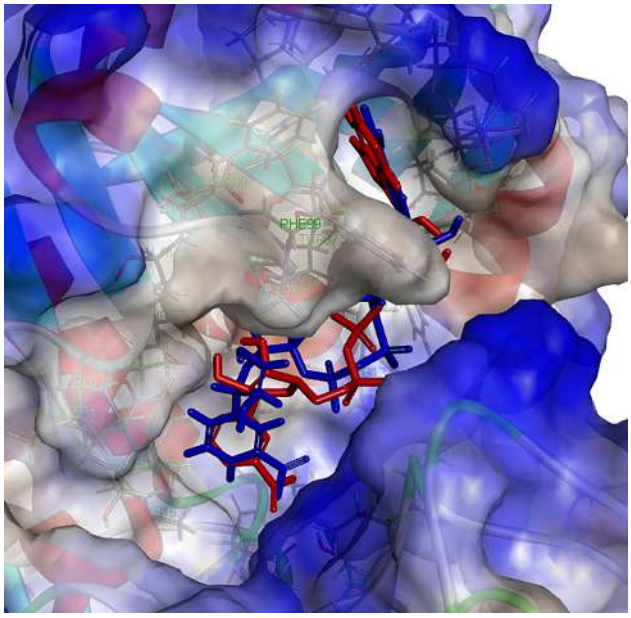

**Figure 1:** Superimposition of the crystallographic (blue) and docked (red) poses of NAD into the NAD binding domain of GAPDH.

# Results

## Preparation of receptor molecule

To ensure the docking protocol was valid, the cocrystalized NAD ligand was extracted from the receptor and redocked within the NAD binding site of GAPDH. The resulting docked conformation with lowest binding energy was then compared to the cocrystalized ligand structure. Using visual inspection, the docked NAD ligand was found to bind at the same binding site as the NAD ligand in crystal structure (Figure 1) with RMSD value of the lowest energy conformation of the redocked NAD ligand being 1.7 Å.

According to a paper by Wang et al. [12], a scoring function used to predict binding affinity between a ligand and a receptor is accurate if RMSD value of the predicted lowest energy conformation of the ligand is less than or equal to 2.0 Å from the experimental crystal structure. Since the RMSD value of the redocked ligand was less than 2.0 Å, the docking protocol used in this study was considered valid, and hence, reliable for docking various other ligands into the binding site of GAPDH.

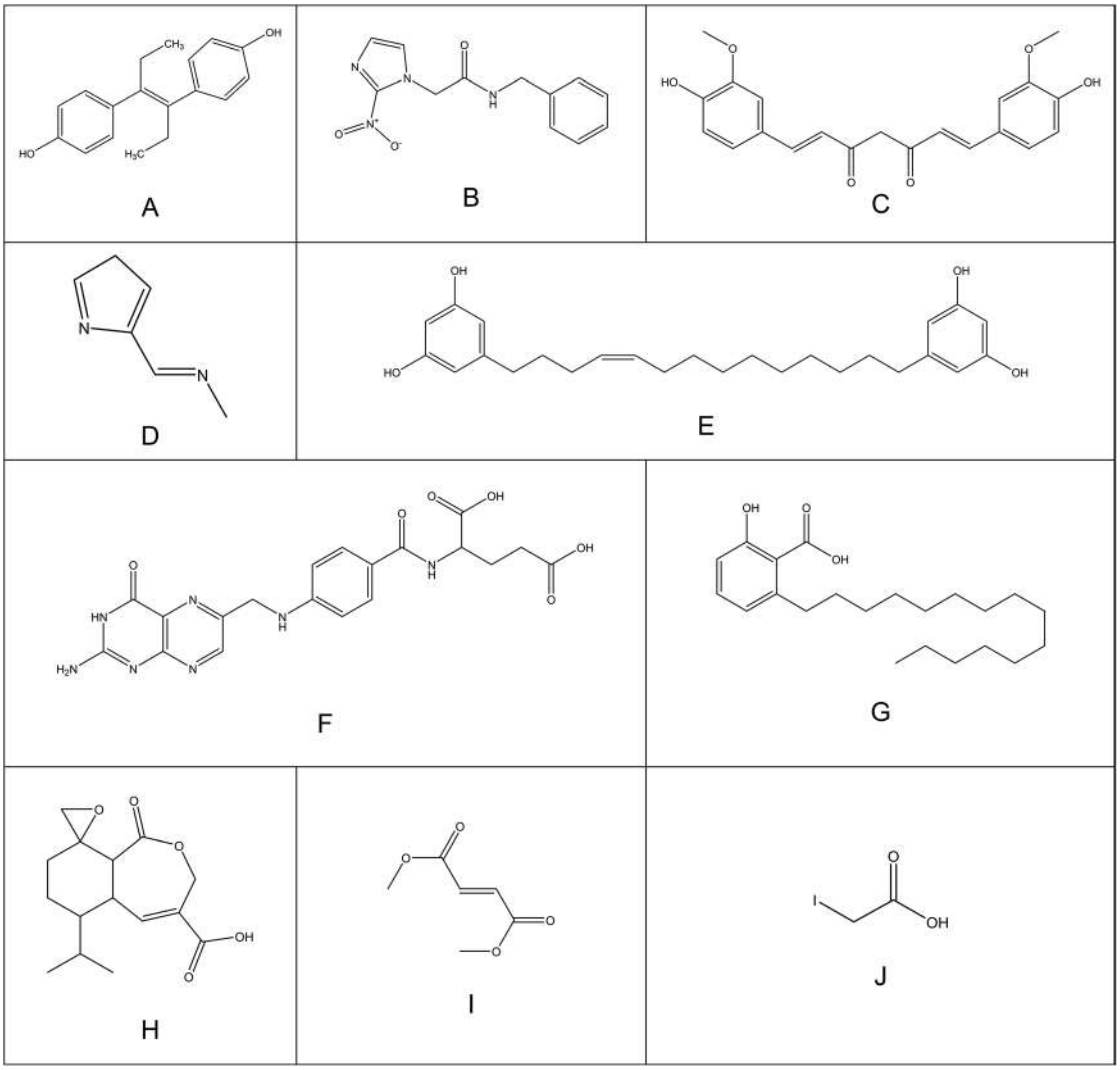


**Figure 2:** Chemical structures of selected GAPDH inhibitors from literature. (A) diethylstilbestrol, (B) benznidazole, (C) curcumin, (D) (*E*)-*N*-methyl-1-(3*H*-pyrrol-5-yl)methanimine, (E) (*Z*)-5,5′-(tetradec-4-ene-1,14-diyl)bis(benzene-1,3-diol), (F) folic acid, (G) anacardic acid, (H) koningic acid, (I) dimethyl fumarate, (J) 2-iodoacetic acid.

**Table 1:** Results obtained after docking of selected ligands (A–J) with GAPDH.

| Compound | Binding free energy, kcal/mol | Docking predicted inhibition constant, μM | Number of hydrogen bond interactions |
|---|---|---|---|
| A | −6.24 | 26.81 | 1 |
| B | −6.26 | 25.88 | 7 |
| C | −7.85 | 1.76 | 6 |
| D | −4.22 | 803.71 | 2 |
| E | −6.86 | 9.37 | 4 |
| F | −7.68 | 2.34 | 10 |
| G | −5.47 | 97.02 | 2 |
| H | −7.62 | 2.62 | 5 |
| I | −4.15 | 908.89 | 3 |
| J | −3.62 | 2,220 | 3 |

## Interaction of selected GAPDH inhibitors with GAPDH

The 10 ligands chosen for the docking studies were illustrated in Figure 2. The binding free energy, predicted Ki and number of hydrogen bonds from each docking were reported in Table 1.

The results showed that C (curcumin), F (folic acid) and H (koningic acid) have the lowest binding energies amongst the selected ligands. Folic acid was then selected for further investigation. As shown in Figure 3, folic acid interacted with Asn-8, Gly-9, Gly-11, Arg-12, Gly-14, Asn-33, Asp-34, Arg-78, Ala-96 and Thr-97 through hydrogen bonding. Besides that, it also formed several other interactions including Leu-35 through *pi*-sigma and *pi*-alkyl interactions, Phe-99 through *pi-pi* stacked interaction, and Phe-10, Ile-13, Glu-77, Asp-79, Pro-80, Ile-83, Gly-98, Phe-100, His-109, Thr-121 and Ala-122 through van der Waals interactions. Despite these interactions, folic acid also had unfavorable hydrogen donor-donor and hydrogen acceptor-acceptor interactions with Asn-33.

## Interaction of folic acid derivatives with GAPDH

To further investigate the feasibility of folic acid as a targeting agent for GAPDH, folic acid was then modified to produce 13 derivatives shown in Figure 4. These derivatives were docked to GAPDH using previous protocols to confirm whether the modifications made were beneficial to the compound's binding affinity.

From the docking results presented in Table 2, it was evident that F7 (folic acid N-hydroxysuccinimide ester) was the most promising folic acid derivative out of the 13 derivatives tested as it has the lowest binding energy among them. F7 interacted with Asn-8, Gly-9, Gly-11, Arg-12, Asp-34, Glu-77, Arg-78, Thr-182 and Gly-183 through hydrogen bonding, Leu-35 and Thr-97 through pi-sigma and pi-alkyl interactions, Phe-99 through pi-pi stacked interaction, and Phe-10, Ile-13, Gly-14, Asn-33, Asp-79, Pro-80, Ile-83, Ala-96, Phe-100, His-109, Asp-184 and Glu-317 through van der Waals interactions (Figure 5).

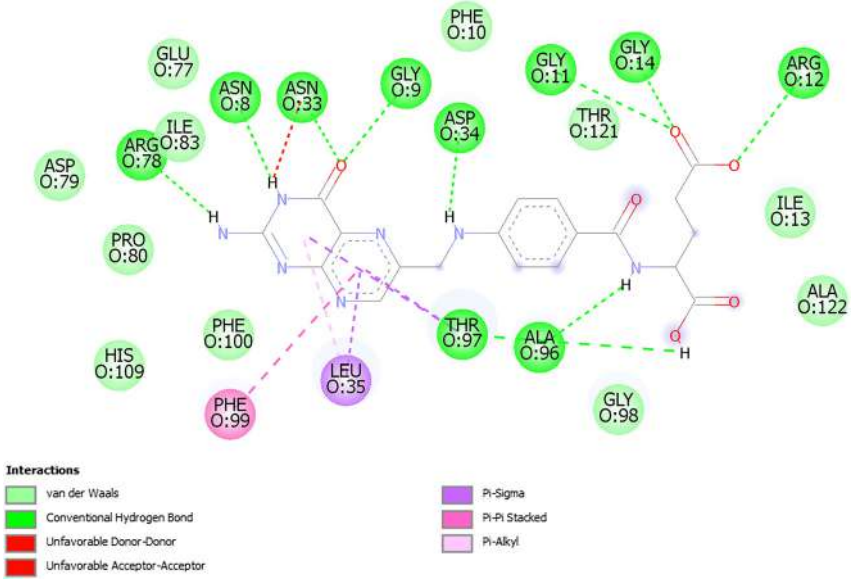


**Figure 3:** 2D diagram of ligand-protein complex of F (folic acid).

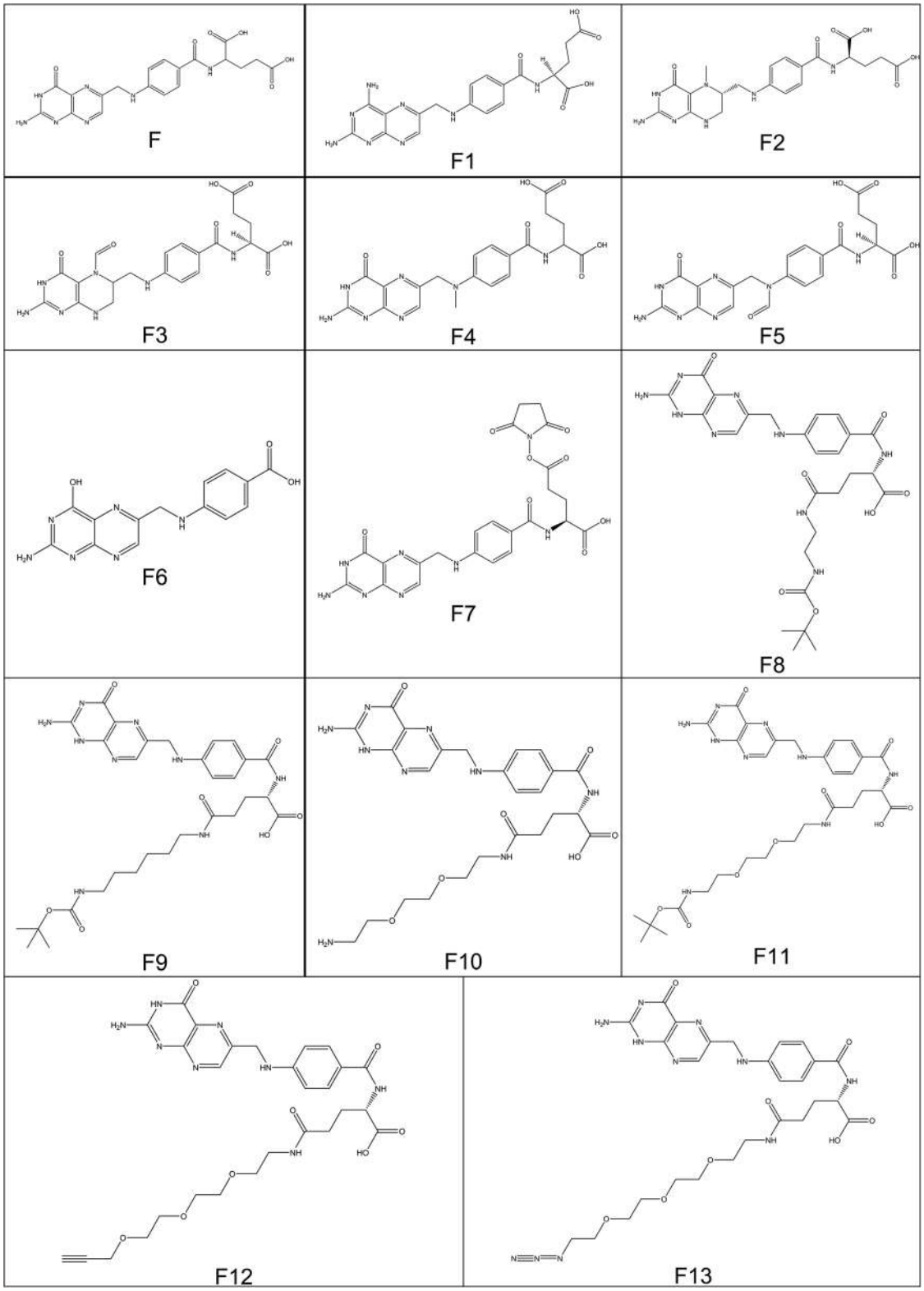


**Figure 4:** Chemical structures of compound F and its derivatives.

**Table 2:** Results obtained after docking of folic acid derivatives with GAPDH.

| Compound | Binding free energy, kcal/mol | Docking predicted inhibition constant, μM | Number of hydrogen bond interactions |
|---|---|---|---|
| F | −7.68 | 2.34 | 10 |
| F1 | −7.43 | 3.56 | 10 |
| F2 | −5.98 | 41.04 | 11 |
| F3 | −6.90 | 8.79 | 8 |
| F4 | −7.73 | 2.14 | 7 |
| F5 | −8.08 | 1.19 | 10 |
| F6 | −8.09 | 1.18 | 10 |
| F7 | −9.22 | 0.173 | 10 |
| F8 | −8.92 | 0.287 | 6 |
| F9 | −6.82 | 10.07 | 7 |
| F10 | −6.50 | 17.29 | 7 |
| F11 | −6.48 | 17.89 | 7 |
| F12 | −5.94 | 44.60 | 9 |

## Discussion

Curcumin showed the highest activity with the estimated $\Delta G_{bind}$ of −7.85 kcal/mol followed by folic acid and koningic acid (−7.68 and −7.62 kcal/mol, respectively). Koningic acid and curcumin has been reported as good GAPDH inhibitors in other studies [13, 14] therefore, these results were expected. Although folic acid has been found as a popular targeting agent for anticancer drugs [15] its binding profile on GAPDH was never reported elsewhere. To our knowledge, this study was the first to investigate the interaction between folic acid with GAPDH. The findings sought in this study would be used to further develop folic acid as a potential targeting agent for GAPDH to treat TB.

To investigate the structure activity relationship of folic acid with GAPDH, 13 derivatives of folic acid were produced using drug optimization strategies. In general, changing or adding substituents to pteridine ring component of folic acid caused the binding affinity to decrease, as in the case of F1, F2 and F3. For F1, changing the double-bonded oxygen atom of the pteridine ring to an amino group caused a small decrease in the binding affinity. This can be attributed to the nitrogen of the amino group being less electronegative than oxygen, resulting in a weaker hydrogen bonding with Gly-9. For F2 and F3, the pyrazine from the pteridine ring was changed into a piperazine. With the loss of aromaticity, no *pi-pi* interaction was observed between this moiety with Leu-35 and Phe-99. This interaction is important in the binding of folic acid with GAPDH, as illustrated in Figure 3. The absence of this interaction has led to a decreased in their binding affinity. The addition of a carbonyl group to the piperazine as in F3 was done with the aim to promote the formation of hydrogen bonds, but this has not offset the previous loss of binding affinity. This observation has demonstrated the importance of this pteridine ring to the folic acid binding mechanism with

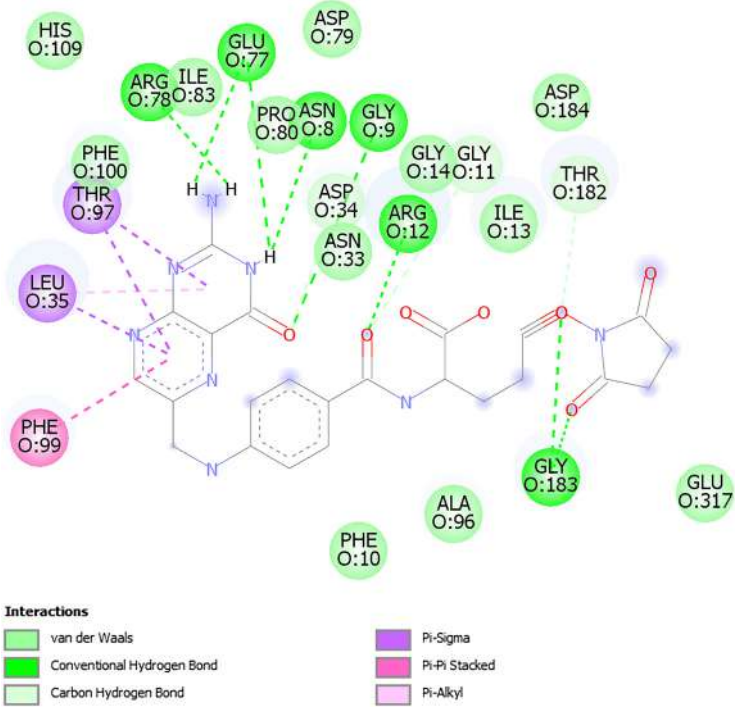


**Figure 5:** 2D diagram of ligand-protein complex of F7.

GAPDH. The addition of a methyl group in F4 and a carbonyl group in F5 to the *para*-aminobenzoic acid moiety in folic acid molecule has resulted in a slight reduction in the $\Delta G_{bind}$ (between 0.05 and 0.40 kcal/mol), which translated into a small increase in binding affinity. The addition of both substituents mentioned above resulted in a marginally better shape complementarity.

The removal of the glutamic acid component from folic acid as in F6 did not result in a decrease in binding affinity but instead the opposite. The hydrogen bonds formed between Arg-12, Gly-14 and Ala-96 with the two carboxylic groups of the glutamic acid moiety were preserved through the formation of hydrogen bonds with the *para*-aminobenzoic acid moiety instead. The small increase in the binding affinity may be due to the reduced steric hindrance, which improved the binding of F6 to GAPDH. It is interesting to note that adding a bulky group such as tert-butyl or N-hydroxysuccinimide at the carboxyl group of glutamic acid has significantly contributed to F7 and F8 performances as the best binding derivatives among the 13 derivatives tested. F7 (folic acid N-hydroxysuccinimide ester) showed the highest activity with the estimated $\Delta G_{bind}$ of −9.22 kcal/mol followed by F8 (γ-{[tert-butyl-N-(6-aminohexyl)]carbamate} folic acid) with estimated $\Delta G_{bind}$ of −8.92 kcal/mol. Figure 5 showed that the addition of N-hydroxysuccinimide has allowed F7 to fill a binding cavity that folic acid could not. This process results in additional van der Waal interactions with Asp-184 and Glu-317 and hydrogen bonds with Thr-182 and Gly-183 that eventually led to an increased binding affinity. However, extension of the glutamic acid moiety produces decreased binding affinity, as evidenced by F9, F10, F11, F12 and F13. The increase in $\Delta G_{bind}$ between 0.7 and 1.74 kcal/mol can be interpreted as a probable limit to the size of molecules allowed to enter the binding cavity mentioned above, and the addition of groups longer than N-hydroxysuccimide may not fit the cavity.

## Conclusions

Folic acid and its two derivatives F7 and F8, have huge potentials to be further developed as targeting agents against the GAPDH receptor. Further study is currently on-going to evaluate the effectiveness of these molecules through ELISA study and subsequently on the M. TB itself.

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**Author contributions:** AMG designed the research project and supervised the progress. MAAN conducted the experiments and wrote the main manuscript text. HAW and SSFR supervised the study progress. All authors have read and have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Not applicable.

**Ethical approval:** Not applicable.

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Iif Hanifa Nurrosyidah, Ni Made Mertaniasih and Isnaeni*

# The effect of red passion fruit (*Passiflora edulis* Sims.) fermentation time on its activity against Extended Strain Methicillin-Resistant (ESBL) *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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**Abstract**

**Objectives:** The purpose of this study is to determine the effect of fermentation techniques on the inhibitory activity of red passion fruit (*Passiflora edulis* Sims.) fermentation filtrate in De Man Rogosa Sharpe-broth (MRS-B) media against Extended Strain Methicillin-Resistant (ESBL) *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* (MRSA).

**Methods:** The fruit pulp was wrapped in banana leaves before compared to direct fermentation processes. This study was divided into three treatment groups. Group 1 was the fruit pulp (5 g) fermented in 45 mL of MRS-B medium for 24 h. Group 2 was the fruit pulp wrapped in banana leaves for 3 days before fermented in MRS-B for 24 h. Group 3 was the fruit pulp wrapped in banana leaves for 3 days before fermentation in MRS-B for 48 h. Fermentation broth of each condition was taken and then filtered using millipore (0.2 μm). As many as 50 μL of filtrates was tested for its inhibitory activity against *E. coli* ESBL and MRSA using the Kirby Bauer method.

**Results:** Group 2 showed the best antibacterial activity against *E. coli* ESBL and MRSA with the average zone of inhibition of 38.3 and 37.6 mm respectively. These values were higher than the first and group 3s activities.

**Conclusions:** The inhibitory activity of group 1s against ESBL and MRSA is categorized as a moderate potency with a diameter of growth inhibition zone of 16–20 mm, whereas the other groups are categorized as strong potency with a diameter higher than 20 mm.

**Keywords:** ESBL; fermentation; inhibitory activity; MRSA; red passion fruit.

## Introduction

*Passiflora edulis* Sims (Passion Fruit) of the Passifloraceae family, has 500 distributed species in areas with warm temperatures and tropical regions. This plant comes from Brazil and has spread to other countries (Asia, Australia, Africa, India, South America, and the Caribbean). Passion fruit has other variants that can be identified from the color of the fruits, such as yellow (*P. edulis* var. *Flavicarpa*), purple (*P. edulis* var. *edulis*), and orange (*P. edulis* var. *Caerulea*). Passion fruit is a good source of ascorbic acid (vitamin C) and carotenoids (vitamin A) [1].

Infectious diseases are still a major health problem in developing tropical countries like Indonesia. Deaths caused by infectious diseases are around 51% [1]. Irrational use of antibiotics worsens this condition. Many bacteria are resistant to some antibiotics, such as Extended Strain Methicillin-Resistant (ESBL) *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) [2].

Passion fruit contains glycoside-flavonoids [3], such as luteolin-6-C-chinovoside, luteolin-6-C-fucoside, cyanogenic glycosides passibiflorine, epipassibiflorin, passicapsin, passicoriacin, epipassicoriacin, epitetraphilin β, amygdalin, prunacin, triterpenoid glycosides, and salicylic glycosides. Other chemical compounds such as the β-carboline alkaloids harman, harmine, harmaline and harmalol, phenols, carotene, and g-lactones are also found in passion fruit. Passion fruit is a fruit that has high nutritional value, many multimineral contents such as magnesium and phosphorus,

***Corresponding author: Dr. Isnaeni**, MS, Apt., Department of Pharmaceutical Chemistry, Universitas Airlangga, Mulyorejo, Surabaya 60115, Indonesia, Phone: +31 8955989, E-mail: isnaeni@ff.unair.ac.id
**Iif Hanifa Nurrosyidah,** Department of Pharmaceutical Chemistry, Universitas Airlangga, Surabaya, Indonesia; and Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Ni Made Mertaniasih,** Department of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

various vitamins, as well as high carbohydrates, and water [4]. Passion fruit is a suitable habitat for the growth of probiotic bacteria because of its adequate nutritional content. Based on previous research, purple passion fruit (*P. edulis* Sims. var. *edulis*) contains LAB (*Lactobacillus bulgaricus* dan *Lactobacillus heterohiochii*) [5].

The cell-free fermentation supernatant from yellow passion fruit (*P. edulis forma flavicarpa* Sims.) fermented in De Man Rogosa Sharpe-Broth (MRS-B) media has been reported can inhibit the growth of *Staphylococcus* spp., MRSA, and *Escherichia coli* Extended Strain Beta-Lactamase (ESBL) [6]. Therefore, this study investigates the effect of fermentation techniques on the inhibitory activity of red passion fruit (*P. edulis* Sims.) fermentation filtrate in MRS-B media against *ESBL E. coli* and MRSA. The fruit pulp was wrapped in banana leaves before compared to direct fermentation processes.

# Materials and methods

### Plant source and determination

The red passion fruits were collected freshly from a local farm in Krembung (Sidoarjo, East Java, Indonesia), harvested in August 2020. They were identified and determined based on the taxonomy character of leaf, flower, fruit, and steam plant and identified on Herbarium Malangensis, Univeristas Negeri Malang (East Java, Indonesia) as *P. edulis* Sims, with the identification number of 'Nomor: 09/25.07.18/herb.malg' (see Figure 1).

### Preparation of fermentation media

The MRS-broth was prepared by dissolving 52.2 g of the MRS-broth powder into 1 L of purified water. Sterilization was performed by autoclaving for 15 min at 121 °C. Nutrient agar (NA) media was prepared by dissolving 20.0 g of the NA powder into 1 L of purified water. Heating in boiling water with stirring constantly was done to obtain all of the powder to completely dissolve and the consistent yellowish liquid was achieved. Ten milliliters of the mixture was then filled into test tubes using a syringe while it was still warm and in liquid form. The test tubes then need to be plugged with cotton and autoclaved for 15 min at 121 °C. Some banana leaves that have been cleaned and sterilized in the autoclave for 15 min at 121 °C were prepared. This NA medium was used to rejuvenate the tested bacterial isolates (ESBL and MRSA) and antibacterial activity test.

**Figure 1:** The red passion fruits (*P. edulis* Sims.).

### Sample preparation, fermentation, and characterization

The passion fruits were washed and dried before they were divided into two parts and the 5 g of passion fruit pulps was weighed and put into 45 mL of MRS-broth media to be fermented with a rotary shaker at 150 rpm, 37 °C for 12 h (group 3/MMB 24). The passion fruits were washed and dried before they were divided into two parts and the 5 g of fruit pulps was weighed and put into banana leaves (the banana leaves were not autoclaved, only cleaned and disinfected with 70% alcohol), and then wrapped in a container in the form of an impermeable jar air and light. The decay was carried out for 3 days at room temperature. After that, group 2 and group 3 were wrapped in banana leaves for 3 days, then the red passion fruit pulp was fermented with MRS-broth media for 24 h (group 2) and 48 h (group 3). The fermentation broth was centrifuged and The supernatant was filtered using a 0.2 µm millipore sieve to remove bacteria [7] (see Figures 2 and 3).

### Inoculum preparation

The selected bacteria strain was transferred aseptically to sterile saline water, vortex and then the turbidity was measured using spectrophotometer against the sterile saline water to obtain 25% Transmittance (about $10^9$ CFU/mL of bacteria) turbidity or optical density at 580 nm [6].

### Antibacterial activity test

The Sterilized NA media were poured into a sterile Petri disk and waited to solidify. Then, the inoculum was swabbed onto the NA

**Figure 2:** The fermentation of red passion fruit pulp wrapped in banana leaves for 3 days (for group 2 and group 3).

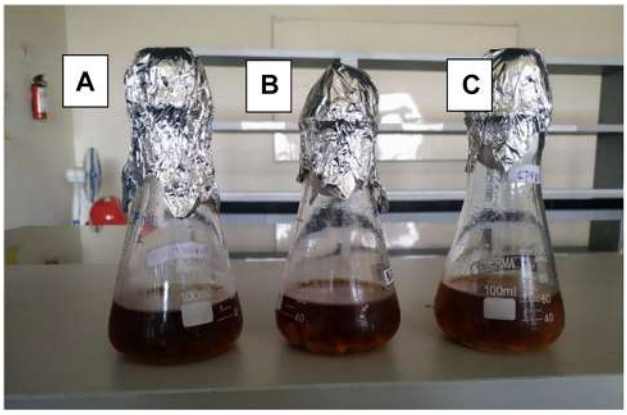


**Figure 3:** (A) Fermented filtrate of red passion fruit pulp with MRS-broth for 24 h (group 1), (B) fermented filtrate of red passion fruit pulp wrapped in banana leaves for 3 days then fermented with MRS-broth for 24 h (group 2), (C) fermented red passion fruit pulp wrapped in banana leaves for 3 days then fermented with MRS-broth for 48 h (group 3).

surface slowly using a sterile swab. The paper disk in the test solution was soaked each as much as 30 µL then the paper disc was placed slowly over the NA surface, and the vancomycin antibiotic paper disc was put slowly as a positive control (comparison standard). There were three times of replication in this study. Then, it was incubated for 24 h at 37 °C. The resulting inhibition zone diameter was observed [8].

## Results

The inhibition zone of *E. coli* ESBL obtained can be seen in Table 1. And Table 2 for the inhibition zone of MRSA. The inhibition zone of *E. coli* ESBL of the red passion fruit pulps was fermented with MRS-broth media (group 3) classified as moderate (16 mm). On the other hand, the treated group was wrapped in banana leaves for 72 h at room temperature before fermented with MRS-broth, group 2 and group 3 had the inhibition zone against *E. coli* ESBL classified as strong (more than 20 mm). The inhibition zone against MRSA of group 3 was classified as weak (13 mm). On the other hand, group 2 and group 3 had the inhibition zone classified as strong (more than 20 mm) (see Figures 4 and 5).

## Discussion

Extended-spectrum beta-lactamase (ESBL) is an enzyme that has the ability in hydrolyzing the antibiotics of the penicillin class, cephalosporin generation one, two, and three, and the monobactam class and cause resistance to all antibiotics. ESBL does not *hydrolyze cephamycin*, which has its own family close to cephalosporin, but it is inhibited by beta-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam. ESBL is generally inactive against carbapenem (imipenem, meropenem, and ertapenem) [9].

The cause of widespread nosocomial infection is MRSA. MRSA is a strain of *S. aures* that is resistant to β-lactam antimicrobials, among them are from the penicillin group. The mechanism of MRSA resistance occurs due to *S. aureus* that produces the gene-encoded Penicillin Binding Proteins

**Table 1:** Inhibition zone diameter for each treatment group against *E. coli* extended-spectrum beta-lactamase (ESBL).

| Treatment group | Inhibition zone diameter, mm | | | | Inhibition classification |
|---|---|---|---|---|---|
| | Replication 1 | Replication 2 | Replication 3 | Average | |
| Group 1 | 17 | 18 | 13 | 16 | Moderate |
| Group 2 | 38 | 39 | 37 | 38 | Strong |
| Group 3 | 33 | 33 | 33 | 33 | Strong |

ESBL, extended spectrum beta-lactamase.

**Table 2:** Inhibition zone diameter for each treatment group against Methicillin-Resistant *S. aureus* (MRSA).

| Treatment group | Inhibition zone diameter, mm | | | | Inhibition classification |
|---|---|---|---|---|---|
| | Replication 1 | Replication 2 | Replication 3 | Average | |
| Group 1 | 9.5 | 13.5 | 16 | 13 | Weak |
| Group 2 | 39 | 37 | 38 | 38 | Strong |
| Group 3 | 39 | 37 | 38 | 38 | Strong |

MRSA, Methicillin-Resistant *S. aureus*.

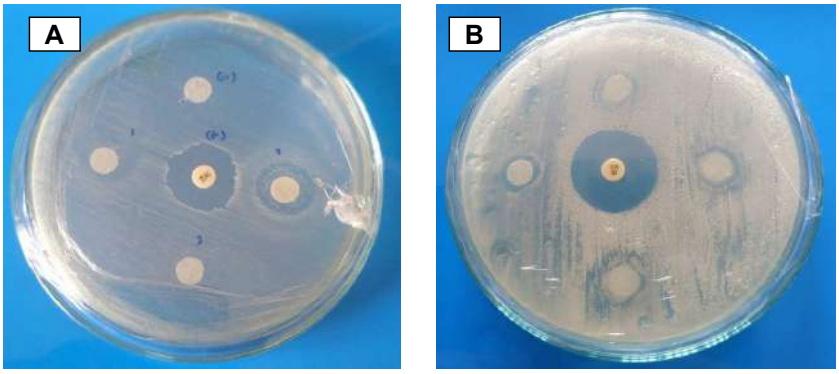


**Figure 4:** Antibacterial activity of group 1 against *Extended Strain Methicillin-Resistant (ESBL) E. coli* with vancomycin 10 µg as a positive control (A), the antibacterial activity of Group 1 methicillin-resistant *S. aureus (MRSA)* with vancomycin 10 µg as a positive control.

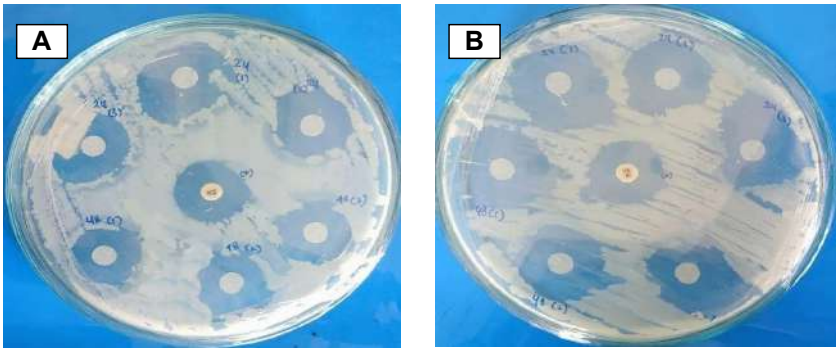


**Figure 5:** Antibacterial activity of group 2 and group 3 against *ESBL E. coli* with vancomycin 10 µg as a positive control (A), the antibacterial activity of group 2 and group 3 against methicillin-resistant *S. aureus (MRSA)* with vancomycin 10 µg as a positive control.

(PBP2 and PBP2a) mecA against all classes of antibiotics. The function of the PBP2 is stopped because giving β-lactam is compensated by PBP 2a resulting in wall synthesis cells on where MRSA takes place [10].

The antibacterial activity of the fermented red passion fruit filtrate is probably derived from organic acids and bacteriocins. It is based on previous research that red passion fruit contains probiotics [11]. Organic acids have been used for many years preservation of food and feed. In addition, the bacteriocin produced by probiotics also has a bactericidal effect. The bacteriostatic effect at similar or near related bacterial strains has already been put into use as an alternative to antibiotics in livestock. The combination of the use of organic acids and bacteriocins is able to produce an optimum inhibitory power against pathogenic bacteria [12]. In this study, the red passion fruit pulp is fermented in banana leaves first and then fermented in MRS-broth resulting in stronger inhibition against ESBL and MRSA compared to fermentation of passion fruit pulp in MRS-broth without fermented in banana leaves. Based on the result from this study, group 2 gives the best inhibition zone compared to group 1 and group 3. The fermentation time in group 3 (48 h) is not directly proportional to the increase in the inhibitory strength of ESBL and MRSA.

## Conclusions

Group 2 shows the best antibacterial activity against *E. coli* ESBL that is higher than group 1 and group 3 with an average inhibition zone of 38 mm classified as strong activity. Group 2 and group 3 show strong activity against MRSA with an average inhibition zone of both 38 mm. The inhibitory activity of group 1 against ESBL is categorized as weak (13 mm) and its activity against MRSA is categorized as a moderate potency with a diameter of growth inhibition zone of 16–20 mm.

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**Author contributions:** All authors contributions can be seen in this table below.

| Type of contribution | Contributors |
|---|---|
| Concept and design | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Data acquisition | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Data analysis/ interpretation | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Drafting manuscript | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Critical revision of the manuscript | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Statistical analysis | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Funding | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Admin, technical, and material support | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Supervision | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |
| Final approval | Iif Hanifa Nurrosyidah, Isnaeni, Ni Made Mertaniasih |

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Rizky Liestya Wardani, Suharjono*, Kuntaman and Agus Widjaja

# Antibiotic use on acute respiratory tract infection nonpneumonia and nonspecific diarrhea in Primary Health Care Centre in Banjarbaru City, South Kalimantan, Indonesia

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**Abstract**

**Objectives:** Acute respiratory tract infection (ARTI) nonpneumonia and nonspecific diarrhea are the most common cases in primary health care centre (PHCC) in Indonesia with the enormous use of antibiotics. The aims of this study were to analyze the antibiotic use and factors affected to the quality of antibiotic use in PHCC in Banjarbaru City, South Kalimantan, Indonesia.

**Methods:** The study was conducted in four PHCCs, two in urban and two in rural areas. All of the patients visited these PHCCs since March to April 2018 were recruited as samples after signing informed consent. Data were analyzed using SPSS version 18.

**Results:** There were no significant difference in antibiotic use between urban and rural PHCC, both on ARTI nonpneumonia and nonspecific diarrhea. The most prescribed antibiotics were amoxycillin and cephadroxil. Based on DDD/1,000 patients-day calculation, the quantity of antibiotics in urban PHCC was 3,544.4 and in rural PHCC was 3,478.6. Physicians with more than seven years of service, both in rural and urban PHCCs, were prescribe the antibiotics higher than who had been working for shorter period. There were no significant difference between physicians who had trained on rational drug use and had not trained yet in urban PHCC (p=0.874), while in rural PHCC there were a significant difference among them.

**Conclusions:** The quantitative analysis showed that the antibiotics use in DDD in urban PHCC was 3,544.416 and in rural PHCC was 3,478.693. Factors affected to the quality of antibiotic use were physician's years of service and rational drug use training's.

**Keywords:** acute respiratory tract infection; antibiotic; care; diarrhea; primary health centre.

## Introduction

Community's health problems in developing countries are dominated by contagious diseases. The data of WHO SEARO shown that 40% a year, the mortality in these area caused by infectious diseases [1]. In Indonesia, acute respiratory tract infection (ARTI) nonpneumonia and nonspecific diarrhea are the most case in primary health care [2]. Based on basic health research 2018 (Riskesdas, Riset Kesehatan Dasar), the period prevalence of ARTI nonpneumonia and nonspecific diarrhea in South Kalimantan were 2.5 and 6.5 [3].

Meanwhile, the antibiotic uses on these diseases in primary health care centres (PHCCs) are still enormous [4]. A study conducted at six PHCCs in South Sumatera found that 49% patients got antibiotic as their therapy, also the antibiotic use on ARTI was 64% and on diarrhea was 79% [5]. It is higher than WHO standard, 20% for ARTI nonpneumonia and 8% for nonspecific diarrhea [6].

Antibiotics are the most frequently used drugs in health service facilities, and they must be used rationally to provide optimal benefits [7]. Rational use of medicines is required so that patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements, for an adequate period of time, and at the lowest cost to them and their community [6]. The rationality of the antibiotic prescriptions was evaluated based on the suitability of the antibiotic selection, dose conformity, and duration and frequency of use, as

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +628121733877, E-mail: suharjono@ff.unair.ac.id
**Rizky Liestya Wardani,** Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Idaman State Hospital of Banjarbaru City, Banjarbaru, South Kalimantan, Indonesia
**Kuntaman,** Department of Clinical Microbiology, School of Medicine Universitas Airlangga-Dr. Soetomo Hospital, Surabaya, Indonesia
**Agus Widjaja,** Health Authority Office of Banjarbaru City, Banjarbaru, Indonesia

recommended in the clinical guidelines [8–10]. The irrational use of antibiotics has negative health consequences, including bacterial antibiotic resistance, inefficiency treatment, increased morbidity and mortality, and increased health care costs [11, 12]. However, the rational use of antibiotics remains a significant problem in many countries, especially in developing countries, so strategies consist of implementation of prudent use of antibiotic and prevention of resistant microbe transmission are needed [4, 13, 14]. The rational drug use is associated with several factors, including health care workers, patients, patient load and health care facilities [15, 16]. Interventions are needed to improve the rational drug use. As a first step is evaluating the antibiotic use in primary care which can be conducted by qualitative and quantitative evaluation to determine the relevant interventions existing problems [17]. The core drug use indicators provide a simple tool for quickly and reliably assessing a few critical aspects of pharmaceutical use in primary health care, commonly used to measure the performance of health care facilities related to utilization of drugs. These indicators are prescribing indicators, patient-care indicators, and facility indicators. Results with these indicators should point to particular drug use issues that need examination in more detail [8].

Banjarbaru is a city bordering with Banjarmasin, the capital of the South Kalimantan Province, with an area of 371.30 $km^2$, a population of 255,597 in 2018. It has nine PHCCs, consists of one inpatient PHCC, and eight non-inpatient PHCCs, divided into five districts. There is no study regarding the antibiotic use at PHCC in Banjarbaru, South Kalimantan yet. Hence, it is needed to be explored in order to evaluate in quality and quantity of antibiotic as well. This study were aimed to analyze the antibiotic use and the affecting factors, as an evidence data for developing recommendation in PHCC's service, also as the evaluation of antibiotic's planning and procurement.

# Materials and methods

This study was conducted by observational analytic about quality, quantity, and factors affected the quality of antibiotic use on ARTI nonpneumonia and nonspecific diarrhea in urban and rural PHCC, with 16 physicians who practicing in there. The samples are patients aged 19–59 years old. This study was conducted after obtaining an ethical clearance from Faculty of Medicine Universitas Airlangga and approval by the Health Authority of Banjarbaru City.

The sampling in this study was conducted by consecutive sampling from March to April 2018. A descriptive and comparative analysis were conducted. The antibiotic usage was analyzed for the quality based on clinical guidelines issued by Ministry of Health of the Republic of Indonesia and the quantity based on DDD calculation [10, 17].

Statistical analysis was performed using SPSS version 18 (SPSS Corp, Chicago, IL, USA). A descriptive analysis, including frequencies and chi square test were performed. The use of antibiotic was calculated the DDD based on the community DDD formula [18]. Furthermore, the logistic regression analysis was used to calculate the Odds Ratios (OR), significances, and 95% confidence interval (95% CI) the factors related with the quality of the antibiotic use. The level of statistical significance was set at 0.05.

# Results

## Demography data

The study was started since March 1 until April 30, 2018. A total of 882 patients, 595 patients with ARTI nonpneumonia and 287 patients with nonspecific diarrhea. These patients were recruited and 16 physicians who practicing in urban and rural PHCC were included. The urban PHCC consists PHCC A and B, and the rural PHCC consists PHCC C and D. The demographic characteristics of sample are shown in Table 1. The majority was female both in urban PHCC (59.2%) and in rural PHCC (58.2%). The majority patients were 19–25 years old in urban PHC (29.8%) and 46–59 years old in rural PHC (33.3%). Both in urban and rural PHC, the majority of the sample's educational background were high school. Generally, there were no significant differences between urban and rural PHC ($p>0.05$).

The total of 16 physicians who practicing in urban and rural PHCC was analyzed, most of the physicians were women both in urban PHCC (100%) and in rural PHCC (87.5%), had trained of rational drug use in urban PHCC (77.8%) and in rural PHCC (57.2%). Meanwhile, the majority physicians in urban PHCC were have experience of service less than seven years (55.5%) but in rural PHCC were more than seven years of service (57.2%) (Table 1).

## Quality of antibiotic use

Of the 882 patients both in urban and rural PHCC, 171 patients (58%) in urban PHCC and 159 patients (53%) in rural PHCC were get antibiotic on ARTI nonpneumonia. Whereas on nonspecific diarrhea, 30 patients (18.6%) in urban PHCC and 14 patients (11.1%) in rural PHCC got antibiotic. There was no significance difference in antibiotic use proportion between urban and rural PHCC ($p=0.223$; $p=0.079$) (Table 2). There was a significant different of antibiotic use in both of ARTI nonpneumonia and nonspecific diarrhea between PHCC A and B in rural PHCC ($p=0.000$), whereas

**Table 1:** Demographic data of the patients and physicians among Primary Health Care Center (PHCC) in Banjarbaru city, South Kalimantan, Indonesia, March–April 2018.

| Subjects | Demographic data, % | | |
|---|---|---|---|
| | Urban PHCC | Rural PHCC | |
| **Patients, n=882** | n=456 | n=426 | |
| 1. Gender | | | p=0.764 |
| Female | 270 (59.2) | 248 (58.2) | |
| Male | 186 (40.8) | 178 (41.8) | |
| 2. Age of patient | | | p=0.140 |
| 19–25 years | 136 (29.8) | 98 (23.0) | |
| 26–35 years | 104 (22.8) | 101 (23.7) | |
| 36–45 years | 81 (17.8) | 85 (20.0) | |
| 46–59 years | 135 (29.6) | 142 (33.3) | |
| 3. Occupation | | | p=0.330 |
| Government employee | 42 (9.2) | 16 (3.8) | |
| Private | 120 (26.3) | 155 (36.4) | |
| Entrepreneur | 23 (5.0) | 35 (8.2) | |
| Unemployed | 271 (59.5) | 220 (51.6) | |
| 4. Education | | | p=0.157 |
| Primary school | 4 (0.9) | 11 (2.6) | |
| Secondary school | 19 (4.2) | 19 (4.5) | |
| High school | 308 (67.5) | 271 (63.6) | |
| Undergraduate | 123 (27.0) | 125 (29.3) | |
| Postgraduate | 2 (0.4) | 0 | |
| 5. History of previous diseases | | | p=0.432 |
| No previous diseases | 411 (90.13) | 377 (88.5) | |
| Has previous diseases | 45 (9.87) | 49 (11.5) | |
| **Physicians, n=16** | | | |
| 1. Gender | | | |
| Female | 9 (100) | 6 (85.7) | |
| Male | 0 | 1 (14.3) | |
| 2. Experience | | | |
| <7 years | 5 (55.5) | 3 (42.8) | |
| >7 years | 4 (45.5) | 4 (57.2) | |
| 3. Training of rational drug use | | | |
| Trained | 7 (77.8) | 4 (57.2) | |
| Not yet training | 2 (22.2) | 3 (42.8) | |

PHCC, Primary Health Care Centre.

no significant different between PHCC C and D in urban area (p=0.897; p=0.245) (Table 3).

## Quantity of antibiotic use

Of the 374 patients who got antibiotic were analyzed further. Amoxycillin was the most frequently used in urban (26.5%) and rural PHCC (22.5%) (Table 4). Overall there was no significance difference in antibiotic use profile between urban and rural PHCC (p>0.05), except fradiomisin-gramisidin (p=0.020). Defined daily dose (DDD) was used to analyze the quantity of antibiotics, which cotrimoxazol had the largest quantity antibiotic in urban PHCC (2,373.310 DDD) and also in rural PHCC (2,349.46 DDD). Total DDD in urban PHCC was 3,544.4 DDD and in rural PHCC was 3,478.7 DDD (p=0.298) (Table 5).

**Table 2:** Antibiotic use on ARTI nonpneumonia and nonspecific diarrhea during March–April 2018.

| Diagnose | Antibiotic use | Urban PHCC, n=456 n, % | Rural PHCC, n=426 n, % | p-Value |
|---|---|---|---|---|
| ARTI | Yes | 171 (58) | 159 (53) | 0.223 |
| nonpneumonia | No | 124 (42) | 141 (47) | |
| Nonspecific | Yes | 30 (18.6) | 14 (11.1) | 0.079 |
| diarrhea | No | 131 (81.4) | 112 (88.9) | |

PHCC, Primary Health Care Centre; ARTI, acute respiratory tract infection.

## Factors affected to antibiotic use

Of the 374 patients who got antibiotic were analyzed further about the multivariant characteristic associated to antibiotic use. Physicians in urban PHCC who have a work experience >7 years were prescribed the antibiotics 3.194 times higher than those who have the experience ≤7 years. Physicians in rural PHCC who have a works experience >7 years were prescribed the antibiotics 3.779 times higher than those who have the experience ≤7 years. Physicians in urban PHCC who had trained the rational drug use were prescribed the antibiotics 1.053 times higher than those who had not trained yet, whereas in rural PHCC they were 2.111 times higher. The antibiotic prescribing in the PHCCs that did not have the clinical guidelines was 6.516 times higher than which have (Table 6).

# Discussion

This study was conducted to analyze the antibiotic use on ARTI nonpneumonia and nonspecific diarrhea in urban and rural PHCC. Generally, the subjects of this study in urban and rural PHCC was homogen. This study shows that the antibiotic use on ARTI nonpneumonia and nonspecific diarrhea was high. WHO has been set the antibiotic use on ARTI nonpneumonia was <20% and on acute diarrhea was <8% [8]. It means that the antibiotic use among PHCC, in both of rural and urban area in Banjarbaru provinces, South Kalimantan were less prudent. As refers to evidence data, showed that ARTI nonpneumonia and nonspecific diarrhea were mostly caused by virus [10]. Even though the antibiotic use in urban PHCC was higher, but there was not

**Table 3:** Antibiotic prescription pattern on ARTI nonpneumonia and nonspecific diarrhea between PHCCs during March–April 2018.

| Diagnose | Urban PHCCs | | p-Value | Rural PHCCs | | p-Value |
|---|---|---|---|---|---|---|
| | PHCC A n, % | PHCC B n, % | | PHCC C n, % | PHCC D n, % | |
| ARTI nonpneumonia | 115 (77.7) (n=148) | 56 (38.1) (n=147) | 0.000 | 79 (53.4) (n=148) | 80 (52.6) (n=152) | 0.897 |
| Nonspecific diarrhea | 28 (43.1) (n=65) | 2 (2.1) (n=96) | 0.000 | 10 (9.6) (n=104) | 4 (18.2) (n=22) | 0.245 |

PHCC, Primary Health Care Centre; ARTI, acute respiratory tract infection.

**Table 4:** Antibiotic prescription pattern on ARTI nonpneumonia and nonspecific diarrhea by physicians in urban and rural PHCCs during March–April 2018.

| Location | PHCC's code | Diagnose | |
|---|---|---|---|
| | | ARTI nonpneumonia, % | Nonspecific diarrhea, % |
| Urban PHCC | PHCC A | 63.9 (n=36) | 21.4 (n=14) |
| | | 76.7 (n=43) | 52.9 (n=17) |
| | | 79.1 (n=43) | 42.3 (n=26) |
| | | 96.2 (n=26) | 62.5 (n=8) |
| | PHCC B | 23.4 (n=47) | 4.3 (n=23) |
| | | 24.3 (n=37) | 0 (n=15) |
| | | 39.0 (n=41) | 0 (n=33) |
| | | 93.3 (n=15) | 14.3 (n=7) |
| | | 10.7 (n=7) | 0 (n=16) |
| Rural PHCC | PHCC C | 85.1 (n=74) | 21.2 (n=33) |
| | | 13.6 (n=59) | 1.8 (n=59) |
| | | 44.4 (n=18) | 14.3 (n=14) |
| | PHCC D | 81.5 (n=27) | 14.3 (n=7) |
| | | 40.0 (n=40) | 12.5 (n=8) |
| | | 56.4 (n=55) | 40.0 (n=5) |
| | | 40.7 (n=27) | 0 (n=2) |

PHCC, Primary Health Care Centre; ARTI, acute respiratory tract infection.

significant different between PHCC in rural and urban (ARTI nonpneumonia p=0.023; nonspecific diarrhea p=0.079). The high use of antibiotic in urban PHCC, especially rolled by PHCC A's antibiotic use profile (p=0.000). The higher use of antibiotic in rural PHC was caused by the higher number of patients that visit PHCC [19].

In this study, the most frequently used in urban and rural PHCC was amoxycillin, cephadroxil, and cotrimoxazol. Amoxycillin and cephadroxil were common used on ARTI nonpneumonia, while cotrimoxazol was frequently used on nonspecific diarrhea. Those antibiotics were available in large quantities of PHCC store, because its lower price than the other antibiotics and also available in all PHCCs as one of the standard drugs in health office. The study of Moore et al. [20] found that no clear evidence of meaningful benefit from amoxicillin even in subgroup patients with acute lower respiratory tract infection. Only those patients with evidence of pneumonia on chest X-ray benefited from amoxicillin treatment [21]. Another study found that no clear evidence of clinically meaningful benefit from amoxicillin treatment in adults presenting to primary care with lower respiratory tract infection for symptom severity or duration, irrespective of etiology or

**Table 5:** Antibiotic use profile in urban and rural PHCCs during March–April 2018.

| Antibiotic | ATC code | Total antibiotic, n=374 | | p-Value | Total DDD | |
|---|---|---|---|---|---|---|
| | | Urban PHCC n, % | Rural PHCC n, % | | Urban PHCC (DDD/1,000 patient-days) | Rural PHCC (DDD/1,000 patient-days) |
| Amoxycillin | J01CR02 | 122 (26.5) | 96 (22.5) | 0.168 | 752.323 | 675.287 |
| Erythromycin | J01FA01 | 2 (0.4) | 0 | 0.171 | 9.798 | 0 |
| Cephadroxil | J01DB05 | 36 (7.9) | 38 (8.9) | 0.583 | 152.479 | 122.845 |
| Thiamphenicol | J01BA02 | 5 (1.1) | 1 (0.2) | 0.120 | 115.30 | 49.751 |
| Cefixime | J01DD08 | 7 (1.5) | 10 (2.3) | 0.381 | 30.795 | 50.287 |
| Cotrimoxazol | J01EE01 | 22 (4.8) | 21 (4.9) | 0.842 | 2,373.3 | 2,349.46 |
| Metronidazol | J01XD01 | 2 (0.4) | 3 (0.7) | 0.600 | 41.929 | 149.253 |
| Ciprofloxacin | J01MA02 | 3 (0.7) | 1 (0.2) | 0.350 | 68.492 | 74.626 |
| Fradiomycin-gramycidin | R02AB30 | 0 | 5 (1.2) | 0.020 | 0 | 7.184 |
| Total | | | | | 3,544.416 | 3,478.693 |

PHCC, Primary Health Care Centre; DDD, defined daily dose.

**Table 6:** Factors affected to antibiotic use.

| Factors affected | Antibiotic use in urban PHCC, n=456 | p-Value | OR | 95% CI | Antibiotic use in rural PHCC, n=426 | p-Value | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|
| | Yes, n=201 n, % | | | | Yes, n=173 n, % | | | |
| **Experience** | | 0.000 | 3.194 | 2.157–4.728 | | 0.000 | 3.779 | 2.488–5.740 |
| ≤7 years | 93 (33.2) | | | | 47 (24.1) | | | |
| >7 years | 108 (61.4) | | | | 126 (54.5) | | | |
| **Rational drug use training** | | 0.874 | 1.053 | 0.556–1.993 | | 0.001 | 2.111 | 1.371–3.250 |
| Had trained | 182 (44.0) | | | | 110 (35.6) | | | |
| Had not trained yet | 19 (45.2) | | | | 63 (53.8) | | | |
| **Clinical guideline** | | 0.000 | 6.516 | 4.321–9.826 | | | | |
| Available | 58 (23.9) | | | | | | | |
| Not available | 143 (67.1) | | | | 173 (40.6) | | | |

PHCC, Primary Health Care Centre; OR, odds ratios; CI, confidence intervals.

biomarker test results [22]. This result was similar with the study of Andrajati et al. [23] in 11 PHCCs in Depok City, Jakarta, Indonesia. Some antibiotic ordered by PHCC would be due to the epidemiology of the disease in that district [7]. Some demographic conditions, pattern of diseases, educational background, health care facility, peers, and workload were also the other factors [24].

The quantity of antibiotic used among PHCC, were 3,544.416 DDD in urban PHCC and 3,478.693 DDD in rural PHCC. DDD is assumed as an average maintenance dose per day for an individual drug's main indication in adults rather than the actual daily dose used in practice, which has changed over time, as has the mix of antimicrobial used, which also affects total DDD [17]. As comparison, antibiotic use in PHCCs in Surabaya in 2013 was 753.84 DDD [25]. In the community, the prevalence of respiratory infection and the percentage of visits in which antibiotics were prescribed, are high [26]. Increases in total DDD could be due to more people being prescribed, more prescriptions for each person, and/or changes in antibiotic choice or dose, therefore it can change without changes in proportion of the population exposed [27]. The study of Neilly et al. [28] showed that the DDD per 1,000 population at Tayside, Scotland increased between 1995 and 2014, from 5,651 to 6,987 per 1,000 population (95% CI 1,309–1,363). The study of Williamson et al. [29] showed that the DDD per 1,000 population per day in New Zealand in 2014 was 25.77 (average annual increase 1.06; 95% CI 0.74–1.38).

According the other factors affecting the antibiotic use, such as physician's years of experience, training of rational drug use, and availability of clinical guideline, it showed any interesting fact in this study. It was found that physicians who have the experience >7 years working as PHCC physician, prescribed antibiotic 3.237 (95% CI 2.450–4.276) times higher than those who have the experience ≤7 years. This result in line with the studies of Cadieux et al. [30], Fahmiani et al. [31], dan Andrajati et al. [23]. The study of Cadieux et al. [30], showed that the physicians in Quebec who were in practice >5 years were more likely to prescribe antibiotic 1.04 times higher (95% CI 1.02–1.05). The study of Fahmiani et al. [31] showed that the health care workers in PHCC in Makassar City with longer years of service were 84.4% prescribed irrationally on ARTI nonpneumonia and 85.9% on nonspecific diarrhea compared with those who have lower years of service. Meanwhile, in study that conducted by Andrajati et al. [23] revealed that the physicians in PHCC in Depok City who have less experience (≤7 years) were 3.952 more rational when prescribing antibiotics than those with more experience (>7 years) (95% 0.158–0.405). This phenomenon would be due to the personal attitudes and knowledge of the physician [32]. Several factors appear to influence prescribing behavior, including demands from patients, pharmaceutical company marketing activities, limited up-to-date information sources. Inappropriate prescribing of antibiotics also appears to be related to inadequate knowledge or training the appropriate prescribing of antibiotics [33].

Physician in urban PHCC who has not trained yet on rational drug use was prescribed antibiotics 1.053 times higher than who has trained (95% CI 0.556–1.993) and there was no significant difference among them (p=0.874). Meanwhile, physician in rural PHCC who has not trained yet on rational drug use was prescribed antibiotics 2.111 times higher than who has trained (95% CI 1.371–3.250) and there was a significant difference among them (p=0.001). A

Cochrane review of various interventions to improve antibiotic prescribing practices treated some, but educational practices as one type of intervention to improve prescriber access [26]. Educational sessions often take place as one-time or recurrent seminars. However, other unique methods of delivery exist, such as discussions, video, online or social media interventions [34–36]. The intensity of educational intervention appears to have an effect on the impact of the intervention, with greater time spent in education and frequent follow-up resulting in better outcomes. Providers who received education monthly for two years decreased their use of antibiotics significantly more than those who received a single 2 h annual education session (20 vs. 16.5% reduction, p<0.0001) [37]. In another trial, a multimodal intervention strategy comprised of peer group education meetings, communication training, monitoring, and feedback on prescribing patterns, initially resulted in a 12% mean decrease in antibiotic prescription rates of respiratory tract infections [38]. Further study should be conducted to perceive whether the education intervention, such as training affects to the antibiotic use pattern [39, 40].

The availability of clinical guideline, highly impacted on the antibiotic prescription. There was a significant difference between PHCC that has the clinical guideline and those that were not (p=0.000). Antibiotic prescribing in PHCCs that were not have the clinical guidelines was 6.516 times higher (95% CI 4.321–9.826). PHCC C was the only one PHCC that has the clinical guideline and shown that the level prescription of antibiotic was low.

The limitation of this study were not any support in laboratory testing, the quantity of the physicians were also not many. But this condition was common in Indonesian PHCC, especially in the islands out of Java. Our results should be explored in larger study to better availability data of antibiotic use in community and in understanding of the determinants that influence prescribing especially in primary health care in Indonesia. This study was put a data base for any intervention in PHCC in this district for improving the performance of antibiotic drug use, especially in the era of antimicrobial resistance.

## Conclusions

The antibiotic use for Acute Respiratory Tract Infection (ARTI) nonpneumonia and nonspecific diarrhea in urban and rural PHCC (Primary Health Care Center) in Banjarbaru City, South Kalimantan was less prudent. The quantitative analysis showed that the antibiotics use in DDD in urban PHCC was 3,544.416 DDD/1,000 person-days and in rural PHCC was 3,478.693 DDD/1,000 person-days. Factors affected to antibiotic use are physician's years of service, training of rational drug use, and availability of clinical guidelines. Appropriate interventions are needed to encourage the improvement of antibiotic use in PHCCs, especially in ARTI nonpneumonia and nonspecific diarrhea. Healthcare authorities can stimulate all PHCCs to compile the clinical guideline and complete the supporting facilities in PHCC.

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Siti Qamariyah Khairunisa*, Dwi Wahyu Indriati, Lidya Tumewu, Aty Widyawaruyanti and Nasronudin Nasronudin

# Screening of anti-HIV activities in ethanol extract and fractions from *Ficus fistulosa* leaves

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**Abstract**

**Objectives:** Human immunodeficiency virus (HIV) infection is considered as a major immunosuppressive disease linked to malignancies and other opportunistic infections. Recently, the high prevalence of HIV drug-resistant strains required a high demand for novel antiviral drug development, especially in herbal medicine approaches. The objective of this study was to evaluate the possibility of *Ficus fistulosa* leaves can inhibit HIV replication in ethanol extract form as well as its fractions using chloroform, ethyl acetate, and butanol solvents.

**Methods:** *F. fistulosa* leaves were extracted using ethanol as a solvent and further gradually fractionated in chloroform, ethyl acetate, and butanol solvents. The targeted persistently infected virus (MT4/HIV) cell lines were cocultured with ethanol extract and fractions at different time points. The syncytium formation and cytotoxicity assays were performed to evaluate the potential antiviral activity of *F. fistulosa* leaves.

**Results:** One of the four tested extract/fractions showed antiviral activity against HIV. The ethanol extract showed weak inhibition with a high level of toxicity ($IC_{50}$ = 8.96 μg/mL, $CC_{50}$ ≥50 μg/mL, and SI = 5.58). Meanwhile, chloroform fraction effectively inhibited the MT4/HIV cell proliferation while keeping the toxicity to a minimal level ($IC_{50}$ = 3.27 μg/mL, $CC_{50}$ = 29.30 μg/mL, and SI = 8.96). In contrast of ethyl acetate fraction and butanol fraction showed no anti HIV activity with a high level of toxicity ($CC_{50}$ ≥50 μg/mL) and low SI value (>2.17 μg/mL and >0.97 μg/mL).

**Conclusions:** Chloroform fraction of *F. fistulosa* leaves showed effectively as anti-viral activity against MT4/HIV cells.

**Keywords:** anti HIV; *Ficus fistulosa*; *in vitro*; medicinal plant.

## Introduction

Since being discovered in 1983, human immunodeficiency virus (HIV-1) has been infecting over 38 million people worldwide based on WHO global data tracking with over 25 million (>65%) infected individuals reside in Africa [1]. The distinctive characteristic of HIV-1 is the substantial genetic diversity that builds up within and between hosts [2]. The high mutation rate in HIV-1 is due to the absence of DNA repair enzymes, creating approximately one nucleotide mutation per cycle during viral replication [3]. This unique characteristic of HIV-1 has become a big obstacle to researchers in studying the new approach of HIV-1 antiviral drug discovery.

Diverse novel approaches to anti HIV treatments are currently being developed worldwide and mostly focusing on the initial step of viral entry. One approach targeted the gp120 receptor on the HIV-1 envelope to create a novel antiviral drug by using boronic acid materials as an anti-retroviral agent (ART) [4, 5]. Another therapeutic method, frequently named "shock and kill" used latency-reversing agents (LRAs) to stimulate pro-viral expression ("shock"), and afterward the latent HIV-infected cells could be exterminated by viral cytopathic effects or host immune responses ("kill") [6, 7]. A recent study showed LRAs extracted from untested marine natural products was efficient to induce antiviral activity *in vitro* [8]. One alternative method is polypharmacology in which two or more multitarget or hybrid drugs can be used simultaneously to increase the antiviral activity against HIV-1 [9, 10]. To date, currently available anti HIV drugs remain nonoptimal

***Corresponding author: Siti Qamariyah Khairunisa**, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, Phone: +6281331843627, E-mail: skhairunisa@staf.unair.ac.id
**Dwi Wahyu Indriati,** HIV Study Group, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Departement of Health, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia
**Lidya Tumewu,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
**Aty Widyawaruyanti,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Nasronudin Nasronudin,** HIV Study Group, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; Department of Internal Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia; and Airlangga University Hospital, Universitas Airlangga, Surabaya, Indonesia

due to complicated procedures, uncontrolled cytotoxicy, and unpredicted side effects.

The failure in HIV-1 treatment especially in low-middle income countries was due to a shortage of drugs and the high cost of treatment [11]. However, the main factor of treatment failure is the lack of patient's discipline to take their prescribed drugs daily in the correct dose or not showing up at doctor's appointments for follow-up checks. The patient's social behavior could impact the development of drug-resistant therapy as well as increasing the fatality rate. The urge for *de novo* anti HIV drug development that is cost-efficient and has fewer side effects has become the priority in AIDS pharmacological research, preferably compounds extracted from local natural sources.

The research about natural products in drug development has been used since centuries ago, pioneered by the Egyptians in the discovery of Penicillin from *Penicillium chrysogenum* recorded in 1928 and was clinically accessible in the 1940s [12, 13]. The medicinal properties of active compounds from plants were derived from the anti inflammatory and protective defense mechanisms of these products [14, 15]. Calanolides from *Calophyllum lanigerum* was one of the first plant-derived compounds discovered to have antiviral activity against HIV-1 [16, 17]. Betulinic acid extracted from the Chinese herb *Syzygium claviflorum* was succeeded to be synthesized as drug, Bevirimat, and continue in clinical trials since 2007 [18]. Other plant-based antiviral candidates have been studied intensively over the years and the results are promising. Additionally, the utilization of locally sourced natural products could be beneficial economically in the development of more affordable medicines.

The genus Ficus has approximately 725 species worldwide and three species namely *Ficus hispida, Ficus septica,* and *F.fistulosa* have been studied, and they exhibited anticancer, anti inflammatory, and antiviral activities [19–21]. However, only Indriati et al. reported the potential anti HIV activity, and this recent study will investigate further the hidden anti-viral activity of *Ficus fistulosa* against HIV-1. In the present study, four samples derived from *F. fistulosa*, ethanol extract, ethyl acetate, chloroform, and butanol fractions were used in *in vitro* assay to assess their antiviral activity.

# Materials and methods

### Cells and viruses

The human acute T-lymphoblastic leukemia cells (MOLT-4/MT-4) were cultured in RPMI-1640 medium and DMEM medium (GIBCO, USA) with the addition of 10% fetal bovine serum (Sigma, USA), 100 U/mL penicillin G, and 100 µg/mL streptomycin. The cells were incubated at 37 °C and 5% $CO_2$ humidity for three days prior to further usage. In addition, the persistently infected cells were previously made from coculture of peripheral blood mononuclear cells (PBMC) from HIV-1 patients and healthy blood donors. Initially, the PBMC were pre activated with 10 µg/mL phytohemagglutinin (PHA), mitogen, and later on, induced with interleukin-2 (IL-2) [22]. The mix of MT-4 and the in-house persistently infected cells HIV-1 (MT-4/HIV-1) were cocultured according to the protocol from Gyuris et al. [23]. All HIV-1 isolates derived from the stock were collected from Surabaya, Indonesia.

### Plant materials

The leaves from *F. fistulosa* were obtained from Cangar Conservation Forest, Malang, West Java, Indonesia. The verification and identification of the plant were supervised by Purwodadi Botanical Garden – Indonesia Institute of Science, East Java, Indonesia. All plant specimens were stored in Natural Product Medicine Research and Development Laboratorium (NPMRD), Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, Indonesia.

### Plant extraction and fractionations

*F. fistulosa* leaves as much as 2.5 kg were drying at room temperature and were powdered into approximately 250 g dry stocks. Later on the 250 g of dry stocks were soaked in 1,250 mL of 80% ethanol and sonicated for 2 min (three times) to extract the constituents. The extract was filtered and residue was extracted again using 1,250 mL of 80% ethanol by the same procedure. The extraction of residue was repeated once again. The extraction was conducted by ultrasonic assisted extraction method for three times and using 3,750 mL of 80% ethanol (3 × 1,250 mL) as a solvent in total. The ethanol extract was further evaporated to obtain 13 g of dried ethanol extract (E). The 10 g of dried ethanol extract was suspended in 100 mL of distilled water and liquid–liquid partitioned successively with chloroform, ethyl acetate, and butanol solvent as much as 3 × 100 mL for each solvent to obtain 4.03 g of chloroform fraction (C), 0.19 g of ethyl acetate fraction (EA) and 0.80 g of butanol fraction (B). The residue was dried to obtain 4.71 g of aqueous fraction (A). All samples were tested for the antiviral activity against HIV-1 except for aqueous fraction was not tested. The stock solutions were stored in –20 °C freezer until being used further.

### Phytochemical analysis

The phytochemical analysis of ethanol extract (E), chloroform fraction (C), ethyl acetate fraction (EA), butanol fraction (B), and aqueous fraction (A) was conducted by thin layer chromatography (TLC) method. Extract and fractions as much as 10 mg were dissolved in 1 mL of methanol. Extract and fractions as much as 3 µL then spotted on silica gel F254 plate (Merck) as stationary phase and chloroform: methanol (Merck) (9:1 v/v) as mobile phase. The spots were identified using $H_2SO_4$ 10% as a spray reagent and observed under UV 254 and 365 nm.

### Analysis of anti HIV potentials of plant extract and fractions

**Anti-viral activity (syncytia formation assay):** After coculturing the MT-4 cells and persistently infected HIV-1 cells derived from PBMC (MT-4/HIV-1), the syncytia formation occurred on the cultured cells. The next step was to apply the coculture cells into syncytia inhibition assay. Two-fold serial dilution of extract/fractions was prepared (50; 25; 12.5; 6.25; 3.13 µg/L). Upon 30 min incubation at 37 °C, MOLT-4 cells (acute lymphoblastic human leukemia cell line) were added into the culture (2.105 cells/mL for a multiplicity of infection (MOI) of 1/20), and the cocultured cells were incubated at 37 °C for one week (seven days). As a negative control (NC), the MT-4/HIV-1 cells were mixed on cultured MOLT-4 cells and incubated at 37 °C for one week (seven days). After incubation, the number of produced syncytia was microscopically counted. The data were furnished in the percent of inhibition compared with negative control by the below equation.

$$\% \text{ Inhibition} = \left[100 - \left(\frac{\text{NC} - \text{Syncitia on sample}}{\text{NC}}\right)\right] \times 100\%$$

Inhibition activity was also evaluated on half concentration of inhibition ($IC_{50}$) value. This assay was also done in duplicate replicates to ensure the valid result of the toxicity effect of these antiviral subtances.

**Cytotoxicity assay:** Follow up the Syncytia assay; the cytotoxicity assay was conducted to measure how potent the tested compound was in terms of antiviral activity against HIV-1. This test only targeted the viable infected cells, not the healthy cells. WST-1 Cell Proliferation Reagent (Roche Applied Science, Switzerland) assay was used in this test by converting tetrazolium salt into a formazan product by viable cells. Serial dilution containing *F. fistulosa* extract/fractions were added into MOLT4 cell culture, using only cell culture without extract/fraction as a positive control and plain RPMI medium as a negative control. The cell culture containing extract/fractions were incubated at 37 °C for seven days. Following the incubation period, the MTT reagent was added into the cell culture and the absorbance was read at 450 nm, measured at two-time points of pre incubation and postincubation at 37 °C for 2 h. The cytotoxicity concentration ($CC_{50}$) score was measured by assessing in which virus dilution that the 50% viable cells detected. This assay was also done in duplicate replicates to ensure the valid result of the toxicity effect of these antiviral subtances.

**Statistical analysis:** In this study, we were used randomized complete block design (RCBD), a design which each experiment unit was divided into randomized block. This design were applied to analyzed the effect of extract and its fractions that were divided into experimental groups (ethanol extract, butanol fraction, ethyl acetate fraction, and chloroform fraction) and the control group on anti HIV effectiveness. Analysis that used for the above design were using two way ANOVA method, which was a type of parametric statistical test that aims to determine whether there were effect differences/effect on two factors (concentration and compound) that cause variations. If the test result showed different effect on each extract and its fraction, further test was needed for the next test step. One of the test methods is using Tuckey test. This test was used when the extract and its fraction were different. $IC_{50}$ and $CC_{50}$ were calculated using probit regression analysis.

## Results

Phytochemical properties from *F. fistulosa* are known to have anti-viral activity, and those subfractions contained flavonoids, terpenoids, and chlorophyll compounds. Genus Ficus have several phytochemical compounds such as triterpenoid; flavonoids; sterols; coumarin; and anthocyanins in every part of the plant [24]. Previous study reported that ethanol extract of *F.fistulosa* leaves was exhibited anti hepatitis C virus activity against JFH1a. The phytochemical identification revealed that the ethanol extract was contained flavonoids, terpenoids, and chlorophyl compounds [25]. Meanwhile, alkaloids compounds as antifungal against *Aspergillus fumigatus* and *Candida albicans* were isolated from *F.fistulosa* stem bark [26]. Phytochemical analysis was conducted in this study by TLC analysis. The result showed that *F. fistulosa* leaves was contained flavonoids, terpenoids and chlorophyl compounds as previous reported [25].The ethanol extract and chloroform fraction showed a similar TLC profile. Chlorophyll was detected in ethanol extract and chloroform fraction under UV light 365 nm which was indicated by red spots. Terpenoids and flavonoids were detected as well after sprayed with $H_2SO_4$ 10% which was indicated by violet spots and yellow–dark brown spots. Meanwhile, ethyl acetate fraction was dominated with flavonoids and contains minor terpenoids. Butanol fraction and aqueous fraction were not eluted well on silica gel due to polar compounds as a dominant content (Figure 1).

In the present study, one extract and three fractions; ethanol extract, chloroform, ethyl acetate, and butanol fractions extracted from *F. fistulosa* leaves were examined for their anti HIV activities *in vitro* (Table 1). Of four samples, only one sample namely chloroform fraction exhibited the antiviral activities against HIV-1. The ethanol extract showed a low $IC_{50}$ value calculated as 8.96 µg/mL and a high $CC_{50}$ of >50 µg/mL while these extract exhibited weak anti HIV activity. Additionally, the chloroform fraction displayed interesting values analyzed as 3.27 and 29.30 µg/mL for $IC_{50}$ and $CC_{50}$, respectively. These high values of SI (8.96) detected in these fraction suggested that the chloroform fraction could be utilized safely under the parameters applied in this study. In contrast of ethyl acetate fraction and butanol fraction showed no anti HIV activity, while we detected the high value of $CC_{50}$ in both fractions (>50).

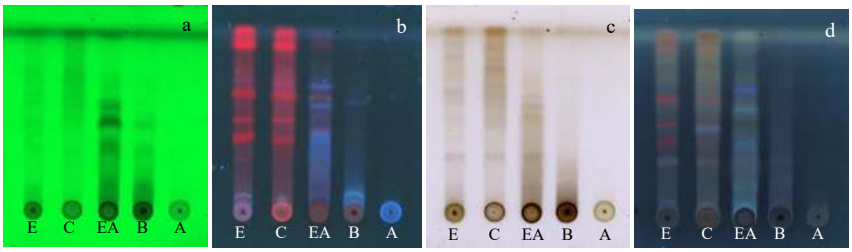


**Figure 1:** Thin layer chromatography (TLC) profile of ethanol extract (E), chloroform fraction (C), ethyl acetate fraction (EA), butanol fraction (B), and aqueous fraction (A) of *Ficus fistulosa* leaves using silica gel as a stationary phase and chloroform-methanol (9/1 v/v) as a mobile phase. The TLC was observed under UV light 254 nm (a), 365 nm (b), white light (c), and UV light 365 nm after sprayed with $H_2SO_4$ 10% and heated at 105 °C for 5 min (d).

**Table 1:** The results of syncytium formation and cytotoxicity assays depicted in calculated $IC_{50}$, $CC_{50}$ and SI values from *Ficus fistulosa* leaves.

| Samples | $IC_{50}$, µg/ml | $CC_{50}$, µg/ml | SI* |
|---|---|---|---|
| Ethanol extract | 8.96 | >50 | >5.58 |
| Chloroform fraction | 3.27 | 29.30 | 8.96 |
| Ethyl acetate fraction | 23.04 | >50 | >2.17 |
| Butanol fraction | 53.98 | >50 | >0.97 |

*SI, selectively index; $CC_{50}$ value was divide by the $IC_{50}$ value.

## Discussion

To date, over 40 currently accessible antiretroviral therapy (ART) drugs have been circulating worldwide converting HIV/AIDS from a lethal infection into a manageable chronic disease [27]. However, due to the high rate of mutations detected in the HIV-1 viral replication cycle, drug resistance mutants have become a big obstacle in HIV-1 treatment accomplishment [28, 29]. The other challenge was to offer more affordable HIV-1 drugs, especially in low-income countries. Therefore, new approaches of using local resources plant-based medicines have been introduced in several Asian countries like Thailand and Indonesia [21, 30].

Indonesia has known for its tropical climate and home of the second-largest biodiversity in the world spreading in over 17,000 islands. Due to its geographical location, the flora of Indonesia displays the Asian, Australian, and native collections. Plant-based medicines using indigenous Indonesian flora have been developed rapidly in recent years. Different studies focusing on antiviral, antibacterial, antimalarial, and antifungal potentials of Indonesian medicinal plants reported promising results to be used further for drugs development Different parts of the plants like stem, leaves, and roots of the following species of native ginger rhizome, *F. fistulosa*, *Garcinia mangostana*, *Melanolepis multiglandulosa*, and *Melicope latifolia* have been studied intensively in the past years [21, 31, 32].

*F. fistulosa* is a part of the genus Ficus in the family of Moraceae commonly found in Asia and New Guinea. In ancient medicine history, Ficus genus species have been used for traditional remedies to cure headaches, breathing problems, diarrhea, cough, toothache, eye infection, and scabies [33]. The extensive pharmacological properties of Ficus have been reported including antimicrobial, antioxidant, antiviral, antiparasitic, anti inflammatory, and anticancer [34].

Wahyuni et al. reported that *F. fistulosa* was efficient in inhibiting the growth of two hepatitis C virus (HCV) strains *in vitro*. The study showed that the ethanol extract from *F. fistulosa* leaves successfully inhibits the virus growth during the initial step of inoculation. The study conducted by Indriati et al. observed that the n-hexane fraction *of F. fistulosa* showed a potent activity as an anti HIV drug candidate. This study result was in accordance with a previously reported study in which n-hexane fraction was exhibited anti HIV activity. Furthermore, crude extract (ethanol extract) showed the highest activity among other samples. The phytochemical content of ethanol extract and chloroform fraction was probably the same due to their similar TLC profile. Both ethanol extract and chloroform fraction contained chlorophyll, terpenoids, and flavonoids compounds which were possible to take a role in their anti HIV activity.

Flavonoids inhibit HIV replication in PBMC in dose dependent manner as it is shown in other herbal medicine known as *Sctellaria* baicalemsis [35]. Other type of flavonoids, gallate ester and quercetin 3-O-(2-galloyl) a-L arbinopyranose inhibit the activity of integrase enzyme. Flavonoids also showed inhibition to RT activity [36]. While triterpenoid acts as anti HIV in several step of HIV cycle such as entry inhibitor which block membrane fusion, inhibit HIV enzymes (protease, reverse transcriptase (RT), and integrase), and viral maturation [37]. Further study needs to be conducted for isolation and identification of active compounds from ethanol extract and chloroform fraction.

## Conclusions

In conclusion, our recent study reported the novel potential of chloroform fraction from *F. fistulosa* as antiviral against HIV-1 in terms of HIV-1 growth inhibition *in vitro* and proven nontoxic to healthy cells since these e fractions contain chlorophyll, terpenoid, and flavonoid which can act as anti HIV.

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**Author contributions:** SQK performed HIV *in vitro* analysis and drafted the manuscript, as well as involved in the design and coordination of the study; DWI performed HIV *in vitro* analysis and helped in drafting the manuscript; LT performed plant extraction and phytochemical analysis; AW and N were involved in the design and coordination of the study. All authors read and approved the final version of the manuscript.
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**Ethical approval:** The local Institutional Review Board deemed the study exempt from review.

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Victoria Yulita Fitriani, Budi Suprapti* and Muhammad Amin

# The characteristics of lactic acid bacteria isolated from fermented food as potential probiotics

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**Abstract**

**Objectives:** This study aims to determine the characteristics of *Lactobacillus acidophilus* and *Lactobacillus reuteri* from fermented soursop fruit juice and cow's milk, respectively as probiotic candidate based on exposure to pH, bile salts, pathogenic bacteria, and antibiotics.

**Methods:** *In vitro* studies were conducted to examine the resistance of *Lactobacillus acidophilus* and *Lactobacillus reuteri* in pH 2, 2.5, 3.2, and 7.2, resistance to bile salts, resistance to pathogenic bacteria *(Escherichia coli, Staphylococcus aureus and Enterococcus faecalis)* and antituberculosis antibiotics.

**Results:** Viability of *Lactobacillus acidophilus* and *Lactobacillus reuteri* isolates remained unchanged ($6.3 \times 10^7$ CFU/mL and $5.03 \times 10^7$ CFU/mL) at various acidic pH, and had a low survival rate in Ox gall 0.3% (bile salts). These isolates also showed antibacterial properties against pathogens in the gastrointestinal tract. Both of these bacteria are quite safe to be used together with ofloxacin, linezolid, moxifloxacin, and levofloxacin, antibiotic for tuberculosis therapy.

**Conclusions:** The results showed that *Lactobacillus acidophilus* and *Lactobacillus reuteri* from fermented soursop fruit juice and cow's milk respectively fulfilled the characteristics of probiotic and could potentially be used as adjunct therapy in tuberculosis drug-resistance.

**Keywords:** antituberculosis; bile acid; *Lactobacillus acidophilus*; *Lactobacillus reuteri*; pathogen; pH.

***Corresponding author: Budi Suprapti**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +628155086694, E-mail: budi-s@ff.unair.ac.id
**Victoria Yulita Fitriani,** Doctoral Programme in Pharmaceutical Science, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Muhammad Amin,** Department of Pulmonology, Faculty of Medicine, Airlangga University, Surabaya, Indonesia

## Introduction

Probiotics are nonpathogenic living microorganisms that provide health benefits to the host when consumed in an adequate amount, by creating microbial balance in the body [1–4]. Other probiotic effects are improvement of the intestinal microbiota, protection against pathogens, immunomodulation, maintenance of intestinal barrier, dan production of short-chain fatty acids and essential vitamins. These effects support the potential use of probiotics in the treatment of various diseases, i.e., diseases of the gastrointestinal tract, allergy, cancer, coronary artery disease, bacterial vaginosis, liver disease, immunity, mental health, hypertension, sepsis, oral health, respiratory infections, including tuberculosis [1, 5, 6].

Recently, research on the role of probiotics in tuberculosis has been growing. *Lactobacillus* strain can modulate the immune system of tuberculosis patients by changing the extent of immune cells ($CD4^+$, $CD8^+$, Treg, and cytokines) involved in tuberculosis. Changes in these immune cells show that the interaction of probiotics with T cells leads to the stimulation of Th1 cells and the production of cytokines which plays an important role in controlling tuberculosis infection [7–10]. Various studies have shown that bacteriocins from probiotic bacteria have potential activity against various *Mycobacterium*. Some of these bacteriocins are nisin, mutacin B-Ny266, lecticin 3147, and bacteriocins from *Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus sakei, Lactobacillus salivarius, Enterococcus faecium, E. faecalis, Aerococcus* sp., and *Pediococcus pentosaceus*. Antimycobacterial activity produced by these bacteriocins are a decrease in internal ATP, leakage of intracellular ATP and inhibition of bacterial growth. Apart from immunomodulation, the antimycobacterial probiotics mechanisms are shown by inhibiting cell wall synthesis and pore formation in bacterial cell membranes by binding to peptidoglycan precursors, reducing proton motive force constituents in *Mycobacterium*, and targeting ATP-dependent bacterial protease [11].

The *Lactobacillus* strain is one of the dominant LABs in fermented foods and has been generally recognized as a probiotics with health benefits either directly or indirectly to its host [6, 12]. To provide these benefits, the *Lactobacillus* strain must be able to adapt to the host environment,

among others, survive and function in the gastrointestinal environment, until it reaches the colon in sufficient numbers [6]. Biological effects of probiotics are strain specific, therefore different strains of bacteria can produce different effects on the host, even though they come from the same genus and species. Therefore it is important to know in depth the specific properties of each strain of bacteria and its effect on health should be tested according to that health condition [13, 14]. Several properties to characterize functional role of probiotic microorganisms, include tolerance to pH and bile salts, adhere to epithelial cells, have antimicrobial activity, as well as their resistance to antibiotics [2–4, 6].

This study is part of a series of studies study series to determine the role of probiotics in immune response of tuberculosis patients, aimed to determine the probiotic characteristics of *Lactobacillus acidophilus* and *Lactobacillus reuteri* isolated from fermented soursop fruit juice and cow's milk respectively towards pH, bile salts, gastrointestinal pathogens, and tuberculosis antibiotics.

# Materials and methods

### Bacteria strains

Two lactic acid bacteria strains of *Lactobacillus* spp. isolated from fermented foods, part of the laboratory collection of Faculty of Sains and Technology, Universitas Airlangga (Surabaya, Indonesia) were selected for this study. *Lactobacillus acidophilus* was isolated from Soursop juice (BioLA246) and *Lactobacillus reuteri* (BioLR321) was isolated from fermented cow's milk. All cultures were stored at −80 °C in MRS (De Man–Rogosa–Sharpe) broth (Hi-Media Pvt. Ltd., India) supplemented with glycerol stock (40% v/v) at a final concentration of 20% [15–17]. Prior to testing, bacteria were precultivated in MRS broth at 37 °C for 24 h.

### Tolerance to pHs and bile salts

Overnight bacterial starter culture cells were standardized to get initial population $10^7$ CFU/mL and inoculated in PBS (Phosphate Buffer Saline), pH value adjusted to 2.0, 2.5, 3.2, 7.2 using hydrochloric acid (HCl) and sodium hydroxide (NaOH), and PBS supplemented with 0.3% Ox gall. PBS adjusted to pH 7.2 was considered a control for pH values and PBS without Ox gall was considered a control for bile salt conditions. The samples of *Lactobacillus* spp. were incubated at 37 °C. For pH tolerance assay was done every 30 min until 180 min and 24 h for bile salts assay using spectrophotometry at a wavelength of 580 nm. All assay has been done triplicate replication with the number of bacteria around $10^7$ cells [18].

### Antimicrobial activity

A disc diffusion assay procedure was used. All tests used MHA (Mueller-Hinton Agar) (Himedia), where overnight cultures of each pathogenic indicator bacteria ($5 \times 10^5$ CFU/mL) were inoculated on the medium. The microorganisms used as pathogenic indicator bacteria are a collection from the Biology laboratory of the Faculty of Science and Technology, Airlangga University, and these microorganisms are *Enterococcus faecalis* (Bio526), *Escherichia coli* (EPEC), *Staphylococcus aureus* (Bio383). The antimicrobial activity was tested by disc diffusion method based on Al-Malkey with modification. One dose from each of the *Lactobacillus* spp. is taken from MRS agar and inoculated into 5.0 mL of sterile MRS broth. The MRS broth culture was then incubated at 37 °C for 24 h. An aliquot of 50 µL of each overnight incubated probiotic bacteria were placed on the disc, then the disc was placed on the MHA. The inoculated agar was then incubated aerobically at 30 °C (Gram-negative) or 37 °C (Gram-positive) for 24 h. The diameters of the inhibition zone were measured using a caliper [19, 20]. There are four inhibition zone criteria based on the diameter of the formed clear area, where these criteria are associated with antimicrobial strength against pathogenic bacteria, namely weak (<5 mm diameter), medium (5–10 mm diameter), strong (10–20 mm diameter), and very strong (diameter> 20 mm) [21].

### Antibiotic susceptibility

The antibiotic susceptibility of the *Lactobacillus* spp. was assessed on MHA agar plates using the antibiotic disc diffusion method. The MHA media was poured and allowed to harden at room temperature. Overnight incubated *Lactobacillus* spp. culture (100 µL) was spread on MHA plates and allowed to dry. The antibiotic discs were placed on inoculated plates and then incubated at 37 °C for 48 h [22]. The antibiotic susceptibility pattern of the isolates was assessed using antituberculosis antibiotics used as second-line or drug-resistant therapy, namely Amikacin (30 µ/disc), Kanamycin (30 µ/disc), Levofloxacin (5 µ/disc), Linezolid (30 µ/disc), Meropenem (10 µ/disc), Moxifloxacin (5 µ/disc), Ofloxacin (5 µ/disc), and Streptomycin (10 µ/disc). The zone of inhibition (ZOI) diameter was measured using the antibiotic zone scale [Clinical and Laboratory Standards Institute (CLSI) scale]. CLSI guidelines (2015) recommend interpretation of results based on breakpoints as follows: isolates with ZOI (zone of inhibition) less than or equal to 14 mm are categorized as resistant (R), isolates with ZOI greater than 20 mm are categorized as susceptible (S) and isolates with ZOI between 15 and 19 mm categorized as intermediate (I) [23].

# Results

## Acid and bile tolerance

The ability of *L. acidophilus* and *L. reuteri* isolates to withstand the test pH after incubation for 180 min at 37 °C are presented in Table 1. *Lactobacillus acidophilus* and *Lactobacillus reuteri* populations have a good tolerance in various pH without a decrease or increase in the number of colonies within 180 min simulate residence time of food in stomach. These data indicate that both *Lactobacillus* spp. very tolerant in acidic and neutral pH environments so that it meets one of the requirements as a probiotic bacteria.

Tolerance to bile salts helps in the evaluation of probiotic bacteria colonization in the small intestine. The

**Table 1:** The number of lactic acid bacteria isolated from food origins in various pH value.

| Lactic acid bacteria | Incubation time, min | Bacterial population (mean ± SD × $10^7$ CFU/mL) | | | | |
|---|---|---|---|---|---|---|
| | | Control | pH 2 | pH 2.5 | pH 3.2 | pH 7.2 |
| *Lactobacillus acidophilus* | 0 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 30 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 60 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 90 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 120 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 150 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| | 180 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 | 6.3 ± 0.00 |
| *Lactobacillus reuteri* | 0 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 30 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 60 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 90 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 120 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 150 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |
| | 180 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 | 5.03 ± 2.19 |

viability of *L. acidophilus* and *L. reuteri* in Ox gall 0.3% after incubated for 24 h at 37 °C are presented in Table 2. The survival rate for each strain was showed in the percentage ratio of the bacterial population before and after incubation. The comparison between the two strains showed that *Lactobacillus acidophilus* tended to have a higher survival rate (2.14 ± 0.54%) than *Lactobacillus reuteri* (1.89 ± 0.15%).

## Antimicrobial activity

The antimicrobial activity of the *Lactobacillus* isolates against bacterial pathogens have been summarized in Table 3. Antimicrobial activity is an important characteristic for probiotic bacteria in demonstrating their natural health benefits as an antagonist to pathogenic bacteria. Based on the pathogenic bacteria used, both *Lactobacillus* strains have antimicrobial activity but between them had significant differences in antimicrobial activity as indicated by their ability to produce clear zones on the MHA plate ($p<0.05$). The antimicrobial strength of *Lactobacillus acidophilus* (BioLA246) against *E. coli* was categorized as very strong, while its strength against *S. aureus* and *E. faecalis* was categorized as strong.

## Antibiotic susceptibility

The resistance of the isolates to tuberculosis antibiotics have been documented in Table 4 and presented as resistant (R), intermediate (I), and susceptible (S).

# Discussion

Bacterial species are categorized as probiotics when they meet several characteristics. They must be able to survive in gastric pH and bile salts environments. Furthermore, they must also manage to attach and colonize epithelial cells, and provide health benefits for the host. The health benefits referred to are antimicrobial activity, anticancer activity, the effect of lowering toxins, and enhancing the immune system [4, 6, 24].

In this study, survival determination of the isolates in a different pH aims to describe the variability acidity condition of stomach. The pH level an adult's empty stomach is around pH 1–3.7 and in jejunum, ileum around pH 5.5–7.7, 6.6–7.9, respectively. Observation bacteria survival in stomach was done for 3 h in accordance the gastric emptying time around 30–180 min depend on food intake. While median value of the transit time in small intestine and colon are 8.5 and 17.5 h, respectively [25, 26]. This study result indicated that both *Lactobacillus* strains (BioLA246 and BioLR321) had a high tolerance to various pH values. The *Lactobacillus* spp. tolerance to acidic pH was also shown by Soliman et al. [27] where *L. acidophilus* persisted at pH 2.0 for an incubation period of 2 h, then after 3 h its growth was delayed compared to controls (pH 6.5). Other study also shows the survival rates of *L. acidophilus* NCIM 2903 and NCIM 2285 reached 95–96% on pH 2.5 after 2 h of incubation [28]. The *Lactobacillus* species can tolerate low pH conditions through maintaining the pH difference between their cytoplasm and the environment by their unique pH homeostasis via $F_0F_1$-ATPase. A low pH environment induces

**Table 2:** The number of *L. acidophilus* and *L. reuteri* in 0.3% Ox gall.

| Incubation time, hours | Bacterial population, CFU/mL | |
|---|---|---|
| | *L. acidophilus* | *L. reuteri* |
| 0 | $3.2 \times 10^7$ | $3.1 \times 10^7$ |
| 24 | $6.83 \times 10^5 \pm 1.72 \times 10^5$ | $5.87 \times 10^5 \pm 0.47 \times 10^5$ |
| $\Delta(P_{24} - P_0)$[a] | $-3.17 \times 10^7 \pm 0.078 \times 10^7$ | $-3.04 \times 10^7 \pm 0.0047 \times 10^7$ |

[a]Differences before and after 0.3% Ox gall exposure.

$F_0F_1$-ATPase resulting in a proton motive force, which increases intracellular pH and results in pH homeostasis [29–33]. In addition, the acid tolerance ability by bacteria is closely related to strain specifications because the bacteria abilities is highly dependent on the strain [34]. In this research, it was thought that the acid tolerance ability of *L. acidophilus* (BioLA246) and *L. reuteri* (BioLR321) in various pH and incubation times due to the source of the isolates used in this study, where the pH of soursop fruit ranging from 3.7 to 5 [35] and the pH of the cow's milk fermentation process which ranges from 5 to 6 [36].

In order to survive passing through the small intestine, probiotic strains of bacteria must live in the presence of bile salts [37]. Bile salts are the result of conjugation bile acids with the amino acids glycine or taurine in the liver [38]. Microorganisms that cannot adapt to intestinal conditions will experience a very toxic effect of bile. Bacteria have cell membranes with the strong lipophilic properties of steroid rings and thus become the main target of bile. Damage to the bacterial cell membrane caused by bile are disruption of the lipid composition and proton motive force, leading to cell death. The unconjugated form of bile acids is weakly acidic, which diffuses passively into the cell, then accumulates in the cytoplasm, causing cytoplasmic acidification. The *Lactobacillus* spp. developed unique defense mechanisms to avoid the damage that bile can inflict. One of which is the increase in $F_0F_1$-ATPase activity to overcome cytoplasmic acidification. Therefore, the increase in $F_0F_1$-ATPase plays a role in the tolerance of *Lactobacillus* spp. to gastric pH and bile. Other detrimental effects are induces oxidative stress and DNA repair mechanisms, impaired sugar metabolism, and misfolded protein [39–41]. In this study, bile salts were stimulated by 0.3% Ox gall and the pH of the test environment was adjusted to the pH of the intestine (7.2) and exposure for 24 h. The survival rate of both LAB in this study was quite low when compared to other studies. Research conducted by Soliman et al. 2015 showed 93.66% *L. acidophilus* survived after being grown for 24 h in MRS broth with 0.3% Ox gall [27]. Some researchers have found that bile tolerance is strain-specific and that bile tolerance cannot be generalized to the same species. The above statement is supported by studies that show heterogeneity in the ability of various strains of lactic acid bacteria to survive in a supportive bile environment [42]. In several studies using intestinal intubation techniques in participants who consumed fermented products, the amount of *live L. acidophilus* in the ileum was 1–10%, and live *L. reuteri* found in feces was around $10^6$ CFU/g [43]. Based on these data, the viability of *L. acidophilus* and *L. reuteri* in this study was lower than other probiotic products, however, the numbers were still within the range of live bacteria found in the intestine. Therefore, further research is needed by making probiotic preparations from the tested lactobacillus strains, to see their viability in dosage forms.

Another main characteristics of a probiotic that must be met are the ability to inhibit and compete with pathogens so that it can maintain gut microbiota balance. In this study,

**Table 3:** Antimicrobial activity of *L. acidophilus* and *L. reuteri* against pathogenic bacteria.

| Pathogenic bacteria | The inhibition zone (mean ± SD) (mm) | | p-Value |
|---|---|---|---|
| | *L. acidophilus* | *L. reuteri* | |
| *E. coli* | 21 ± 0.5 | 17.33 ± 0.58 | 0.002 |
| *S. aureus* | 19.83 ± 0.58 | 17.67 ± 0.29 | 0.006 |
| *E. faecalis* | 18.67 ± 0.58 | 16.50 ± 0.50 | 0.006 |

**Table 4:** Antibiotic resistance pattern of the *L. acidophilus* and *L. reuteri* towards tuberculosis antibiotics.

| Antibiotics | *L. acidophilus* | *L. reuteri* |
|---|---|---|
| Streptomycin (10 µg/disc) | R | R |
| Ofloxacin (5 µg/disc) | I | S |
| Kanamycin (30 µg/disc) | R | R |
| Amikacin (30 µg/disc) | R | R |
| Meropenem (10 µg/disc) | R | R |
| Linezolid (30 µg/disc) | S | S |
| Moxifloxacin (5 µg/disc) | S | S |
| Levofloxacin (5 µg/disc) | S | S |

R, resistant; S, sensitive; I, intermediate; as per CLSI guidelines. The breakpoints for the antibiotic sensitivity/resistant in mm zone of inhibition: R (≤14 mm), I (15–19 mm), S (>20 mm).

*Lactobacillus acidophilus* and *Lactobacillus reuteri* had a wider inhibition zone in *Escherichia coli* than *S. aureus* and *E. faecalis*. These results indicate their effectiveness in inhibiting the growth of Gram-negative bacteria, compared to Gram-positive bacteria. Based on CLSI (2015), the tested bacteria in this study were able to inhibit the growth of pathogenic bacteria by referring to the resulting inhibition zone diameter > 14 mm [23]. *Lactobacillus acidophilus* was most effective in inhibiting the growth of *E. coli* as indicated by the inhibition zone diameter >20 mm. *Lactobacillus* spp. and *Bifidobacterium* spp. has antimicrobial activity through their ability to compete competitively with pathogens for attachment to the epithelium and mucosa, and inhibits epithelial invasion by pathogens. Other antimicrobial abilities are acquired through the production of antimicrobial substances or metabolites (hydrogen peroxide, lactic acid, bacteriocins), competition for nutrients, and/or stimulating mucosal immunity [44, 45]. Most of the antimicrobial activity against Gram-positive pathogens is the bactericidal effect of protease-sensitive bacteriocins, and the production of organic acids and hydrogen peroxide is an antagonistic effect against Gram-negative pathogens [46]. The increase in lactic acid production that occurs through fermentation causes a decrease in media pH. This condition will prevent pathogens by lowering the intracellular pH, resulting in disruption of vital cell function. The antimicrobial effect is also caused by inseparable acid form and its reducing capacity intracellular pH, causing inhibition of vital cells function [4, 46].

The benefits of *Lactobacillus* in modulating the immune system in tuberculosis conditions are proven by changes in the levels of $CD4^+$, $CD8^+$ T cells, Tregs, and cytokines (IFN-α, TNF-γ, IL-10, IL-12) in drug-sensitive tuberculosis [7–10]. Therefore, for the development of its use in tuberculosis conditions, it is necessary to test the resistance of probiotic bacteria against antibiotics commonly used in these conditions to ensure the effectiveness of their application. The main requirement for probiotic strains is that they do not carry antibiotic resistance genes that can be transmitted by horizontal gene transfer to bacterial recipients in the gut, because it may promote the development of new antibiotic-resistant pathogens [4, 23]. Antibiotic resistance is a concern and is undesirable because it is suspected to be genetically based and there is the potential for transfer to other microorganisms. Lactic acid bacteria, including *Lactobacillus* spp., can experience intrinsic and acquired resistance. Intrinsic resistance to antibiotics is natural resistance, which is encoded in chromosomes, is passed down at the time of division, and is not transferred to other species. This type of resistance can be caused by a certain composition on the cell wall so that it is not easily weakened by antibiotics. In contrast to intrinsic resistance, acquired resistance is encoded in plasmids and can be transferred horizontally to other species. *Lactobacillus* is known to have plasmids, so that antibiotic resistance that occurs in *Lactobacillus* can be transferred simultaneously to other species [4, 22, 23, 46]. Results (Table 4) showed that *Lactobacillus acidophilus* and *Lactobacillus reuteri* were susceptible to linezolid, moxifloxacin and levofloxacin, as well as ofloxacin (only for *Lactobacillus reuteri*), yet resistant to streptomycin, kanamycin, amikacin, and meropenem. *Lactobacillus* spp. resistance to tetracyclines, erythromycin, rifampin, ciprofloxacin, and trimethoprim/sulfamethoxazole has been widely reported [23, 46, 47]. In this research, it is not known whether the resistance is intrinsic or acquired. To ensure both *Lactobacillus* spp. strain are safe for tuberculosis infection, in terms of antibiotic resistance, further research is necessary to look at the potential for resistance gene transfer on other species.

## Conclusions

Based on the acid and bile tolerance test, and their antimicrobial activity and resistance to tuberculosis antibiotics, *Lactobacillus acidophilus* from fermented soursop juice (BioLA246) and *Lactobacillus reuteri* from fermented cow's milk (BioLR321) fulfilled the characteristics as a probiotic candidate and was potentially used as adjunct therapy in tuberculosis drug-resistant.

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Alifia Risma Fahmi, Suharjono* and Kuntaman

# Profile of gyrA gene mutation in clinical isolate of levofloxacin resistant *Escherichia coli*

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**Abstract**

**Objectives:** *Escherichia coli* is one of the pathogenic bacteria that caused a nosocomial infection. Levofloxacin is one of the fluoroquinolones group antibiotics which is a broad-spectrum antibiotic that works effectively against *E. coli*. The mutation can happen in the bacteria which caused a resistant effect in the use of antibacterial. This study aimed at identifying mutation in gene *gyrA* among *E. coli* that were resistant to levofloxacin.
**Methods:** The susceptibility of *E. coli* was determined by disk diffusion. PCR and sequencing were performed to identify the mutation in *gyrA*.
**Results:** A total of 10 isolates showed result resistance to levofloxacin and gyrA gene mutation in the amino acid changes. Nucleotide sequence analysis revealed a point mutation in QRDR (quinolone resistance determining region) of gyrA Ser83→Leu, Asp87→Asn. The silent mutation was also found at codon Val85, Arg91, Ser111, Thr123.
**Conclusions:** Mutation in the *gyrA* gene affect the occurrence of bacterial resistance of *E. coli* to levofloxacin.

**Keywords:** codon; *Escherichia coli*; *gyrA*; levofloxacin; mutation.

## Introduction

A hospital-acquired infection (HAIs) is an infection that patients get while receiving treatment in a hospital or other health unit for 48 h or more after entering the hospital or between 30 days after the patient is discharged from the hospital [1]. According to WHO, nearly 15% of patients treated have nosocomial infections. This infection is the cause of death in neonates by 4–56% [2]. The incidence of this infection is also high in developed countries, around 3.5–12%. One of the bacteria that causes the nosocomial infection is *Escherichia coli*. It plays a role in several diseases such as urinary tract infections, septicemia, pneumonia, neonatal meningitis, peritonitis, and gastroenteritis. Virulence factors of this pathogen are endotoxins, capsules, adhesions, and three types of secretion systems [3]. One of the antibiotics that is frequently used as a therapy for nosocomial infections is the fluoroquinolones in which Levofloxacin is a broad-spectrum antibiotic that works effectively against *E. coli* bacteria [4]. Levofloxacin has been reported to have excellent antimicrobial activity and to be effective for both Gram-positive bacteria and anaerobes, compared to ciprofloxacin [5] Quinolones target two essential enzymes from bacteria: topoisomerase II which is composed of DNA gyrase and DNA topoisomerase IV. Each enzyme is a heterotetramer, with gyrase consisting of *gyrA* and *gyrB* subunits and topoisomerase IV consisting of *parC* and *parE* subunits [6]. Although widely used, levofloxacin resistance is also high. From a study conducted by Jang et al., in 2011 examining the resistance of levofloxacin in *E. coli*, the results were 29.49% in 2005, 26.51% in 2006, 40.21% in 2007, 43, 20% in 2008, and 31.75% in 2009 [7]. The changes to one amino acid either gyrase or topoisomerase IV can cause quinolone resistance [6]. The region where mutations occur in genes that encode fluoroquinolone resistance is short DNA sequences known as quinolone resistance-determining regions (QRDR). The mutations in the QRDR of these genes produce amino acid substitution, change the structure of the target protein and subsequently the affinity of the fluoroquinolone binding enzyme, leading to the occurrence of drug resistance [8]. DNA gyrase mutations in *E. coli* bacteria can occur in *gyrA* and *gyrB*, where mutations in the *gyrA* gene are more common than mutations in the *gyrB* gene. The most common gyrA gene mutation is amino acid substitution Ser83→Leu and Asp87→Asn [4, 9, 10]. In this study, we focused on getting data about the profile gyrA gene mutation from *E. coli* in Indonesia because not much data provide the profile mutation.

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 812 1733 877, E-mail: suharjono@ff.unair.ac.id
**Alifia Risma Fahmi,** Faculty of Pharmacy, Universitas Airlngga, Surabaya, Indonesia
**Kuntaman,** Departement of Clinical Microbiology, Faculty of Medcine, Universitas Airlangga, Surabaya, Indonesia

## Methods

The clinical sample provides from the Microbiology Laboratory of Dr. Soetomo Hospital Surabaya. The sample collected from urine, blood, wound, and sputum sample. Each sample then screens for susceptibility. The susceptibility test of *E. coli* isolates to levofloxacin was performed using BD Phoenix automatic microbiology and continue with the standard disk diffusion method according to the CLSI guideline 2007. *E. coli* ATCC 25922 strain was used as the quality control. The range of diameter zone inhibition according to CLSI was resistance ≤13 mm, intermediate 14–16 mm and susceptible ≥17 mm. The procedure to determine the profile gyrA gene mutation from *E. coli* clinical isolate was an extraction of DNA using the Extra-N ampiTM Blood PCR Kit, then followed by Polymerase Chain Reaction (PCR) for gene gyrA. The primer used for gyrA were F-5′TGC CAG ATG TCC GAG AT3′ and R-5′GTA TAA CGC ATT GCC GC3′. Then the process of electrophoresis was performed on 2% agarose in Tris Borate solution. Labeling was using PCR QiaQuick Column (QIAGEN, Valecia, CA) and DNA sequencing was using the ABI Prism 310 Genetic Analyzer (Applied Biosystem, Forter City, USA) [11].

## Results

There are 10 clinical isolates of *E. coli* that are resistant to levofloxacin. The antibiotic susceptibility and zone diameter if inhibition of levofloxacin for 10 isolates are presented in Table 1. All the sample tests used the electrophoresis method. Gel electrophoresis is a technique used to separate DNA fragments according to their size. DNA samples are loaded into wells (indentations) at one end of a gel, and an electric current is applied to pull them through the gel. DNA fragments are negatively charged, so they move toward the positive electrode. The results of the gyrA gene electrophoresis produces a single band at 268 bp. The amplification result of gyrA is displayed in Figure 1.

**Table 1:** Sensitivity test results.

| No | Sample code | Isolate code | Susceptibility test Zone diameter of inhibition, mm |
|---|---|---|---|
| 1 | 1-gyr | 364 | R (6) |
| 2 | 2-gyr | 392 | R (7) |
| 3 | 3-gyr | 449 | R (9) |
| 4 | 4-gyr | 612 | R (12) |
| 5 | 5-gyr | 446 | R (9) |
| 6 | 6-gyr | 10,365 | R (7) |
| 7 | 7-gyr | 10,370 | R (8) |
| 8 | 8-gyr | 10,737 | R (6) |
| 9 | 10-gyr | 10,909 | R (8) |
| 10 | 11-gyr | 10,925 | R (5) |
| 11 | 9-gyr | ATCC *E. coli* | S (3)6 |

R, resistance; S, susceptible.

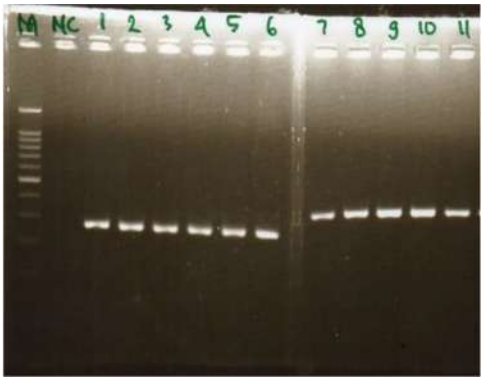


**Figure 1:** Electrophoresis result gyrA gene produce single band ar 268 bp.

The results of sequencing analysis demonstrate mutations from 10 *E. coli* isolates that are resistant to levofloxacin showed in Table 2. Mutations that show amino acid changes (missense mutation) occur in all isolates that happen in codon 83 (C→T transition codon TCG) where there is a change in serine to leucine and codon 87 (G→A transition codon GAC) where there is a change in aspartic acid to asparagine. The silent mutation where there is no change in amino acids is also found in sample number 1,2,3,4,6,7,8,10,11 (Val85, Arg91 and Ser111) and sample number 1,2,3,4,5,6,7,8,10,11 (Tre123) [Table 2].

## Discussion

Based on the research on *E. coli* bacteria for quinolone resistance determining regions (QRDR) for the gyrA gene, amino acid substitution occurs in codon 67–106. The occurrence of amino acid substitution in these areas contributes to the occurrence of resistance in *E. coli* [12, 13]. In gyrA *E. coli*, mutations in two codons, 83 and 87, show the most frequent results [14]. A previous study conducted by Wang et al. in 2001 revealed that all 30 *E. coli* clinical isolates studied experienced mutations in S83L and D87N [15]. Also, the research conducted by El-Sokkary et al. in 2015 exhibited that all of 17 isolates with high resistance to levofloxacin had mutations in the S83L code and 99.3% had mutations in the D87N code. Another study was also conducted by Zayed et al. in 2015, which showed the results of 130 samples studied, 80% of the samples experienced mutations in S83L, 70% experienced mutations in D87N and mutations outside the QRDR area were also found [9]. The publication in 2018 researched 240 samples of which 27.5% were positive ESBL. All isolates showed mutations in codon 83 (C→T in the nucleotide base TCG). 93.3% showed mutations in codon 87 (G→A in the nucleotide base GAC) [16].

**Table 2:** Mutation in gyrA gene.

| No | Isolate number | Codon position | Change in nucleotide base | Amino acid change | Percentage, % |
|---|---|---|---|---|---|
| 1 | 1,2,3,4,5,6,7,8,10,11 | 83 | TCG→TTG | Serine→Leucine | 100 |
| 2 | 1,2,3,4,6,7,8,10,11 | 85 | GTC→GTT | Valine→Valine | 90 |
| 3 | 1,2,3,4,5,6,7,8,10,11 | 87 | GAC→AAC | Asparatic acid → Asparagine | 100 |
| 4 | 1,2,3,4,6,7,8,10,11 | 91 | CGC→CGT | Arginin→Arginin | 90 |
| 5 | 1,2,3,4,6,7,8,10,11 | 111 | TCT→TCC | Serine→Serine | 90 |
| 6 | 1,2,3,4,5,6,7,8,10,11 | 123 | ACG→ACA | Treonin→Treonin | 100 |

In this study, the results of sequencing of the gyrA gene QRDR show mutations in two positions, serine-83 and aspartic acid-87, which are similar to previous studies. The mutations in codons 83 and 87 in gyrA are the mutations that mostly occur in *E. coli* which are resistant to fluoroquinolones [17]. Multiple mutations can cause increased resistance than a single mutation. It happens because of the loss of hydrogen bonds due to reduced binding energy in bacteria [10]

This research discovers two types of mutations, namely missense mutation in codons 83 and 87, and silent mutations in codons 85, 91, 111 and 123. A silent mutation occurs when there is the change of a single DNA nucleotide within a protein-coding portion of a gene that does not affect the sequence of amino acids that make up the gene's protein. No research certainly mentions that silent mutation has a role in the occurrence of resistance in bacteria in mutations in the form of silent mutations where nucleotide changes occur but there is no change in amino. However, there is a possibility that this mutation has a role in the occurrence of resistance as in the research conducted by Kimchi and Sarfaty which affirms that silent mutation occupies a position where nucleotide triplets are not frequently used by cells. It causes a slowdown in the process of making proteins by the cells. The slow process of protein making can cause changes in the final result. The cell itself can compensate if there is only one silent mutation that occurs but not if there is more [18].

## Conclusion

All 10 isolates of *E. coli* phenotypically show resistance to levofloxacin and experience missense mutation in the gyrA gene, namely Ser83 to Leu (100%) and Asp87 to Asn (100%). There are also silent mutations on codons Val85, Arg92, Ser111, and Tre12. Levofloxacin is one of the antibacterial that used to treat *E. coli* infection. This study demonstrates that resistance to these drugs occurs through mutation in this bacteria.

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Siti Mudaliana*

# Antimicrobial activity of *Centella asiatica* and *Gigantochloa apus*

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**Abstract**

**Objectives:** Antibiotic treatments can create multi-drug resistance among several pathogens. There is a need for an antibiotic alternative to overcome this problem. In Indonesia, *Centella asiatica* (Asiatic pennywort) and *Gigantochloa apus* (string bamboo) are two common medicinal plants used to treat tuberculosis, diarrhea, and other symptoms. This study was done to compare the antimicrobial activity of *C. asiatica* and *G. apus* against five pathogenic bacteria, i.e., *Mycobacterium tuberculosis* H37Rv strain, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhi*.

**Methods:** The ethanol extracts of *C. asiatica*, and *G. apus* shoot were obtained by using speed extractor, pressure, and temperature extraction. The phytochemical contents of each extract were screened. The ethanol extract's antimycobacterial activity was determined using Lowenstein Jensen (LJ) medium and antibacterial activity was determined using Kirby–Bauer methods on Mueller Hinton agar (MHA).

**Results:** The phytochemical analysis showed that *G. apus* extract contains alkaloids and tannins, whereas *C. asiatica* extract contains flavonoids, alkaloids, saponins, and tannins. This study showed that *G. apus* inhibited the growth of *M. tuberculosis* H37Rv strain and *S. typhi*. *C. asiatica* showed antimicrobial activity against all pathogenic bacteria tested, except *B. subtilis*.

**Conclusions:** Both medicinal plants extract can inhibit the growth of five pathogenic bacteria tested, thus, have the potential as an alternative treatment, or complementary, to treat the pathogenic bacterial infection.

**Keywords:** antibacterial; antimycobacterium; *C. asiatica*; drug; *G. apus*.

***Corresponding author: Siti mudaliana**, Laboratory Herbal of Materia Medica Batu, Public Health Office of East Java Province, Batu, Indonesia, Phone: +62 812 8012 1028, E-mail: mudaliana@gmail.com

## Introduction

Tuberculosis (TB), a respiratory disease caused by a pathogenic bacterial infection, remains a serious health problem, especially in developing countries. In 2017, WHO showed increasing TB cases worldwide compared to the last five years with total 10 million cases, mainly occurred in Southeast Asia (44.4%) and Africa (24.8%). Indonesia is the country with the fourth-largest TB cases incidence after India, China, and South Africa. In their 2017 report, WHO estimated 318 TB incidences per 100,000 populations, with a death rate of 40 per 100,000 people in Indonesia [1–23]. The Ministry of Health of the Republic of Indonesia through Basic Health Research Riskesdas [2] also showed that other infectious diseases, particularly respiratory tract disease and diarrhea, are still a serious health problem in Indonesia.

The high incidence of TB was partly due to the emergence of drug-resistant strains of pathogenic bacteria *Mycobacterium* (MDR-TB). This situation calls for new "drug" alternatives to treat TB effectively. For centuries, Indonesian people have been using medicinal plants to treat various diseases, including the ones caused by bacterial infections such as TB. Often, people use medicinal plants to treat mild diseases, e.g., diarrhea and eczema, since it is easily accessible and cheap, particularly for people living in a remote area and lower-middle-class family [2]. Several medicinal plants have been used to treat or reduce TB symptoms, including the herb Asian pennywort (Indonesian: pegagan, gotu kola, cica) or *Centella asiatica* (L.) Urb. and shoots of string bamboo (Indonesian: bambu apus, bung abus) or *Gigantochloa apus* (Schult.) Kurz. In Indonesia, e.g., at East Java, both plants are used as TB medicine by boiling it and drinking the aqueous extract or cooking it and consuming it as a side dish.

A study of *C. asiatica* showed the cognitive enhancing effect and antioxidant activity of its aqueous extract [3]. Another experiment showed the antibacterial and antifungal activity of *C. asiatica* extract [4]. However, these studies did not mention exactly the concentration administered for the antibacterial treatment. *C. asiatica* extract also showed better performance against MDR strains compared to antibiotic rifampicin [5]. On the other hand, only a few recent studies of antimicrobial activity of

*G. apus*, e.g., Indriatie et al. [6] showed the effect of ethanolic extract of *G. apus* in inhibiting the growth of *Escherichia coli* and *Staphylococcus aureus*. This research was performed to explore and compare the antibacterial and antimycobacterial effects of *C. asiatica* and *G. apus*.

# Materials and methods

### Extract preparation

The ethanol extracts of the herb *C. asiatica* (L.) Urb. and shoots of bamboo *G. apus* (Schult.) Kurz were obtained using fast pressurized solvent extraction (Buchi, Switzerland). Fine plant powder was extracted in 100 mL absolute ethanol (Merck, Germany) at a temperature of 50 °C (100 bar). Based on the previous experiment, the use of absolute ethanol as a solvent in a fast pressurized solvent extraction has resulted in a high extract yield. Besides that, other studies also showed ethanol as the best solvent to extract antimicrobial properties of *C. asiatica* [7, 8]. A rotary evaporator (Buchi, Switzerland) was used to distill the extract in a water bath set at 60 °C. The dried extracts obtained were stored in a refrigerator at 4 °C until being used. Dried extracts were dissolved and diluted with 1% DMSO (Merck, Germany) to make a 50 mg/mL of stock solution, then sterilized by filtration through a 0.2 µm cellulose acetate membrane filter (Whatman, USA and Sartorius, USA). Three different concentrations, i.e., 1.25, 2.5, and 5.0 mg/mL of each extracts were prepared.

### Phytochemicals analysis

The plant extract was screened for the presence of flavonoids, alkaloids, saponins, and tannins using the standard procedures as described previously [9]. The dried extract was dissolved in distilled water. Then, 1.0 mL of sample (dissolved extract) was added with a reagent according to the subjected test, as described below.

**Flavonoid analysis:** The sample was heated for 5 min then added with 10 mg of magnesium, followed by dropwise addition of 38% HCl (Merck, Germany). The positive flavonoid was detected due to the change of color in the solution from orange to red.

**Alkaloid analysis:** The sample in a reaction tube was added with Mayer's reagent (Merck, Germany). The different tubes were also prepared and added each with Dragendorf reagent (Merck, Germany) and Bouchardat's reagent (Merck, Germany), respectively. The formation of precipitate indicated alkaloid presence in the sample.

**Saponin analysis:** The sample was added with 1.0 mL of hot water and three drops of 35% HCl (Merck, Germany), then shaken hardly. The formation of stable foam indicated the presence of saponin.

**Tannin analysis:** A 1.0 mL of sample was heated for 5 min and a sufficient amount of 1.0% of iron (III) chloride solution (Merck, Germany) was added. The color of greenish brown or blackish-blue indicated the presence of tannin.

### Antibacterial activity test

Antibacterial activity test was carried out with the Kirby–Bauer disk diffusion susceptibility test as described previously. Bacteria used were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and two other bacteria obtained from FK UB (Malang, Indonesia), i.e., *Bacillus subtilis* and *Salmonella typhi*. Paper discs (Oxoid, UK) were immersed into samples with a concentration of 1.25; 2.5; 5.0 mg/mL, a 5 mg/mL gentamicin (Indofarma, Indonesia) as a positive control (PC), and 1% DMSO in sterilized $dH_2O$ as a negative control (NC), then placed onto Mueller Hinton agar (MHA; Oxoid, UK) inoculated with tested bacteria. Experiments were done in triplicate. Incubation was performed at 37 °C for 24 h. Inhibition zones around the paper disc were measured using a digital caliper [10].

### Antimycobacterial activity test

Antimycobacterial activity tests were conducted at The Institute of Tropical Diseases, Universitas Airlangga (ITD). Three different stock solution of extracts were added with medium Lowenstein Jensen (LJ; Oxoid, UK), i.e., 250 µL stock and 9.75 mL LJ; 500 µL stock and 9.5 mL LJ; and 1 mL stock and 9 mL LJ to create a concentration of 1.25; 2.5; and 5.0 mg/mL respectively. As a positive control, 40 µg/mL rifampicin (Sanbe, Indonesia) on LJ was used. Then, 7 mL of mixed extract-LJ were poured into a sterilized McCartney bottle, coagulated at 85 °C for 45 min, and stored at room temperature for 24 h. Then, extracts were challenged against *Mycobacterium tuberculosis* strain H37Rv. Incubation was performed at the temperature of 37 °C for four weeks [5].

# Results

The identification of phytochemical constituents from the tested extracts showed that *C. asiatica* contained flavonoids, alkaloids, saponins, and tannins (Table 1). Meanwhile, the shoot extract of *G. apus* only consisted of alkaloids and tannins (Table 1). The antibacterial activity test of *C. asiatica* showed growth inhibition of *E. coli* and *S. typhi* at a concentration of 5 mg/mL and *S. aureus* at a concentration ≥2.5 mg/mL (Table 2). Among all bacteria tested, *G. apus* only showed inhibition effect against *S. typhi*, however, this effect appears in a lower *G. apus* concentration (1.25; 2.5; and 5 mg/mL) compared to *C. asiatica* (5 mg/mL; Table 2).

**Table 1:** Phytochemical contents of *C. asiatica* and *G. apus* ethanolic extract.

| Chemical constituents | *C. asiatica* | *G. apus* |
|---|---|---|
| Flavonoids | Yes | No |
| Alkaloids | Yes | Yes |
| Saponins | Yes | No |
| Tannins | Yes | Yes |

**Table 2:** Antibacterial activity of *C. asiatica* and *G. apus* ethanolic extract.

| Concentrations of extract | Average inhibition zone, mm[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *C. asiatica* | | | | *G. apus* | | | |
| | *E. coli* | *S. aureus* | *B. subtilis* | *S. typhi* | *E. coli* | *S. aureus* | *B. subtilis* | *S. typhi* |
| 1.25, mg/mL | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.55 |
| 2.5, mg/mL | 0 | 2.39 | 0 | 0 | 0 | 0 | 0 | 3.30 |
| 5, mg/mL | 3.63 | 3.60 | 0 | 8.54 | 0 | 0 | 0 | 4.29 |
| PC[b] | 14.96 | 11.81 | 18.71 | 19.12 | 11.53 | 10.32 | 19.41 | 19.46 |
| NC[c] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]n=3; [b]PC, positive control, gentamicin 5 mg/mL; [c]NC, negative control, 1% DMSO in sterilized $dH_2O$.

The result from the antimycobacterial activity test showed that both LJ-medium supplemented with *C. asiatica* and *G. apus* inhibit the growth of *Mycobacterium* (Table 3). A better performance was shown by *C. asiatica* extract where the growth of *Mycobacterium* was inhibited even in the lowest dose used. *G. apus* extract only inhibited the growth of *Mycobacterium* when the highest dose of 5 mg/mL was administered. These results shown that the minimum dose to inhibit the *Mycobacterium* growth was 1.25 mg/mL for *C. asiatica* and 5 mg/mL for *G. apus*.

## Discussion

*C. asiatica* has been known for its wide use from medicinal properties to treat several diseases to bioactive substances used in the cosmetics industry [11]. Possibly, the chemical properties of *C. asiatica* that contain relatively complete phytochemical constituents contribute to its wide purposes. Indeed, this study showed various secondary metabolites contents of *C. asiatica* such as flavonoids, alkaloids, saponins, and tannins. Because of its chemical contents, *C. asiatica* has been used over decades to cure various diseases and health symptoms, e.g., wound healing, gastric ulcer, diarrhea, antidepressant, cognitive enhancement, and TB. The use of *C. asiatica* in East Java for wound healing has been scientifically supported by some studies [12, 13]. In this study, *C. asiatica* inhibits the growth of *S. aureus*, which is a bacterium present in the human skin (microbiome) and can cause skin infection [14]. In some Indonesia areas, *C. asiatica* has been traditionally used to treat mild diarrhea symptoms [15]. Indeed, our study also shows *C. asiatica* antibacterial activity against *E. coli*, a common pathogenic bacterium causing diarrhea. A study by Nasution et al. (2018) also showed that *C. asiatica* successfully inhibited the growth of six bacteria, three fungi, and one yeast, i.e., *E. coli*, *S. aureus*, *Staphylococcus albus*, *Staphylococcus pyogenes*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia*, *Aspergillus niger*, *Aspergillus flavus*, *Microsporium boulardii*, and *Candida albicans* [7]. For typhoid disease, *C. asiatica* is rarely found to manage the *Salmonella* infection. A study in animal models conducted by Besung et al. [16] showed that *C. asiatica* treatment significantly increased the level of IL-6, which is related to typhoid disease. In this study, *C. asiatica* did not show any effect on *B. subtilis* growth. Another relevant study showed that *C. asiatica* effectively inhibited the *B. subtilis* growth [7]. However, a study by Idris et al. [8] did not mention the strain of bacteria tested and *C. asiatica* was also administered in a higher concentration (30, 70, and 100%). Even though the antibacterial activity of *C. asiatica* is relatively lower compared to antibiotic gentamicin, but the practical function of *C. asiatica* cannot be arbitrarily neglected. Regarding its property in the ethnomedicine studies, which is widely used to cure some infectious diseases, especially in the remote area or middle-lower class family, a scientific study of *C. asiatica* is needed [17, 18].

Commonly, antibiotic rifampicin is administered to treat TB patients. However, this antibiotic has been known to cause several disadvantage side effects, including resistance, gastric ulcer, etc. To counter this side effect, some Indonesian people prefer to use medicinal plants

**Table 3:** Antimycobacterial activity of *C. asiatica* and *G. apus* ethanolic extract.

| Concentrations of extract | Mycobacterial growth[a] | |
|---|---|---|
| | *C. asiatica* | *G. apus* |
| 1.25, mg/mL | No | Yes |
| 2.5, mg/mL | No | Yes |
| 5, mg/mL | No | No |
| PC[b] | No | No |
| NC[c] | Yes | Yes |

[a]n=2; [b]PC, positive control, rifampicin 40 µg/mL; [c]NC, negative control, medium LJ only.

instead. Several studies showed that *C. asiatica* can be used to reduce the symptoms of TB. The nonpolar fraction of methanol extract of *C. asiatica* presented significant growth inhibition against *M. chelonae*, a nonrelevant tuberculosis *Mycobacterium* [19], also the aqueous extract of *C. asiatica* significantly demonstrated an anti-mycobacterial activity against standard strain H37Rv and/or MDR strain of *Mycobacterium tuberculosis* [5].

Phytochemical screening of *G. apus* shoot revealed that this plant only contains alkaloids and tannins. This study showed that *G. apus* has the potential to treat the *Mycobacterium tuberculosis* infection, even though it needs to be administered in a higher concentration than *C. asiatica*. Further study on antibacterial test showed its efficacy to inhibit only *S. typhi* from three other bacteria tested. This suggests that *G. apus* can be used for treating typhoid disease besides TB. This is a novel finding since the previous study of *G. apus* and its activity against *Salmonella* or *Mycobacterium* did not exist.

The antimicrobial activity of *C. asiatica* and *G. apus* is possibly related to their chemical constituents, i.e., alkaloids and tannins. A recent study of alkaloids derived from plants demonstrated an insightful and promising result [20]. Tannins acted as an antibacterial by their capability to bind iron. The aerobic microbial activity needs iron to perform variety of functions, e.g., reduction of the precursor of DNA formation; formation of heme, an iron-containing cofactor essentially important for protein function; etc. Moreover, tannic acid present in the plant extract may inhibit the growth of bacteria through its strong iron-binding capacity [21]. Furthermore, flavonoids and alkaloids, which were both phytochemical constituents of *C. asiatica* and *G. apus*, evidently have antibacterial activity. Flavonoids and alkaloids were found to exhibit antibacterial and antibiofilm activity against bacteria *S. aureus* and *B. subtilis* [22], also against *P. aeruginosa* and *B. subtilis* [23].

## Conclusions

In conclusion, extract of *C. asiatica* and *G. apus* can inhibit the growth of five pathogenic bacteria tested, thus, have the potential as an alternative treatment, or complementary, to treat the pathogenic bacterial infection.

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**Ethical approval:** This research conducted *in vitro* and did not involve any animals or human or any respondents.

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Fivy Kurniawati*, Nanang Munif Yasin, Farida Aulia and Gidfrie Vinanda Krisha

# Drug-related problems of antibiotic use in gastroenteritis related to patient therapy outcomes at Universitas Gadjah Mada Hospital

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**Abstract**

**Objectives:** Gastroenteritis is a disease of digestive system commonly occur among the people. Some cases of gastroenteritis are caused by bacteria, so it is treated by using antibiotics. Inappropriate use of antibiotics can be associated to Drug-Related Problems (DRPs). This study aims to identify patterns of potential DRPs of antibiotic use and analyze the effect of potential DRPs of antibiotic use toward the patient's therapeutic outcomes and length of stay.
**Methods:** This is a retrospective cross-sectional study carried out by using patient's medical record. The study population was gastroenteritis patients at the inpatient ward of Universitas Gadjah Mada Hospital during January 2018–June 2019. Then, SPSS was employed to analyze the data and the effect of potential DRPs toward therapeutic outcomes was analyzed by utilizing the chi-square method.
**Results:** More than half of gastroenteritis patients in Universitas Gadjah Mada Hospital were identified to have potential DRPs of antibiotic use. The most identified of potential DRPs was problems related to drug selection. Based on the chi-square analysis, there was no relationship between potential DRPs of antibiotic use and the therapeutic outcome. In addition, there was also no relationship between potential DRPs of antibiotic use and patient's length of stay.
**Conclusions:** The potential DRPs of antibiotics use do not have a significant effect on the therapeutic outcome and length of stay of the gastroenteritis patients in Universitas Gadjah Mada Hospital.

***Corresponding author: Fivy Kurniawati,** Pharmacology and Clinical Pharmacy Department, Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia, E-mail: fivy_k@ugm.ac.id. https://orcid.org/0000-0002-6748-9504
**Nanang Munif Yasin,** Pharmacology and Clinical Pharmacy Department, Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia
**Farida Aulia and Gidfrie Vinanda Krisha,** Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia

**Keywords:** antibiotic; DRPs; gastroenteritis; Universitas Gadjah Mada Hospital.

## Introduction

Gastroenteritis is a disease of the digestive system which is commonly found both in developed and developing countries. This disease can be transmitted through contaminated food and drink, contaminated water, travel to and from affected areas, interactions with animals, and transmission obtained at health care facilities [1]. This disease is one of the highest contributors to morbidity in various countries, especially in developing countries with relatively low levels of sanitation like Indonesia [2].

Gastroenteritis is also regarded as an infectious disease that can be caused by viruses, bacteria, or protozoa [1]. Thus, the primary management of infectious gastroenteritis includes identification of infectious microbes, empirical therapy with antimicrobials, and monitoring of the patient's response to infection [3]. Antibiotics are the most widely used antimicrobials as empirical therapy in various health services and has become a major concern of the medical world [4].

Inappropriate antibiotic use can cause Adverse Drug Reactions (ADRs); for instance, aminoglycoside can cause nephrotoxicity, can block the neuromuscular activity, and ototoxicity [5]. The high risk for resistance, ADRs, and the decreasing effectiveness of antibiotics can be associated with the Drug-Related Problems (DRPs) [6]. Drugs with special administration rules, such as antibiotics, opioids, diuretics, anticoagulants, and cardiovascular drugs, are prone to DRPs cases [7].

DRPs are unwanted occurrence related to drug use, both actual and potential, which affect the patient's health outcomes. Most cases of DRPs are predictable and preventable. The frequency of DRPs cases can be minimized through pharmacist intervention [8]. Therefore, a number of developing countries have begun to increase the role of clinical pharmacists and communities to increase the rationality of antibiotic use [9].

This study highlights the patterns of potential DRPs of antibiotic use and its effects toward the therapeutic

outcomes of pediatric and adult gastroenteritis patients. The data from this research is expected to be an evaluation material for related parties in treating gastroenteritis by using antibiotics. Thus, adverse effects of DRPs antibiotic use can be suppressed.

## Materials and methods

### Study design

This is a retrospective cross-sectional study carried out by using the patient's medical record. The sampling was performed by applying purposive nonrandom sampling method. Ethical approval of this study was obtained from Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada – Dr. Sardjito General Hospital, with approval number KE/FK/0405/EC/2019.

### Clinical samples

The population of this study was gastroenteritis patients at the inpatient ward, Universitas Gadjah Mada Hospital Yogyakarta during January 2018–June 2019. The subjects of this study were gastroenteritis patients diagnosed by clinician with ICD 10 A09.9 (gastroenteritis and colitis of unspecified origin) and received empirical antibiotic therapy. ICD 10 is the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD), a medical classification list by the World Health Organization (WHO). Patients diagnosed with other bacterial infections and incomplete medical record related to socio-demographic characteristics, disease's characteristics, drug administration lists, and patient's vital signs during hospitalization were excluded from the study.

### Data analysis

The data were analyzed by using Statistical Package for the Social Sciences (SPSS). The patient's characteristics consisting of socio-demographic characteristics and medication characteristics were analyzed descriptively and were presented in the form of frequency and percentage. Types of potential DRPs were classified by using Classification for Drug Related Problems V8.03 by Pharmaceutical Care Network Europe and were presented descriptively in the form of percentage. The gastroenteritis management guidelines applied in this study were the 2017 Infectious Diseases Society of America (IDSA) Clinical Practice Guidelines for the Diagnosis and Management of Infectious Diarrhea [1] and Clinical Practice Guidance from Universitas Gadjah Mada Hospital on 2015. The therapeutic outcome of antibiotic use was analyzed descriptively by utilizing the vital sign data (heart rate, body temperature, and respiration rate) and patient's discharge status as enforced by clinician. The therapeutic outcome was presented in the form of frequency and percentage. The relation of potential DRPs of antibiotic use toward patients' therapeutic outcomes was analyzed by employing the chi-square method.

## Results

The number of samples in this study was 191 patients. Socio-demographics characteristics of research subjects can be seen in Table 1. The results shows that 101 subjects (52.9%) were male; the total of 71 subjects (37.2%) were 0–5 years old; and the majority of the research subjects (82.2%) were participants of the national health insurance.

Table 1 also shows that there were 86 patients who underwent hospitalization for 4–5 days (46.39%). More than half of the subjects (65.5%) used 4–7 items of medicine and 144 subjects (75.4%) received one item of antibiotics. The most nonantibiotic drugs prescribed were supplement (21.78%), antiemetic (16.78%), and antipyretic (14.17%), which can be seen in Table 2. Meanwhile, Table 3 shows that the proportions of the most commonly used antibiotics were ceftriaxone (20.1%), levofloxacin (16.9%), and cefotaxime (15.3%).

Table 4 presents that at least there were 133 of 191 patients (69.6%) identified with potential DRPs of antibiotic use. The number of identified potential DRPs was 139 cases. The types of DRPs that can be analyzed from the

**Table 1:** Socio-demographic and treatment characteristics of research subjects.

| Variable | Category | Frequency | Percentage, % |
|---|---|---|---|
| Sex | Male | 101 | 52.9 |
| | Female | 90 | 37.1 |
| Age category, years | 0–5 | 71 | 37.2 |
| | 6–11 | 18 | 9.4 |
| | 12–16 | 4 | 2.1 |
| | 17 | 3 | 1.6 |
| | 18–35 | 26 | 13.6 |
| | 36–64 | 46 | 24.1 |
| | 65–74 | 10 | 5.2 |
| | ≥75 | 13 | 6.8 |
| Health payment | Health insurance | 157 | 82.2 |
| | Non health insurance | 34 | 17.8 |
| Length of stay, days | 2–3 | 75 | 39.3 |
| | 4–5 | 86 | 45.0 |
| | 6–7 | 26 | 13.6 |
| | ≥8 | 4 | 2.1 |
| Total drugs used (items) | <4 | 8 | 4.2 |
| | 4–7 | 125 | 65.5 |
| | 8–11 | 52 | 27.2 |
| | >11 | 6 | 3.1 |
| Total antibiotic used (items) | 1 | 144 | 75.4 |
| | 2 | 38 | 19.9 |
| | 3 | 7 | 3.7 |
| | 4 | 2 | 1.0 |

**Table 2:** Distribution of nonantibiotic drugs prescribed.

| Variable | Frequency | Percentage, % |
|---|---|---|
| Supplement | 186 | 21.78 |
| Antiemetic | 139 | 16.28 |
| Antipyretic | 121 | 14.17 |
| Antidiarrheal | 111 | 13.00 |
| Antiulcer | 104 | 12.18 |
| Antiasthma | 20 | 2.34 |
| Oral rehydration | 19 | 2.22 |
| Anticonvulsant | 18 | 2.11 |
| Corticosteroid | 14 | 1.64 |
| Antihistamine | 9 | 1.05 |
| Others | 113 | 13.23 |
| Total | 854 | 100 |

patients' medical record were the domain of drug selection, the drug form, the drug dose selection. The drug selection domain included drug discrepancies according to the guidelines or formularies, patients contraindicated with drugs, and drug administration without indication. Potential DRPs in the drug form included inappropriate drug forms. Meanwhile the drug dose selection included too high dose and too low dose. Most of the patients had the potential to experience DRPs from the drug selection domain as many as 98 cases (70.5%), followed by potential DRPs from the dose selection domain as many as 29 cases (20.9%), and the drug form domain as many as 12 cases (8.6%).

Table 5 shows the majority of potential DRPs in the drug selection domain were caused by drug discrepancies according to guidelines or formularies 93 subjects (66.9%), drug administration without indication four subjects (2.9%), drug inappropriate due to patient contraindications one subject (0.7%). Table 5 also shows the potential causes of DRPs in the dose selection domain can be grouped into too low dose and too high dose. Potential DRPs due to too low dose occurred in 14 subjects (10.1%), whereas potential DRPs due to too high dose occurred in 15 subjects (10.8%).

**Table 4:** The percentage of potential DRPs of antibiotic use.

| Variable | Frequency | Percentage, % |
|---|---|---|
| Drug selection | 98 | 70.5 |
| Drug form | 12 | 8.6 |
| Dose selection | 29 | 20.9 |
| Total | 139 | 100 |

**Table 5:** The percentage of causes of potential DRPs in antibiotic use.

| Domain | Variable | Frequency | Percentage, % |
|---|---|---|---|
| Drug selection | Drug discrepancies according to guideline or formulary | 93 | 66.9 |
| | Drug administration without indication | 4 | 2.9 |
| | Patients contraindicated with drugs | 1 | 0.7 |
| Drug form | Inappropriate drug form | 12 | 8.6 |
| Dose selection | Drug dose too high | 15 | 10.8 |
| | Drug dose too low | 14 | 10.1 |

**Table 3:** The distribution of antibiotic use.

| Antibiotic classification | Category | Frequency | Percentage, % |
|---|---|---|---|
| Second generation cephalosporin | Ceftriaxone | 50 | 20.1 |
| | Cefotaxime | 38 | 15.3 |
| | Cefuroxime | 2 | 0.8 |
| Third generation cephalosporin | Cefixime | 21 | 8.4 |
| | Cefoperazone sublactam | 6 | 2.4 |
| | Cefoperazone | 1 | 0.4 |
| | Ceftazidime | 1 | 0.4 |
| Penicillin | Ampicillin sublactam | 12 | 4.8 |
| | Ampicillin | 11 | 4.4 |
| | Amoxicillin | 2 | 0.8 |
| Fluoroquinolone | Levofloxacin | 42 | 16.9 |
| | Ciprofloxacin | 7 | 2.8 |
| Aminoglycoside | Gentamicin | 9 | 3.6 |
| | Amikacin | 1 | 0.4 |
| Nitroimidazole | Metronidazole | 28 | 11.2 |
| Sulfamethoxazole + trimethoprime | Cotrimoxazole | 18 | 7.2 |

The effect of potential DRPs on antibiotic use on gastroenteritis patients' treatment outcome was analyzed by chi-square in 191 subjects. The result can be seen in Table 6. The patient's therapeutic outcome was categorized into two groups: improving and not improving, while potential DRPs was also categorized into two groups: DRPs and no DRPs. Based on the chi-square analysis, the p-value is 0.204. This study also investigated the effect of potential DRPs on antibiotic use on length of stay in gastroenteritis patients by chi-square analysis. The length of stay was classified into two categories, namely ≤4 days and >4 days. The grouping is based on the average length of stay of patients. Based on the chi-square analysis, the p-value is 0.235.

## Discussion

This study indicates that at least 133 of 191 subjects (69.6%) experienced DRPs. The number of potential DRPs cases observed was 139 cases. Some gastroenteritis patients had more than one potential of DRPs cases. This result is in line with a similar study in Indonesia, stating there are about 40–62% of inappropriate use of antibiotics, some of which are related to diseases that do not need antibiotics. In research related to the quality of antibiotic use in various parts of the hospital, 30–80% of problems with antibiotic use are indicated [10]. This result is lower than a similar study conducted in Ethiopia which stated that the number of DRPs using antimicrobials reached 75.7% [11]. The high number of potential DRPs cases in this study can be attributed to the fact that antibiotics are drugs with special administration rules [7].

Based on appropriateness toward gastroenteritis guideline by IDSA in 2017 and Clinical Practice Guidelines Universitas Gadjah Mada Hospital in 2015, there were 93 of 139 (66.9%) potential DRPs of antibiotic use. These results are comparable to that of a retrospective study in Cape Town, South Africa which stated that the noncompliance rate of antibiotic prescribing based on guidelines or formularies reached 67.9% of 449 patients (95%, CI: 27.90–36.55). Several factors can influence antibiotic mismatch based on guidelines, including the availability of related facilities or drugs, the patient's physiological condition related to the type of antibiotic, and a nonspecific diagnosis [12].

**Table 6:** Relationship of potential DRPs of antibiotic use toward patients therapy outcomes and length of stay.

| Variable | Category | Potential DRPs | | p-Value |
|---|---|---|---|---|
| | | DRPs | No DRPs | |
| Outcome | Improving | 97 | 37 | 0.204 |
| | Not improving | 36 | 21 | |
| Length of stay | ≤4 days | 92 | 45 | 0.235 |
| | ≥4 days | 41 | 13 | |

DRPs, Drug-Related Problems.

As many as four out of 139 DRPs cases (2.9%) were DRPs caused by drug administration without indication. Gastroenteritis patients who can be given empiric antibiotic therapy are patients with the following indications: 1) symptoms of Shigellosis (high-frequency bloody stool, fever, abdominal pain, tenesmus), 2) traveler's diarrhea accompanied by high fever more than 38.5 °C, 3) symptoms suggestive of sepsis, 4) patients with low immune systems, 5) the presence of mucus in the stool, 6) the presence of leukocytes in the stool, 7) leukocytes in the abnormal hematological test, out of range 4.0–11.0 $10^3$/μL 8) Neutrophils are abnormal, outside the range 40.0–75.0%. Not all gastroenteritis cases are caused by bacteria, therefore limiting the use of antibiotics can reduce the risk of resistance and ADR [1, 13]. This study's results were lower compared to those of studies in Ethiopia, which stated that one form of DRPs that often appeared in antibiotic use was the use of antibiotics without indication (28.9% of 152 patients) [11].

The results showed that 12 patients experienced potential DRPs from the aspect of antibiotic drug forms. The most common form of DRPs is the administration of levofloxacin in the form of an injection dosage of 500 mg. At the same time, the patient can still use oral antibiotics because the patient does not experience extreme nausea and vomiting. The use of IV parenteral antibiotics can cause pain and discomfort in the patient, phlebitis, thrombosis, and local or systemic infections. The use of parenteral antibiotics is only indicated in patients who have difficulty swallowing or absorbing oral antibiotics, low bioavailability of antibiotics, and cases of severe infection [14].

The main causes of potential DRPs in the antibiotic dose selection domain were categorized into two, namely too low dose and too high dose. Assessment of antibiotic dose suitability in pediatric patients (0–17 years) is based on the patient's weight. Meanwhile, dose suitability in adult patients (≥18 years) was based on the estimated creatinine clearance value based on the Cockcroft and Gault equation. A total of 14 patients (10.1%) experienced potential DRPs due to too low doses, with details of one adult patient and 13 pediatric patients. The dose is closely related to the minimum inhibitory concentration (MIC). If the dose fails to reach a level higher than MIC at the

infection or tissue site, treatment failure will result. This situation then became one of the causes of resistance [10].

Meanwhile, as many as 15 patients (10.8%) experienced potential DRPs due to too high a dose, with details of nine adult patients and six pediatric patients. The nine adult patients received levofloxacin. Levofloxacin is a broad-spectrum fluoroquinolone antibiotic that is lipophilic with kidney as the main eliminating organ. Levofloxacin is eliminated by the kidneys in its intact form. The toxicity of levofloxacin is known to be dose-dependent. Therefore, it is necessary to adjust the levofloxacin dose in patients with decreased renal function (characterized by a decrease in the estimated creatinine clearance value) to avoid toxic effects [15].

Based on the chi-square analysis between potential DRPs and the outcome of antibiotic therapy, the p-value was 0.204. The p-value >0.05 indicates that potential DRPs cases that occur with antibiotic use do not significantly relate to the outcome of gastroenteritis patients' therapy. Empirical antibiotics are also not recommended in every patient with gastroenteritis, only patients with specific indications [1]. Based on a review study conducted in South Korea, antibiotic therapy shortens the duration of symptoms of Campylobacter infection. However, the level of significance is not large [13]. This indicates that the use of empirical antibiotics in gastroenteritis patients, whether there is potential DRPs or not, does not significantly relate to the patient's treatment outcome.

Based on the DRPs chi-square analysis of the potential use of antibiotics and patients' length of stay, the p-value was 0.235. The p-value > 0.05 indicates that the potential DRPs for using antibiotics does not significantly impact the patients' length of stay. Conditions that show no relationship between potential DRPs and treatment outcomes and length of stay can occur because, in addition to the incidence of DRPs, other factors can affect patient treatment outcomes, such as disease severity, payment status, and disease complications experienced by patients [16].

## Conclusions

In conclusion, the most potential types of DRPs were drug selection problems in 98 of 139 DRPs cases (70.5%). Then, potential DRPs in using antibiotics for gastroenteritis does not significantly relate to the therapy outcome and the gastroenteritis patients' length of stay.

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Fivy Kurniawati*, Nanang Munif Yasin, Safina Nur Azizah and Silvia Ayu Purbaningtyas

# The impact of suitability of empirical antibiotics use on therapeutic outcome of respiratory tract infection patients at inpatient wards of Universitas Gadjah Mada Academic Hospital

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**Abstract**

**Objectives:** Currently, respiratory infection is regarded as one of the most common infectious diseases. This study aims to find out the impact of appropriate use of empirical antibiotic on therapeutic outcomes of patients with respiratory infections at inpatient wards of UGM Academic Hospital.

**Methods:** This is a cross-sectional study that uses retrospective data through patient medical records. The population was all patients who received empirical antibiotic therapy for respiratory infections at inpatient ward from July 2018 to July 2019. The sample was collected using the purposive sampling method, and the total number of samples was 192. The appropriate use of empirical antibiotic including the correct type, dosage, route, duration, and frequency was evaluated according to the Antibiotic Guidelines of UGM Academic Hospital 2018, Drug Information Handbook, Frank Shann Drug Doses, Infectious Disease Society of America (IDSA)/American Thoracic Society (ATS) 2016 & 2019, Pharmacotherapy Handbook 2015, and Pharmaceutical Care for Respiratory Tract Infections 2005. The data was analyzed descriptively by using Chi-square bivariate analysis.

**Results:** The result shows that 47.9% of 192 patients have received antibiotics properly according to the type, route, dose, frequency, and duration. The results of empirical antibiotic therapy have improved the repair of vital signs in 37.5% of patients. Meanwhile, the result of Chi-square bivariate analysis between the suitability of empirical antibiotic use and the improvement of therapeutic outcome is p=0.478 (p>0.05), which means that it is not statically significant.

**Conclusions:** It can be concluded that there is no correlation between the suitability of empirical antibiotics use and the improvement of the therapy outcomes. Thus, the use of empirical antibiotics based on the guidelines does not always have an impact on the improvement of the treatment outcomes of the patients with respiratory infection at inpatient wards of UGM Academic Hospital.

**Keywords:** empirical antibiotic; infection; outcome therapy; respiratory; suitability.

## Introduction

Respiratory tract infections are infections that occur in the lungs, chest, sinuses, nose, and throat caused by bacteria or viruses [1]. It can attack children and adults with a high risk of mortality and morbidity, causing almost four million people to die from respiratory infections [2]. The use of antibiotic is the main treatment in the management of respiratory infection. However, many types, classifications, bacterial sensitivity patterns, and discovery of new antibiotics often make it difficult for clinicians to choose the right antibiotics, which then resulting in antibiotic resistance. This condition has caused several fatal consequences, especially the patient outcomes, including prolonged illness, increased risk of death, and prolonged stays in hospital [3].

One way to ensure and figure out the appropriateness of antibiotic use in hospitalized patients is by evaluating the use of antibiotics compared to the existing guidelines. The evaluation of the quality of antibiotic use is seen from the type, duration, dose, frequency, and administration route based on the designated guidelines [4]. The purpose of this study is to determine the effect of empirical antibiotics use according to the designated guidelines for the treatment outcomes of respiratory infections at inpatient wards of UGM Academic Hospital.

***Corresponding author: Fivy Kurniawati**, Pharmacology and Clinical Pharmacy, Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia, E-mail: fivy_k@ugm.ac.id. https://orcid.org/0000-0002-6748-9504
**Nanang Munif Yasin,** Pharmacology and Clinical Pharmacy, Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia
**Safina Nur Azizah and Silvia Ayu Purbaningtyas,** Universitas Gadjah Mada Fakultas Farmasi, Yogyakarta, Indonesia

# Materials and methods

## Study design

This is a cross-sectional study with retrospective data collection through the medical records of patients suffering from respiratory tract infections at the inpatient ward of UGM Academic Hospital, Yogyakarta. Ethical clearance was obtained from the Medical and Health Research Committee of the Faculty of Medicine, Public Health, and Nursing, UGM (Ref. No: KE/FK/0455/EC/2019). The unit of analysis of this study was the medical records.

## Clinical samples

The population of this research was the medical records of all patients with respiratory tract infection who received empirical antibiotics in the inpatient ward of the UGM Academic Hospital from July 2018 to July 2019. This study was carried out by using purposive sampling method with retrospective data collection. The sample was part of the population that meets the inclusion criteria. The inclusion criteria of samples were complete medical record data, including the age, antibiotic use (type, dose, route, frequency, and duration), the record of clinical conditions monitoring, and the patient's vital signs. From the calculation carried out, the minimum number of the samples was 84 patients.

## Data analysis

Assessment of the suitability of empiric antibiotic therapy was conducted based on several books (Guidelines for Antibiotics Use in UGM Academic Hospital 2018, Infectious Diseases Society of America/American Thoracic Society (ATS) Guidelines 2016 and 2019, Pharmacotherapy Handbook 2015, Pharmaceutical Care for Diseases Respiratory Tract Infections 2005, Drug Information Handbook, and Drug Doses Frank Shann). The data analysis was performed by describing the patient's characteristics, suitability of empirical antibiotic use, and discharge status as carried out by the clinician. The empiric antibiotic therapy outcomes were analyzed descriptively and presented in terms of frequency and percentage. The correlation between suitability of empiric antibiotic use and therapeutic outcome was analyzed with Chi-square test by using Statistical Package for the Social Sciences (SPSS).

# Results

The samples in this study were 192 patients. Patient characteristics in Table 1 indicated that the percentage of male patients (66.1%) is more than female patients (33.9%). The age ranges of patients were infants (24.0%), toddlers (22.9%), and seniors (25.0%). The patients who were hospitalized for less than 7 days were 162 of 192 patients (84.4%). The type of respiratory tract infection that most patients suffered was unspecified pneumonia (37.5%)

**Table 1:** Patient characteristics (n=192).

| Characteristic | Category | n(%) |
|---|---|---|
| Gender | Males | 127 (66.1) |
| | Females | 65 (33.9) |
| Age | >0–30 days | 1 (0.5) |
| | >1 month–2 years | 46 (24.0) |
| | >2–6 years | 44 (22.9) |
| | >6–12 years | 9 (4.7) |
| | >12–18 years | 1 (0.5) |
| | >18–25 years | 5 (2.6) |
| | >26–35 years | 4 (2.1) |
| | >35–45 years | 5 (2.6) |
| | >45–55 years | 8 (4.2) |
| | >55–65 years | 21 (10.9) |
| | >65 years | 48 (25.0) |
| Length of stay, days | ≤7 days | 162 (84.4) |
| | >7 days | 30 (15.6) |
| Diagnosis | Acute RTI, unspecified | 16 (8.3) |
| | Tonsillopharyngitis | 4 (2.1) |
| | Pneumonia, unspecified | 72 (37.5) |
| | CA pneumonia | 54 (28.1) |
| | HA pneumonia | 3 (1.6) |
| | Aspiration pneumonia | 4 (2.1) |
| | Bronchopneumonia | 35 (17.2) |
| | Chronic bronchitis | 5 (2.6) |
| | Acute bronchitis | 1 (0.5) |

The suitability of antibiotic usage for patients with respiratory infections treated at the inpatient ward of the UGM Academic Hospital was considered based on five categories of suitability (type, route, dose, frequency, and duration). The results showed that 92 of 192 patients (47.92%) had received empiric antibiotics appropriately. The patients who received suitable types of antibiotic also had appropriate routes. Analysis of the suitability type and route showed that 175 of 192 patients (91.15%) received antibiotics appropriately, as shown in Table 2.

The following analysis was an analysis of the dose, frequency, and duration of empirical antibiotics sequentially. The result showed that 160 of 192 patients (83.33%) of patients had applied appropriate dose, 135 of 192 patients (70.31%) had received appropriate doses and frequency, 92 of 192 patients (47.92%) had received the proper doses, frequency, and duration. Analysis of dosage, frequency, and duration of antibiotic administration can be seen in Table 3.

Patient's therapy outcome determined by noting the patient's vital signs during empirical antibiotic administration which included pulse rate, body temperature, respiratory rate, and oxygen saturation and matched with normal ranges based on the guidelines. The therapeutic outcome shown in Table 4 indicates that 146 of 192 patients (76.04%) improved and 46 of 192 patients (23.96%) did not improve.

**Table 2:** Appropriability of the antibiotic's type.

| Literature | n(%) | Information |
|---|---|---|
| UGM Academic Hospital Guidelines for Respiratory Infection in Children | 5 (2.6) | Inappropriate |
| | 96 (50) | Appropriate |
| UGM Academic Hospital Guidelines for Community-acquired pneumonia (CAP) | 6 (3.13) | Inappropriate |
| | 48 (25) | Appropriate |
| UGM Academic Hospital Guidelines for Hospital-acquired pneumonia (HAP) | 2 (1.04) | Inappropriate |
| | 1 (0.52) | Appropriate |
| UGM Academic Hospital Guidelines for Aspiration pneumonia | 1 (0.52) | Inappropriate |
| | 3 (1.56) | Appropriate |
| UGM Academic Hospital Guidelines for bronchitis | 3 (1.56) | Inappropriate |
| | 3 (1.56) | Appropriate |
| Pharmacotherapy Handbook for bronchopneumonia | 24 (12.5) | Appropriate |

The analysis results reveal no correlation between the suitability of empirical antibiotic use and the improvement of the therapeutic outcome, as seen in Table 5.

## Discussion

Empirical antibiotics for pediatrics use first-line antibiotics, ampicillin, or second-line antibiotics, cephalosporins [5]. While the use of the amikacin antibiotic for pediatric patients is classified as a definitive antibiotic. Three adult patients with bronchitis received ceftriaxone antibiotics that were not suggested by the hospital's guidelines. The application of single ceftazidime antibiotic in patients with Community-acquired pneumonia (CAP) considered inappropriate. According to the IDSA and ATS Guidelines in 2019, the combination of beta-lactam antibiotics with macrolides can be used [6]. Combination of ceftazidime with macrolides can improve patient clinical outcomes because of its immunomodulatory effects [7]. Two patients with CAP also received a combination of beta-lactam with quinolones that were incompatible with the patient's heart disease history. One patient with CAP received three combinations of the antibiotics, which were ceftriaxone, metronidazole, and meropenem. The administration did not recommend it because it would not increase the effect of the antibiotics [8].

In Hospital-acquired Pneumonia (HAP), one patient received the combination of ceftazidime and metronidazole antibiotics. This case was considered inappropriate since the use of metronidazole was not compatible with the patient's comorbid (thrombocytopenia) [9]. Other HAP patients received single meropenem antibiotics. This case was inappropriate because the patients had a late-onset poststroke with multidrug-resistant organisms (MDRO) risk factors. Therefore, the patient should be given a combination of carbapenem and quinolone antibiotics. Aspiration pneumonia patients' treatment using the combination of levofloxacin and clindamycin antibiotics was considered unnecessary because the Infectious Disease Society of America (2016) recommended using single empirical antibiotic therapy to increase the effectiveness of the treatment. Clindamycin is the first-line of empiric antibiotic therapy in aspiration pneumonia [10].

Analysis of antibiotic dose calculation was adjusted to each guideline. The dose administration of antibiotics in the pediatric patient was given according to the patient's body weight calculation. Seven pneumonia adult patients suffered kidney problems, so the drug dosage clearance was made by considering the kidney problem, by using creatinine clearance or glomerular filtration rate (GFR). The dose calculation employed the Cockroft Gault formula. After being analyzed, the antibiotic dose was matched with the frequency and duration of antibiotic administration based on the guidelines.

**Table 3:** Appropriability of the antibiotic's dose, frequency, and duration.

| Literature | Information | Pediatric patients (n=101) | Adult patients (n=91) | n(%) |
|---|---|---|---|---|
| UGM Academic Hospital Guidelines, Drug Information Handbook, Drug Doses Frank Shann, Pharmaceutical Care for Respiratory Infection, Pharmacotheraphy Handbook | **Dose** | | | |
| | Appropriate | 85 | 75 | 160 (83.33) |
| | Inappropriate | 16 | 16 | 32 (16.67) |
| | **Dose and frequency** | | | |
| | Appropriate | 71 | 64 | 135 (70.31) |
| | Inappropriate | 33 | 24 | 57 (29.69) |
| | **Dose, frequency, and duration** | | | |
| | Appropriate | 57 | 35 | 92 (47.92) |
| | Inappropriate | 66 | 44 | 100 (52.08) |

**Table 4:** Therapy outcome.

| Vital sign | Improved | | Not improved | | Total patients |
|---|---|---|---|---|---|
| | n | % | n | % | |
| Temperature | 177 | 92.2 | 15 | 7.8 | 192 |
| Heart rate | 181 | 94.3 | 11 | 5.7 | 192 |
| Respiratory rate | 175 | 91.1 | 17 | 8.9 | 192 |
| $SpO_2$ | 176 | 91.7 | 16 | 8.3 | 192 |

**Table 5:** Correlation of antibiotic suitability with therapeutic outcomes.

| Antibiotic suitability | Therapeutic outcomes | | | | Total patients | p-Value |
|---|---|---|---|---|---|---|
| | Improved | | Not improved | | | |
| | n | % | n | % | | |
| Appropriate | 72 | 37.50 | 20 | 10.42 | 92 | 0.478 |
| Inappropriate | 74 | 38.54 | 26 | 13.54 | 100 | |

The therapeutic outcome of RTI patient was observed from the improvement of the patient's vital signs. Patients with bronchitis and pneumonia usually have improvement on their temperature, pulse rate, and frequency of breath. These indicate the degradation of lung function, affecting oxygen availability in the blood [11].

The suitable antibiotics use, yet it still did not improve patient outcome could be allegedly due to co-diagnosis such as heart disease disorders; stroke; or asthma. Those could inhibit the improvement of the patient's vital signs. Another cause of the unimprovement in therapy was probably due to a history of previous respiratory diseases such as chronic obstructive pulmonary disease (COPD) and pulmonary infections. Thus, the use of antibiotics had not been effective enough in treating the infections.

The use of antibiotics that were considered inappropriate but still provided improvement for patient's vital signs, probably because antibiotics were quite sensitive to the kind of bacteria that cause infection. Patients also received supportive therapy such as antipyretics to decrease patient's body temperature, bronchodilators to help improve respiratory rate, and oxygen administration to improve oxygen saturation in patients.

Result of another study regarding the use of empirical antibiotics to the achievement of clinical outcome in pediatric pneumonia patients showed a significance value of $p=0.368$ ($p>0.05$) which indicated that there was no significant correlation between types of antibiotics and clinical outcome factors [12]. No correlation of suitability of antibiotic use with the outcome therapy is influenced by many factors, including consideration of antibiotic selection, disease severity, comorbidity, condition of immunity, and nutritional status of the patient [13].

# Conclusions

The use of empirical antibiotics under the guidelines does not always impact on improving treatment outcomes for respiratory infection patients in the UGM Academic Hospital inpatient wards.

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Risa Zulfiana, Suharjono* and Kuntaman

# Genetic profile mutation *rpoB* in clinical isolate of rifampicin-resistant *Staphylococcus aureus*

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**Abstract**

**Objectives:** *Staphylococcus aureus* is one of the bacteria which causes nosocomial infection. *Methicillin-Resistant Staphylococcus Aureus* eradication using antibiotics combined with rifampicin has shown good results, whereas, adjuvant rifampicin has long been hypothesized to improve the outcome of *S. aureus* infection treatment. Resistant-rifampicin *S. aureus* mutates in *rpoB* gene at some codons. This study was conducted to identify the mutation of *rpoB* gene in *S. aureus* which was resistant toward rifampicin.

**Methods:** In this study, isolates collected in the Microbiology Laboratory of Dr. Seotomo Surabaya Hospital during May–September 2019. Then, the dilution method was carried out to determine the minimum inhibition concentration for resistant-rifampicin and dilution to determine the inhibition zone diameter. After that, DNA extraction was carried out from rifampicin-susceptible isolates as a control and resistant-rifampicin isolates followed by identification of *rpoB* gene mutations by Polymerase Chain Reaction (PCR) and sequencing.

**Results:** There were nine isolates studied. They were four resistant-rifampicin isolates and four susceptible-rifampicin isolates. In four rifampicin-resistant isolates, the most frequent mutations that occurred was His-481 codon (75%) followed by the Ile-527 codon (25%). Rifampicin-susceptible isolates mutated in Pro-475 and Asn-474 codons. One rifampicin-resistant isolate had two mutations in codons Ile-527 and Asn-474.

**Conclusions:** The type of mutation that causes the most rifampicin resistance was a missense mutation. The susceptible-rifampicin isolate experienced silent mutations. There was a relation between the type of missense mutation of *rpoB* gene and rifampin resistance.

**Keywords:** codon; mutation; rifampicin; *rpoB*; *Staphylococcus aureus*.

## Introduction

*Staphylococcus aureus* is one of the Gram-positive bacteria which causes nosocomial infection in the blood flow, urinary tract, respiratory tract, pus, gastrointestinal tract, soft tissue, as well as on the skin [1, 2]. Metastatic infection which occurs almost in all organs causes mortality approximately 20% [3]. The existing eradication method of *Methicillin-Resistant Staphylococcus Aureus* (MRSA) is to use oral antibiotic with the addition of rifampicin [4]. Rifampicin works by inhibiting RNA synthesis and binding beta subunit of DNA-RNA polymerase, thereby it inhibits RNA transcription process [5, 6]. Rifampicin resistance which occurs on *S. aureus* is due to the changes of its target leads to reduced affinity for the antibiotic enzyme [7]. The resistance is associated with the mutation in the main region (cluster I and II) of *rpoB* gene [8]. MRSA rates in the world are increasing but limited data on MRSA in Indonesia, and data MRSA which is less susceptible to rifampicin is 75.7% [9].

Rifampicin resistance is typically based on the amino acid substitution in three specific clusters of binding rifampicin region in the beta subunit of bacterial RNA polymerase [7, 10]. The internal sequence in 460 bp *rpoB* gene was amplified by Polymerase Chain Reaction (PCR). This area determined the rifampicin resistance in cluster I (Amino Acid 462–488) and cluster II (Amino Acid 515–530). The results of this study obtained high resistance mutations in codons 468Gln3Lys (T38a) and 481His3Tyr (T38b) [10].

The aim of the present study was to determine genetic mutation profile of *rpoB* gene toward *S. aureus* isolate which was proven to be resistant to rifampicin by using PCR technique and sequencing technique in Indonesia, thus providing preliminary data the relation among the profile of mutation area, minimum inhibition level, and the resistance *S. aureus* toward rifampicin.

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 812 1733 877, E-mail: suharjono@ff.unair.ac.id
**Risa Zulfiana,** Department of Pharmacy, Hajj Hospital, Surabaya, Indonesia; and Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Kuntaman,** Department of Clinical Microbiology, Dr. Soetomo General Hospital, Surabaya, Indonesia

# Materials and methods

### Samples

Clinical isolates and strain ATCC 25923 as a control obtained from installation laboratory/clinical microbiology SMF RSUD Dr. Soetomo Surabaya which has received an ethical clearance through a hospital research and development institute during May–September 2019.

### The bacterial sensitivity testing

The sensitivity testing used was BD Phoenix™ automatic microbiology method. The colonies isolated on Mueller-Hinton agarose were suspended into BD Phoenix™ broth, identified with a turbidity standard of 0.5 McFarland ($1.5 \times 10^8$ CFU/mL) using a *CrystalSpec™ nephelometer* (Becton Dickinson). DNA was extracted by using Extract N-ampi™ Blood PCR Kit.

### Detection of *rpoB* gene mutations associated with rifampicin resistance

PCR examination was used to detect the mutations in the region of *rpoB* gene (460 bp, nuclotides 1,354–1,814) using *rpoB* primer with the sequence: rpoB1-F (5′-ACC GTC GTT TAC GTT CTG TA) and rpoB2-R (5′-TCA GTG ATA GCA TGT GTA TC) [10–12]. The amplification used Bio Radicycler thermal machine with the sample of 5 µL (DNA Ekstract), 12.5 µL of PCR mix, and 1 µL of each primer forward and reverse. Followed by denaturation at 95 °C for 15 min and then annealing at 53 °C for 30 s. Thereafter extension at 70 °C for 45 s, in 35 cycles, and final extension at 72 °C for 8 min. The next stage was electrophoresis which was carried out at 2% agarose in Tris Borate solution. Before entering the labeling stage, first, purify PCR products both from agarose gel after the electrophoresis process and PCR residual solution by using *QiaQuick PCR column* (*QIAGEN, Valecia, CA*). After purification, labeling was carried out for 35 cycles, with the condition of denaturation of PCR at 95 °C for 15 min, annealing at 53 °C for 30 s, extension at 70 °C for 45 s, in 35 cycles, and the final extension *at* 72 °C for 8 min. After that, the sequencing was carried out the procedure of PCR purified by using the ABI Prism 310 Genetic Analyzer (Applied Biosystem, Forter City, USA).

# Results

There were nine isolates of *S. aureus* which were examined, including four resistant-rifampicin (MRSA) isolates, four rifampicin-susceptible (MSSA) isolates, and ATCC 25923 strain as control. Minimum Inhibitory Concentration (MIC) isolate MSSA and ATCC≤0.5 µg/L while MRSA≥2 µg/L, and the MRSA inhibition zone diameter was ≤16 mm according to the Clinical and Laboratory Standards Institute (Table 1). There were seven isolates which mutated in one codon and one of them mutated in two codons. There were three MRSA isolates mutated at codon 481 which generally changed from histidine into asparagine His481 → Asn. MRSA isolate mutated at two codon at codon 527 with the changing of

**Table 1:** The results of susceptibility test and the mutation *rpoB* gene *S. aureus* isolates.

| No | Sample code | Isolate code | Patterns of resistance to rifampicin (R: Resistant; S: Susceptible) | Minimum inhibitory concentration, µg/L[a] | Inhibition zone diameter, mm[b] | Codon position | Mutation code | Type mutation[c] | Number of mutations |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2-RPOB-F | 14755 (MRSA) | R | ≥2 | 14 | 481 | His481(CAT) → Asn(AAT) | MM | 1 |
| 2 | 3-RPOB-F | 15095 (MRSA) | S | ≤0.5 | 23 | 475 | Pro475(CCA) → Pro(CCG) | SM | 1 |
| 3 | 4-RPOB-F | 15043 (MSSA) | S | ≤0.5 | 23 | 474 | Asn474(AAC) → Asn(AAT) | SM | 1 |
| 4 | 5-RPOB-F | 15292 (MSSA) | S | ≤0.5 | 25 | 474 | Asn474(AAC) → Asn(AAT) | SM | 1 |
| 5 | 7-RPOB-F | 16170 (MSSA) | S | ≤0.5 | 24 | 474 | Asn474(AAC) → Asn(AAT) | SM | 1 |
| 6 | 8-RPOB-F | 14996 (MRSA) | R | ≥2 | 16 | 481 | His481(CAT) → Asn(AAT) | MM | 1 |
| 7 | 9-RPOB-F | ICN 25 (MRSA) | R | ≥2 | 13 | 481 | His481(CAT) → Asn(AAT) | MM | 1 |
| 8 | 10-RPOB-F | ICN 38 (MRSA) | R | ≥2 | 10 | 474<br>527 | Asn474(AAC) → Asn(AAT)<br>Ile527(ATT) → Phe(TTT) | SM<br>MM | 2 |

[a]Rifampicin-resistant *S. aureus* with MIC ≥4 µg/L and rifampicin-susceptible ≤1 µg/L (CLSI). [b]Inhibition Zone Diameter rifampicin-resistant obtained through the diffusion method is ≤16 mm (CLSI). [c] Type Mutation = MM, Missense Mutation; SM, Silent Mutation; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MRSA, *Methicillin-Resistant Staphylococcus Aureus*.

isoleusin to phenilalanin Ile527 → Phe and codon 474 asparagine Asn474 → Asn. The three MRSA isolate mutated at codon 474 asparagine Asn474 → Asn, and one mutated at codon 475 proline Pro475 → Pro.

## Discussion

Aubry-Damon et al. [7] obtained mutations with high resistance to codons Gln468Arg, His481Tyr, Arg484His, and Asp550Gly. Wichelhaus et al. [9] obtained high resistance mutations at codons 468Gln3Lys (T38a) and 481His3Tyr (T38b). Meanwhile Tang et al. [13] conducted research during 2006–2010, getting results from 39 isolates that there was only a change in amino acids in cluster I, namely His481Tyr/Leu/Asp 84.6%, Ser486Leu 33.3%, Ala477Asp 30.8%, Gln468Lys/Leu/Arg 20.5%, and Arg484His 17.9% and in cluster II there was no mutation because low resistance mutations were only present in three isolates with one amino acid Pro519Leu, Ile527Phe, and Ser529Leu. These studies show that most mutations occur in codons 481 and 468.

There were two types of mutation that occurred in this study. There were silent mutation and the other was missense mutation. Silent mutation occurred in four rifampicin-susceptible samples and one rifampicin-resistant sample, namely one proline at codon 475, and three asparagine at codon 474. Meanwhile, in missense mutation, there was a changing of three histidine into asparagine at codon 481 (75%, n=4) and one isoleusin changed to phenylalanine at codon 527 (25%). Nonsense mutation was not seen in this study.

Rifampicin-resistant isolates mutated at cluster I approximately three samples, namely His481 → Asn and mutated at cluster II approximately one sample, namely Ile527 → Phe. Due to the limitation of MIC reading device, it was not possible to determine which cluster had the greatest MIC resistance.

Rifampicin-susceptible isolates experienced silent mutation and isolates which experienced rifampicin resistance based on minimum inhibition level and the diameter of inhibition zone, all of them, experienced missense mutation. The study conducted by Aubry-Damon et al. [7] showed that missense mutation occurred in all *S. aureus* isolates which was resistant toward rifampicin. This can be related to the mutation results in this study.

The data obtained in this study showed that the rifampicin-resistant *S. aureus* occurred in Indonesia mutated like common mutation in other study. However, it should be noted that mutations in the codon cluster II 527 are rarely found in other countries, further research with a larger sample size is mandatory to determine the number of mutation and its potential to cause resistance.

## Conclusions

It can be concluded that the type of mutation that causes the most rifampicin resistance was a missense mutation. The susceptible-rifampicin isolate experienced silent mutations. There was a relation between the type of missense mutation of *rpoB* gene and rifampicin resistance.

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Novan Yusuf Indra Pratama, Bambang Subakti Zulkarnain*, Soedarsono and Umi Fatmawati

# Hematological side effect analysis of linezolid in MDR-TB patients with individual therapy

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**Abstract**

**Objectives:** This study aimed to estimate the prevalence and analyze the risk factors for linezolid-induced hematological side effects in multidrug-resistant tuberculosis (MDR-TB) patients.

**Methods:** Data were collected from medical records of MDR-TB patients who received linezolid between January 2018 and May 2020. Statistical significance analysis and multivariate analysis were performed with SPSS version 24 software.

**Results:** Hematological side effects were identified in 27 out of 93 patients (29.0%). The most prevalent effect was anemia (29.0%), while the less prevalent effects were thrombocytopenia (3.2%) and leukopenia (2.2%). These side effects were reported after 2 weeks of linezolid treatment. The drug dose was more than 11 mg/kgBW/day or patient weighing less than 54 kg was identified as an independent risk factor for anemia in multivariate analysis.

**Conclusions:** Anemia was the most prevalent of linezolid-induced hematological side effects in MDR-TB patients. Therefore, hemoglobin monitoring might be recommended in patients weighing less than 54 kg and after receiving linezolid therapy for at least 2 weeks.

**Keywords:** adverse drug reactions; hematologic; linezolid; MDR-TB.

## Introduction

Linezolid is an antibiotic of the oxazolidinone class that is sensitive to Gram-positive bacteria, including *Mycobacterium tuberculosis* [1, 2]. In multidrug-resistant tuberculosis (MDR-TB) therapy, linezolid is included in group A, namely the drug that is prioritized to be administered together with 3–4 other antibiotic combinations for 18–20 months [3]. In general, linezolid has a good tolerance for short-term use, but side effects will arise with increasing use and increasing dose [4]. In its use as MDR-TB, linezolid therapy will be administered for 6–18 months at a dose of 600 mg/day [5]. Hematological side effects are one of the most common side effects to occur, characterized by anemia, leukopenia, and thrombocytopenia. The mechanisms underlying these side effects are bone marrow suppression and immune system-mediated phenomena. Toxicity caused by linezolid results in suppression of platelet production by the bone marrow and hematopoietic cells and a known short-term effect affecting erythropoiesis [6–8] Meta-analysis of 12 studies stated that the average incidence of anemia was 28.47% [9]. Meanwhile, a meta-analysis conducted by Zhang stated that anemia occurred in 25% of patients from 15 studies [10]. The decline in the success rate of therapy is the side effect results of the drug resulting from discontinuation of therapy. The risk factors for side effects need to be known so that clinicians are more aware by increasing the monitoring of side effects; this is expected to reduce the incidence of side effects. Based on a lot of studies that have been conducted, there have not been consistent results, including research by Hanai, which stated that the duration of using linezolid is the only factor causing anemia [11]. Takahashi mentioned the duration of use and creatinine clearance as risk factors for thrombocytopenia [11]. A study conducted by Natsumoto stated that dosage per kilograms body weight per day (DKPD) and serum creatinine are risk factors for thrombocytopenia [12]. This study aimed to estimate the incidence and analyze the risk factors for linezolid-induced hematological side effects in MDR-TB patients. This study is important to be conducted because there is no data on the incidence of side effects of linezolid in Indonesia and there are no studies that examine the risk factors for side effects of linezolid in tuberculosis (TB) patients.

***Corresponding author: Bambang Subakti Zulkarnain**, Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62811 3419 355, E-mail: bambang-s-z@ff.unair.ac.id
**Novan Yusuf Indra Pratama,** Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Soedarsono,** Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
**Umi Fatmawati,** Department of Pharmacy, Dr Soetomo General Hospital, Surabaya, Indonesia

## Materials and methods

This study was designed to comply with the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, with reference number No.0034/LOE/301.4.2/VI/2020. This research was conducted using a retrospective observational method from January 2018 to May 2020 in MDR-TB Outpatient at Dr. Soetomo General Hospital. The inclusion criteria in this study included patients with a diagnosis of adult MDR-TB (aged over 18 years) and had undergone a complete blood count at least two times, before receiving linezolid and after receiving linezolid. While the exclusion criteria included patients with human immunodeficiency virus (HIV), end-stage chronic kidney disease, hepatitis, cirrhosis and spinal cord cancer, blood cancer, patients with a history of bleeding during TB therapy, patients who underwent surgery and transplants during the treatment period in MDR-TB clinic, and patients who are taking immunosuppressive drugs while undergoing TB therapy such as corticosteroids and calcineurin inhibitors. Descriptive analysis was in the form of prevalence and onset of side effects. Statistical analysis was performed using the SPSS ver. Twenty six in the form of univariate and multivariate binary logistic regression tests on risk factors for hematological side effects. Significant value is indicated by p value <0.05 and the results of the odds ratio.

## Results

There were 124 patients screened for eligibility and 104 patients included in the study, while 11 patients excluded due to HIV with zidovudine therapy and two patients with acute kidney injury. Therefore, 93 patients were further analyzed. There were more male (54.8%) than female patients. Meanwhile, the majority of ages were in the age range of 41–60 years which were 51 patients (63.4%). Diabetes mellitus was the most common comorbid found in 44 patients (47.3%). GeneXpert was the main examination to diagnose MDR-TB. In this study, the highest number was medium which was 51 patients (54.8%). A lot of MDR-TB patients came from patients who relapse from previous TB therapy as many as 33 patients (35.4%). The second highest number was 22 new patients who had close contact with MDR-TB patients (23.6%). Furthermore, there were 18 patients who dropped out of category 1 tuberculosis therapy (19.4%) (Table 1). The side effects were attained from patient medical records, which are obtained from surveillance forms and doctor's integrated records. The most common side effect experienced by patients was anemia with 27 cases (29.03%) with severity ranging from mild to life-threatening. Thrombocytopenia occurred in three cases (3.22%) and leukopenia in two cases (2.15%). The mean reduction in hemoglobin levels of the 27 patients was 3.54 g/dL with the onset of side effects 91 days after starting linezolid therapy. The earliest onset of side effects was on day 12 and the longest appeared on day 243. Meanwhile, thrombocytopenia and leukopenia had an average onset of 105 and 96 days, respectively (Table 2). The results of the binary logistic regression analysis showed that there was only one risk factor that affected the emergence of anemia side effects from linezolid, namely the dose per kg per day of linezolid with a p value of 0.015 and a crude odd ratio of 4.237 in univariate analysis. While the multivariate results obtained an adjusted odd ratio of 5.509 (Table 3).

**Table 1:** Sociodemographic and clinical characteristics of multi-drug-resistant tuberculosis (MDR-TB) patients.

| Sociodemographic and clinical characteristics | n, % |
|---|---|
| **Gender** | |
| Male | 51 (54.8) |
| Female | 42 (45.2) |
| **Age** | |
| <20 | 2 (2.1) |
| 21–40 | 23 (24.8) |
| 41–60 | 57 (63.4) |
| >60 | 11 (9.7) |
| **Comorbid** | |
| Diabetes mellitus | 44 (47.3) |
| Hypertension | 5 (5.4) |
| Cataract | 1 (1.2) |
| Cardiovascular | 2 (2.2) |
| Stroke | 1 (1.2) |
| Hyperthyroid | 1 (1.2) |
| No comorbid | 39 (41.9) |
| GeneXpert **results** | |
| High | 19 (20.4) |
| Medium | 51 (54.8) |
| Low | 15 (16.1) |
| Very low | 7 (7.5) |
| Negative | 1 (1.2) |
| **History of TB treatment** | |
| Relapse | 33 (35.4) |
| Drop out | 18 (19.4) |
| Not conversion on 3-month therapy | 1 (1.2) |
| Fail on category 1 therapy | 14 (15.0) |
| NonDOTS | 5 (5.4) |
| New patients | 22 (23.6) |

**Table 2:** Prevalence and onset of linezolid side effects (n=93).

| Hematologic adverse effect | Case, n, % | Onset, day | Grade of severity, n |
|---|---|---|---|
| Anemia | 27 (29.03) | 91 (12–243) | Threatening life: 3<br>Severe: 8<br>Moderate: 12<br>Mild: 4 |
| Thrombocytopenia | 3 (3.22) | 105 (94–122) | Mild: 3 |
| Leukopenia | 2 (2.15) | 96 (94–99) | Mild: 2 |

**Table 3:** Univariate and multivariate analysis of anemia risk factor.

| Risk factor | ADE (n=27) | No ADE (n=66) | *Crude* OR (95% CI) | p-Value | *Adjusted* OR (95% CI) | p-Value |
|---|---|---|---|---|---|---|
| **Patients factor** | | | | | | |
| Age | 50.52[a] | 44.92[a] | 2.122 (0.52–8.60) | 0.292 | | |
| Gender | 11[a] | 40[a] | 0.447 (0.18–1.11) | 0.084 | | |
| Creatinine serum | 0.91[a] | 0.88[a] | 2.122 (0.52–8.60) | 0.292 | | |
| Creatinine clearance | 62.90[a] | 79.94[a] | 2.358 (0.81–6.84) | 0.115 | | |
| **Comorbid** | | | | | | |
| Diabetes melitus | 13[b] | 31[b] | 1.048 (0.43–2.57) | 0.918 | | |
| Coronary arterial disease | 1[b] | 1[b] | 1.000 (0.0-0.0) | 1.000 | | |
| Hypertension | 1[b] | 4[b] | 2.500 (0.15–41.5) | 0.523 | | |
| Stroke | 1[b] | 0[b] | 1.000 (0.0-0.0) | 1.000 | | |
| **Drug factor** | | | | | | |
| Dosage per kg body weight per day | 13.95[a] | 12.28[a] | **4.237 (1.32–13.62)** | **0.015** | **5.509 (1.51-20.2)** | **0.010** |
| **Hematological baseline** | | | | | | |
| Baseline hemoglobin | 11.69[a] | 12.82[a] | 1.556 (0.63–3.83) | 0.336 | | |
| Baseline thrombocyte | 395.96[a] | 416.53[a] | 5.200 (0.45–59.9) | 0.186 | 12.1 (0.80–182.5) | 0.072 |

[a]Mean. [b]total. ADE, adverse event; OR, Odd Ratio; p value, nilai dari tes Wald; CI, *Confidence Interval*. The eliminated data were obtained from the results of the stepwise forward and backward LR in multivariate regression analysis.

## Discussion

Linezolid has good therapeutic effectiveness to eradicate TB germs, it can be seen from the good therapeutic success rate of the WHO meta-analysis, but at the same time linezolid is the drug with the highest incidence of side effects when compared to other antituberculosis drugs, this of course can be one of the factors that can increase the drug dropout rate of TB patients and the failure of TB therapy [3, 13]. In this study conducted at Dr. Soetomo, the incidence of side effects was noted to be lower than other similar studies such as the study by Tang in China which showed that 51.5% (vs. 29.03%) of MDR-TB patients on linezolid therapy were anemic, 12.1% (vs. 3.22%) had thrombocytopenia, and 15.2% (vs. 2.17%) had leukopenia [14]. Lee in South Korea said that 60% had anemia and 17.1% had leukopenia. The difference in results could be due to differences in patient characteristics, which resulted in different risk factors for exposure to side effects [2]. Patients affected by anemia side effects have an onset that varies between 12 and 243 days with an average onset of 91 days, these results are consistent with a similar study, a study by Singh stated that the side effects of linezolid myelosuppression occurred after patients took linezolid for 2 weeks [4]. Ramachandran in his study also stated that hematological side effects appeared at 2 weeks after using linezolid [15]. The Food Drug Administration received reports that side effects occurred after patients had taken linezolid on the average for more than 4 weeks [4]. It was found that anemic patients also experienced a trend of decreasing platelets and leukocytes, but it was not as significant as a decrease in hemoglobin. The side effects of linezolid hematology are thought to be reversible due to the process of bone marrow suppression/myelosuppression or can also be caused by the immune-mediated system [12, 16]. The results of the multivariate analysis showed that the only risk factor for side effects was the DKPD which was more than 11 mg/kg/day. It can be seen in Figure 1, that patients who received a dose of more than 11 mg/kg/day had three times more anemic than patients who received a dose of less than 11 mg/kg/day, because the dose received by patients was even, namely 600 mg/day. Therefore, if converted to body weight, patients weighing less than 54 kg are patients who are at risk for side effects. Body weight is affected by the distribution of water and fat in patients which will affect the volume of drug distribution [17]. Linezolid has a protein drug binding of 30% and a distribution volume of 40–50 L, the structure of linezolid which tends to be polar causing high levels of free drugs in

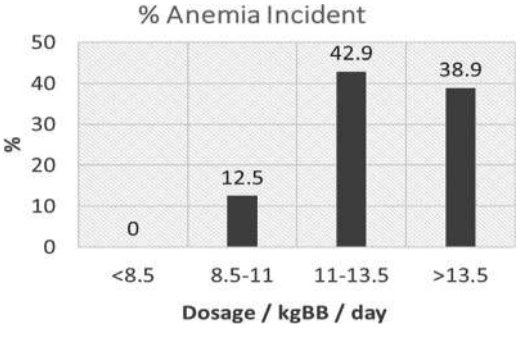


**Figure 1:** The incidence of anemia increased significantly in patients who received doses >11 mg/kg/day.

the blood and higher water solubility [18]. Consequently, in patients with lower body weight, linezolid will have a higher area under curve (AUC) as well as plasma concentration so that there is more potential for side effects. In a study conducted by Natsumoto, patients who were over 80 years old and weighing <40 kg had a high AUC linezolid (>800 ug h/mL) and was also accompanied by a high percentage of side effects (63.6%) [12]. In a study conducted by Nukui, it was also stated that high linezolid plasma concentrations were a risk factor for side effects of linezolid [19]. These results suggest that linezolid dosing should be adjusted according to the patient's body weight. Research related to the effectiveness and safety of linezolid conducted by Milliard showed that 300 mg/12 h was the optimal dose which shows the best level of effectiveness with minimal potential side effects so that it can be considered as input as the next linezolid dosing scenario [20]. This supports the results of previous studies that the linezolid dosage should be adjusted according to the patient's body weight to minimize side effects. As in previous studies that patients with a low body weight (<54 kg) had a higher tendency to develop side effects [12]. This study has limitations, including retrospective data collection so that data is limited from medical records, other than that is the limited number of patients and time of the study, the duration of therapy is not a risk factor variable in this study. This study was single center, so that was difficult to generalize the results to the Indonesian population.

## Conclusions

Anemia was the most prevalent of linezolid-induced hematological side effects in MDR-TB patients followed by thrombocytopenia and leukopenia. Therefore, hematologic monitoring is recommended in patients receiving a dose more than 11 mg/kgBW/day or weighing less than 54 kg and after at least 2 weeks of therapy.

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**Informed consent:** There is no informed consent needed because this study was retrospective.

**Ethical approval:** This study was designed to comply with the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, with reference number No.0034/LOE/301.4.2/VI/2020.

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Wenny Putri Nilamsari*, Muhammad Fajar Rizqi, Natasya Olga Regina, Prastuti Asta Wulaningrum and Umi Fatmawati

# Adverse drug reaction and its management in tuberculosis patients with multidrug resistance: a retrospective study

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**Abstract**

**Objectives:** This study was conducted to assess adverse drug reactions and their management in MDR-TB patients. Indonesia is the fifth highest country with multidrug-resistant tuberculosis (MDR-TB) high burden around the world. The number of MDR-TB patients in Indonesia is increasing every year, but the data regarding ADRs are still limited. Therefore, more data on their characteristics and their management is very valuable for clinicians and pharmacists.

**Methods:** The study is a descriptive study, using retrospective data of MDR-TB patients who completed therapy from January 1st, 2015 to December 31st, 2015 at the Tuberculosis Outpatient unit at the Dr. Soetomo Teaching Hospital Indonesia. Each adverse effect was judged with standards of the clinic and was documented in patients' medical records.

**Results:** There were 40 patients included in this study. During therapy, 70% of patients developed at least one adverse drug reaction. The five most prevalent adverse effects found in this study were hyperuricemia (52.5%) followed by gastrointestinal (GI) disturbances (40%), ototoxicity (37.5%), hypokalemia (27.5%), and athralgia (12.5%). Managements that were undertaken to overcome the adverse drug reactions were adding symptomatic drugs and/or modifying the treatment regimen.

**Conclusions:** Because of the small samples we cannot attain a general conclusion. However, the result of this study is very imperative as this data gives us insight regarding adverse effects in MDR-TB patients in Indonesia.

**Keywords:** adverse drug reactions; Kanamycin; management; multidrug resistant tuberculosis; second line anti TB drugs.

## Introduction

Tuberculosis (TB) is a transmissible disease that has been categorized as one of the 10 major deadly diseases around the world. Based on WHO global report in 2019, Indonesia is the third highest TB burden country (8%), after India (24%) and China (10%) [1]. Strains of *Mycobacterium tuberculosis* resistant *in vitro* to rifampicin and isoniazid are the cause of multidrug-resistant tuberculosis (MDR-TB) [2]. On a global scale, 3.4% of current TB cases and 18% of previously-treated cases possess MDR-TB or rifampicin-resistant TB. Universally, Indonesia is the fifth country with the highest MDR-TB with approximately 24,000 patients entitled to MDR-TB treatment in 2018. The national MDR-TB prevalence is estimated at 2.8% among new TB cases and 16% among previously-treated TB cases. The occurrence of MDR-TB makes treatment more complicated. The lengthy therapies and the adverse drug reactions (ADR) that occur often affect the adherence of TB patients. A cross-sectional study in Ethiopia showed that ADRs significantly impacted the failure of tuberculosis treatment [3]. Timely recognition of ADRs and understanding their management are necessary to prevent unsuccessful therapies. Although MDR-TB patients in Indonesia are increasing every year, data regard to ADRs is still limited. Therefore, more data on their characteristics and their management is very valuable to clinicians and pharmacists. The purpose of this study is to examine ADR characteristics of MDR-TB regimen and their management in MDR-TB patients in Dr. Soetomo Teaching Hospital, Surabaya, Indonesia.

***Corresponding author: Wenny Putri Nilamsari**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +62859 6422 7396, E-mail: wenny-p-n@ff.unair.ac.id
**Muhammad Fajar Rizqi and Natasya Olga Regina,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Prastuti Asta Wulaningrum,** Department of Pulmonology, Dr. Soetomo Teaching Hospital, Surabaya, Indonesia
**Umi Fatmawati,** Department of Pharmacy, Dr. Soetomo Teaching Hospital, Surabaya, Indonesia

## Materials and methods

This study has attained approval from the Research and Ethics Committee of Dr. Soetomo Teaching Hospital before being conducted. This study was a retrospective review conducted at TB outpatients Dr. Soetomo Teaching Hospital, Surabaya Indonesia. Inclusion criteria consisted of MDR-TB patients who finished treatment from January 2015 to December 2015. Patients with incomplete data regarding ADR were excluded. All patients received regimens for MDR-TB patients according to Indonesian Ministry of Health guidelines which were Pyrazinamide–Levofloxacin–Kanamycin–Cycloserin–Ethambutol–Ethionamide during intensive phase and maintenance phase except Kanamycin. Para Amino Salicylic acid was given as the substitute for any other drugs that cannot be tolerated. All patients being treated were in the intensive phase for 6–8 months and the maintenance phase for 10–16 months. Data regarding patients' demographics such as gender, age, type of resistance, duration of therapy, and previous TB status was collected from the medical records. We reviewed the entire chart in the medical record to determine ADRs and their management. ADRs were defined clinically using clinical criteria or laboratory using laboratory data assessment. Ototoxicity was specified as tinnitus or hearing loss confirmed by audiometry. Hypokalemia was specified as serum kalium below normal value. Hyperuricemia was specified as uric acid serum level more than normal value. The gastrointestinal disturbance was specified as nausea vomiting or any abdominal discomfort. A psychiatric disorder was diagnosed by psychiatrist including anxiety and tendency for suicidal. The visual disorder was specified as visual change diagnosed by an ophthalmologist. In addition, we also reviewed the medication chart to document if there were any changes in the MDR-TB regimen or symptomatic drugs added.

## Results

From January to December 2015, we collected 40 medical records of patients who had completed therapy for this study, consisting of 50% male patients and 50% female patients. All treatment started in 2013 and finished in 2015. The majority of MDR-TB patients were within the age group of 25–44. Among MDR-TB cases, 97.5% of them had TB treatment history (relapse, default, failure, and unknown causes) and 2.5% cases were new ones. Below are patients' clinical characteristics and sociodemographic data (Table 1). During therapy, a lot of patients developed more than one ADR. The total adverse drug reaction characteristics were 83 cases. The majority of ADRs were hyperuricemia (52.5%), gastrointestinal (GI) disturbances (40%), ototoxicity (37.5%), and hypokalemia (27.5%). Less frequently reported ADRs were athralgia (12.5%), rash (12.5%), headache (10%), psychiatric disorder (7.5%), visual disorder (5%), and nephrotoxicity (2.5%) (Table 2). Although the majority of patients were cured at the end of treatment, clinicians and pharmacists should be aware of the life-threatening adverse events such as hypokalemia and nephrotoxicity. Clinicians managed the adverse events by adding symptomatic drugs and or modifying the treatment regimen including dose reduction, temporary or permanent discontinuation of the offending drugs. The study found that 14 patients whose offending drugs were permanently stopped due to ototoxicity (8 patients), hyperuricemia (3), and psychiatric disorder (3).

## Discussion

In this study, most patients were aged between 25 and 44 years, which was in line with the study by Skrahina that showed that the number of MDR-TB patients was the highest in the age range of 24–44 years, compared to patients aged 45–64 years [4]. Factors that were thought to cause this were that people of those ages were more productive and socialized more with the surrounding so they were more easily infected with TB.

With regard to the previous TB treatment status, there were patients who experienced treatment failure as many as 42.5%, followed by a recurrence of 37.5% (Table 1). The high rate of treatment failure was an early identification of

**Table 1:** Sociodemographic and clinical characteristics of MDR-TB patients.

| Sociodemographic and clinical characteristics | N | % |
|---|---|---|
| **Age, years** | | |
| ≤24 | 3 | 7.5 |
| 25–44 | 19 | 47.5 |
| 45–64 | 17 | 42.5 |
| ≥65 | 1 | 2.5 |
| **Gender** | | |
| Male | 20 | 50 |
| Female | 20 | 50 |
| **History of TB sensitive treatment (before diagnosed MDR-TB)** | | |
| Yes | 39 | 97.5 |
| No | 1 | 2.5 |
| **Previous TB treatment status** | | |
| Relapse | 15 | 37.5 |
| Failed | 17 | 42.5 |
| Default | 2 | 5 |
| Unknown | 5 | 12.5 |
| **Type of resistance** | | |
| 2 drugs | 20 | 50 |
| ≥2 drugs | 20 | 50 |
| **Duration of therapy, month** | | |
| Mean (min–max) | 26 (22–32) | |
| **Outcome** | | |
| Cure | 39 | 97.5 |
| Completed | 1 | 2.5 |

**Table 2:** Adverse drug reactions characteristic and incidence (n=83).

| Characteristic of ADRs | N | % | Possible causes |
|---|---|---|---|
| Hyperuricemia | 21 | 52.5 | Pyrazinamide<br>Ethambutol (less) |
| Hypokalemia | 11 | 27.5 | Kanamycin<br>Capreomycin |
| Gastrointestinal disturbances | 16 | 40 | Pyrazinamide<br>Ethambutol<br>Levofloksasin<br>Ethionamide |
| Ototoxicity | 15 | 37.5 | Kanamycin<br>Capreomycin |
| Nephrotoxicity | 1 | 2.5 | Kanamycin<br>Capreomycin |
| Athralgia | 5 | 12.5 | Pyrazinamide |
| Headache | 4 | 10 | Cycloserine |
| Psychiatric disorder | 3 | 7.5 | Cycloserine<br>Ethionamide |
| Allergy | 5 | 12.5 | All TB drugs |
| Visual disorder | 2 | 5 | Ethambutol |

drug resistance. Treatment failure could be influenced by patients' noncompliances, the high burden of mycobacterial infection as indicated by cavities, inappropriate treatment regimens, socioeconomic problems, lack of knowledge, adverse drug reactions, and comorbidities [5, 6]. Meanwhile, recurrence could be affected by patients' low immune systems due to lack of environmental hygiene and contact with other TB and MDR-TB patients [7].

The result of this study indicates that 20 patients (50%) had resistance to 2 types of drugs, and 20 patients (50%) had resistance to more than 2 types of drugs. Factors leading to TB drug resistance were dose mismatch, nonadherence to medication, and inappropriate regimen. Before starting MDR-TB treatment, the type of TB drug resistance in patients must be known for the selection of an appropriate combination [6, 8].

The first rank of ADR during MDR-TB treatment was hyperuricemia in 21 patients (52.5%). Suspicious drugs causing this effect were Pyrazinamide and to less extent ethambutol. Pyrazinamide is a strong uric acid retention agent that causes more than 80% reduction of uric acid renal clearance at a dose of 300 mg daily, whereas Ethambutol increases uric acid by decreasing uric acid clearance. The effect of hyperuricemia was observed by giving Ethambutol therapy in the second, third, and fourth weeks. However, Ethambutol had less consistency in the increase of uric acid when compared to Pyrazinamide [9]. Compared to a study in Cameroon (58.3%) and Thailand (81.25%), the prevalence of hyperuricemia in our study was lower [10, 11]. Hyperuricemia was managed by discontinuing pyrazinamide in 3 patients. Meanwhile, the rest of them received a symptomatic drug, Allopurinol.

The second highest ADR found was GI disturbances that occurred in 16 patients (40%) which was less prevalent compared to a study in China (65.4%) but similar to a study in Nepal (40%). Pyrazinamide, Ethambutol, Levofloxacin, and Ethionamide were associated with this effect [12, 13]. There was no patient that experienced permanent discontinuation of offending drugs. Patients developing GI disturbances were treated symptomatically by administering symptomatic drugs such as H2 blocker, proton pump inhibitor, and or antivomiting.

Ototoxicity occurred in 15 patients (37.5%). Kanamycin and Capreomycin were suspected to cause this effect. Aminoglycoside-induced ototoxic mechanisms generally include drugs that pass through the endothelial and epithelial barrier layers resulting in intracellular sensory physiological disruption. The mechanism of molecules was not clearly explained, but some subjects were associated with A1555G polymorphism on 12S rRNA resulting in the breakdown of protein synthesis which was the main mechanism for ototoxicity that called ototoxicity induced with aminoglycoside [14–16]. The prevalence of ototoxicity in this study was higher than in other studies. In Ethiopia, it was shown that 4.8% of patients were clinically diagnosed with ototoxic symptoms and in Botswana, it was shown that 10% of patients showed ototoxic symptoms [17, 18]. Ototoxicity led to permanent discontinuation of offending in 8 patients. Meanwhile, the others were managed by temporary discontinuation, substituted with capreomycin, and added with supporting drugs.

Hypokalemia is a potentially life-threatening adverse effect. In this study, the prevalence of hypokalemia in 11 patients (27.5%) was more prevalent than in a study in Egypt (25.4%) and Pakistan (2.8%) [19, 20]. Hypokalemia was possibly caused by Kanamycin and Capreomycin. Kanamycin and Capreomycin cause secondary hyperaldosteronism which increases urinary excretion of potassium, magnesium, and calcium [21–23]. Hypokalemia was successfully managed by adjusting the dose of Kanamycin and administering a potassium supplement.

Athralgia (12.5%) was managed by the temporary pause of the possible cause (pyrazinamide) and the administering of symptomatic drugs. Meanwhile, allergy (12.5%) possibly caused by all TB drugs was managed with symptomatic drugs. Cycloserine also caused 10% of patients headache and it was managed by administering symptomatic drugs. Although psychiatric disorder was only experienced by 7.5% of patients, it led to permanent

discontinuation of cycloserine in all patients to develop this negative effect. The current study showed nephrotoxicity was only experienced by 2.5% of patients. However, clinicians and pharmacists should be aware of this potentially life-threatening effect.

The average length of treatment for patients in this study was 26 months (Table 1). There were some patients with prolonged duration of therapy. Prolonged treatment can be caused by a lack of knowledge about the dangers of TB, the occurrence of ADRs, lack of family support, and comorbidities that can lead to noncompliance. A study in China revealed ADRs were significantly associated with the discontinuation of medication leading to nonadherence in the drug consumption and prolonged treatment time [24]. A similar result also occurred in the Netherland in which TB treatment was associated with a higher frequency of symptoms, presumed to be due to adverse drug reactions [25].

This study had some limitations. Because of the retrospective nature of the study, data on causality and severity assessment was not well documented. In addition, we might have underestimated or overestimated the number of ADRs. Nevertheless, our data is important because it provides data on ADR and its management in Indonesia. Even though most of the ADRs in this study were managed successfully, there were some life-threatening ADRs such as hypokalemia and nephrotoxicity. Thus, timely recognition and appropriate management were needed to avoid unwanted outcomes.

## Conclusions

Due to the limited number of research samples and the retrospective method, further research with a higher number of samples and a better research design is needed to attain a general conclusion. However, the result of this study is very important because it gives preliminary information on the adverse effects of second-line anti TB drugs in MDR-TB patients in Indonesia.

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Ratri Rokhani, Suharjono*, Kuntaman and Mohammad Akram

# Analysis of prophylactic antibiotic use and risk factor of postoperative infection in urological surgery patients

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**Abstract**

**Objectives:** The widespread use of inappropriate prophylactic antibiotics in urological surgery patients can increase the risk of resistance and development of postoperative infection. This study was aimed to analyze the quality of prophylactic antibiotics use and identify the risk factor of postoperative nosocomial infection in urological surgery patients.

**Methods:** Observational prospective data were obtained from patients' medical records. Data were the pattern of prophylactic antibiotic use in surgical patients' urology in Dr. H. Slamet Martodirdjo Hospital, Pamekasan, for the period of April–June 2020. Inclusion criteria included patients hospitalized with urological surgery and received prophylactic antibiotics before surgery. Exclusion criteria consisted of medical records that were incomplete, and the patient disagreed to participate in the research. Analysis qualitative antibiotic prophylactic used the Gyssens method and risk factor used Chi square.

**Results:** Seventeen patients were not administered for antibiotic prophylactic and nine patients with skin incision were observed to determine the incidence of surgical site infection (SSI) and 55 patients with urethral incision were observed to determine the incidence of urinary tract infection (UTI) postoperative. There was no incidence of SSI and there were three incidences of UTI. The qualitative analysis of the Gyssens method showed that category-0 was of 51 (79.7%) and category-I was of 13 (20.31%).

**Conclusions:** The quality of the use of prophylactic antibiotics with the Gyssens method shows that there is an appropriate category (category-0) and a few are in category-I (inappropriate administration time) and no incidence of surgical wound infection.

**Keywords:** antibiotic prophylactic; Gyssens analysis; risk factors infection.

***Corresponding author: Suharjono**, Department of Clinical Pharmacy, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia, Phone: +62 (031)5033710, E-mail: suharjono@ff.unair.ac.id
**Ratri Rokhani,** Clinical Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia
**Kuntaman,** Department of Clinical Microbiology, School of Medicine-Dr. Soetomo Hospital, Universitas Airlangga, Surabaya, East Java, Indonesia
**Mohammad Akram,** Surgical Department, Slamet Martodirdjo Hospital, Pamekasan, East Java, Indonesia

## Introduction

The widespread use of inappropriate antibiotics is a major issue in public health and patient safety [1]. Inappropriate use of antibiotics can cause various problems, including more expensive treatment, more toxic side effects, widespread resistance, and the emergence of superinfection events that are difficult to treat [2]. The epidemiology of antibiotics was shown in a study conducted at a hospital in Istanbul in 2011, showing that out of the 553 hospitalized patients, 199 of them received antibiotic therapy and 109 of them were patients in the surgery unit. These antibiotic prescriptions showed as many as 70.9% were for therapeutic antibiotics and 29.1% were for prophylactic antibiotics [3].

Infections that often occur primarily in urological surgery and are the main causes of postsurgical nosocomial infections are surgical site infection (SSI) and urinary tract infections (UTI) [4]. SSI is a serious complication of surgical procedures that occur in surgery and ones of the major hospital acquired infections highly associated with prolonged hospitalization, additional costs to health systems, morbidity, and mortality [5]. Based on studies in hospitals in the Southeastern United States, it is noted that out of 532,694 surgical procedures performed, 3,988 of them have complex SSI (prevalence rate of 0.7 infections per 100 procedures) [6] and in Indonesia, it is 2–18%. In urological surgery, SSI occurs after major surgery while UTI occurs after an endourological procedure.

The global problem that is currently being faced is the growing number of bacteria resistant to antibiotics. The use of antibiotics appropriately in the hospital is an important factor in dealing with this problem. One way to overcome

this is by using antibiotics rationally, intervening to optimize the use of antibiotics, and monitoring and evaluating the use of antibiotics in hospitals [7].

The number of patients at Dr. Slamet Martodirjo Hospital who undergoes urological surgery each month ranges from 80–120 patients. Until now, there has never been an evaluation regarding the rationality of the use of prophylactic antibiotics and the incidence of postoperative nosocomial infections in urological surgery patients at the hospital.

## Materials and methods

This study was designed to comply with the criteria for ethical conduct and was approved by the Health Research Ethics Committee of Dr. Slamet Martodirjo Hospital, with reference number of 070/251/432.603/KEPK/2020. This research was an analytic and observational study using a cross-sectional design. Prospective data collection to analyze the rationality of prophylactic antibiotics and to analyze whether there was a relationship between risk factors and postoperative nosocomial infections at Dr. Slamet Martodirdjo Hospital. Research was conducted from April to June in the surgical inpatient room (surgery B) of Dr. Slamet Martodirjo Hospital.

Data obtained from observation and assessment of the patient's medical record and the patient's condition with monitor acetylsalicylic acid (ASA) score. Inclusion criteria included patients hospitalized with urological surgery and received prophylactic antibiotics before surgery. Exclusion criteria consisted of urological surgery patient data from medical records that were incomplete, patients with HIV/AIDS and decreased awareness, and the patient/patient's family who were not willing to sign the inform for consent to participate in the research.

Assessment of the quality of the use of prophylactic antibiotics in urological surgery patients was carried out using the Gyssens method. This assessment was carried out by two reviewers. The results of the Gyssens analysis were grouped into seven categories, namely categories-0 to VI [2].

Postoperative nosocomial infections in this study were incidence evidences of SSI and UTI. The risk factors for SSI assessed in this study included: type of prophylactic antibiotics, time of administration, duration of administration, plan of surgery, type of surgery, duration of surgery, length of stay before surgery, antibiotics before surgery, ASA value, and the presence or absence of comorbid in the form of diabetic mellitus (DM). The risk factor for UTI assessed in this study included: type of prophylactic antibiotics, time of administration, duration of administration, plan of surgery, type of surgery, duration of surgery, length of stay before surgery, antibiotics before surgery, ASA value, the presence or absence of comorbid in the form of DM, gender, and the use of catheter. To determine the risk factors of postoperative nosocomial infection in urological surgery patient, Chi square method was used.

## Results

During the study, there were 81 patients who underwent urological surgery, who were in accordance with inclusion and exclusion criteria and received prophylactic antibiotics, 64 patients who were then used as study samples. The percentage of patients consisted of 58 males (90.62%) and six females (9.38%). The most common type of urological disease was benign prostate hyperplasia, transurethral rejection prostate, and other urological surgery cases that required incisions through the urethra and skin.

Based on the results of the Gyssens analysis, it was known that as many as 79.68% of the use of prophylactic antibiotics in the urology surgery included in category-0 meaning that it was correct – rational, and 20.31% to category-I meaning that the administration was not on time.

The risk factors for infection in the area of surgery that were assessed in this study included: the type of prophylactic antibiotics, the time of administration, the administration, the nature of the operation, the type of surgery, the duration of surgery, the length of stay before surgery, the antibiotics before surgery, the ASA value, and the presence or absence of comorbid in the form of diabetes. During the study period, more surgery was performed through urethral incisions rather than skin incisions. According to the European Urological Association, urological surgery that has the risk of causing infection of the operating area is open surgery or through skin incisions. Meanwhile, endourological procedures performed through urethral incisions have a higher risk of causing UTI. A total of operations performed through skin incisions were nine procedures, while through the urethra there were 55 procedures. The following were the results of observations on the risk factors for the incidence of infection in the surgical area in urological surgery patients (Table 1).

During the study period, there was no incidence of SSI in urological surgery patients. Observation of the incidence of SSI was carried out on nine patients who underwent surgery through skin incisions until the 30th day after surgery (Table 2). Three incidences of UTI were found in urological surgery patients after surgery. Observation of the incidence of UTI was carried out in 55 patients who underwent surgery through urethral incisions until the 30th day after surgery. UTI diagnosis was carried out by a clinician who checked it after examining signs and symptoms and a complete urine check. A total of three patients who experienced this UTI were identified after the patients were controlled on the seventh day after being discharged from the hospital. Gender and catheter use had risk factors showing a significant effect on the incidence of UTI ($p<0.05$) (Table 3).

**Table 1:** The quality of prophylactic antibiotic use in urological surgery patients using Gyssens method.

| Antibiotic | Category | | | | | | | Amount |
|---|---|---|---|---|---|---|---|---|
| | VI | V | IV | III | II | I | 0 | (n=64) |
| Cefuroxime | 0 | 0 | 0 | 0 | 0 | 7 | 25 | 32 |
| Cefoperazone sulbactam | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| Ceftriaxone | 0 | 0 | 0 | 0 | 0 | 3 | 10 | 13 |
| Amoxicillin + Clavulanic acid | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 3 |
| Ampicilin | 0 | 0 | 0 | 0 | 0 | 2 | 12 | 14 |
| Amount | 0 | 0 | 0 | 0 | 0 | 13 | 51 | 64 |
| Percentage, % | 0 | 0 | 0 | 0 | 0 | 20.31 | 79.68 | 100 |

Gyssens category: 0 = appropriate and rational, I = not on time, II = inappropriate dose/interval/route, III = duration is too long/too short, IV = some are more effective/less toxic/less expensive/a narrow spectrum, V = no indication and VI = incomplete data.

**Table 2:** Risk factor of surgical site infection.

| Risk factor | Category | SSI Incidence | | No SSI Incidence | | Amount (n=64) |
|---|---|---|---|---|---|---|
| | | n | % | n | % | |
| Type of prophylactic antibiotic | Cefuroxime | 0 | 0 | 5 | 55.55 | 5 |
| | Cefoperazone sulbactam | 0 | 0 | 0 | 0 | 0 |
| | Ceftriaxone | 0 | 0 | 1 | 11.11 | 1 |
| | Amoxicillin + Clavulanic acid | 0 | 0 | 0 | 0 | 0 |
| | Ampicilin | 0 | 0 | 3 | 33.33 | 3 |
| Time of administration, min | <30 | 0 | 0 | 4 | 44.44 | 4 |
| | 30–60 | 0 | 0 | 4 | 44.44 | 4 |
| | >60 | 0 | 0 | 1 | 11.11 | 1 |
| Duration of administration, h | <24 | 0 | 0 | 9 | 100 | 9 |
| | >24 | 0 | 0 | 0 | 0 | 0 |
| Plan of surgery | Elective | 0 | 0 | 9 | 100 | 9 |
| | Cito | 0 | 0 | 0 | 0 | 0 |
| Type of surgery | Clean | 0 | 0 | 0 | 0 | 0 |
| | Clean contaminated | 0 | 0 | 9 | 100 | 9 |
| | Contaminated | 0 | 0 | 0 | 0 | 0 |
| | Dirty | 0 | 0 | 0 | 0 | 0 |
| Duration of surgery, h | <1 | 0 | 0 | 9 | 100 | 9 |
| | >1 | 0 | 0 | 0 | 0 | 0 |
| Duration of inpatient before surgery, days | <3 | 0 | 0 | 9 | 100 | 9 |
| | >3 | 0 | 0 | 0 | 0 | 0 |
| Preoperative antibiotic use | Yes | 0 | 0 | 0 | 0 | 0 |
| | No | 0 | 0 | 9 | 100 | 9 |
| ASA score | I | 0 | 0 | 0 | 0 | 0 |
| | II | 0 | 0 | 9 | 100 | 9 |
| | III | 0 | 0 | 0 | 0 | 0 |
| | IV | 0 | 0 | 0 | 0 | 0 |
| Comorbid, DM | Yes | 0 | 0 | 1 | 11.11 | 1 |
| | No | 0 | 0 | 8 | 88.88 | 8 |

ASA score: I = normal healthy patient, II = patient with mild systemic disease, III = patient with severe systemic disease, IV = patient with severe systemic disease that is a threat to life.

**Table 3:** Risk factor of urinary tract infection.

| Risk factor | Category | UTI incidence | | No UTI incidence | | Amount (n=55) | p-Value |
|---|---|---|---|---|---|---|---|
| | | n | % | n | % | | |
| Type of prophylactic antibiotic | Cefuroxime | 2 | 3.63 | 25 | 45.45 | 27 | 0.878 |
| | Cefoperazone sulbactam | 0 | 0 | 2 | 3.63 | 2 | |
| | Ceftriaxone | 1 | 1.81 | 11 | 20 | 1 | |
| | Amoxicillin + Clavulanic acid | 0 | 0 | 3 | 5.45 | 3 | |
| | Ampicilin | 0 | 0 | 14 | 25.45 | 14 | |
| Time of administration, min | <30 | 0 | 0 | 13 | 23.63 | 13 | 0.631 |
| | 30–60 | 2 | 3.63 | 29 | 60 | 31 | |
| | >60 | 1 | 1.81 | 9 | 10.90 | 10 | |
| Duration of administration, h | <24 | 3 | 5.45 | 52 | 94.54 | 55 | 0.061 |
| | >24 | 0 | 0 | 0 | 0 | 0 | |
| Plan of surgery | Elective | 3 | 5.45 | 52 | 94.54 | 55 | 0.089 |
| | Cito | 0 | 0 | 0 | 0 | 0 | |
| Type of surgery | Clean | 0 | 0 | 0 | 0 | 0 | 0.076 |
| | Clean contaminated | 3 | 5.45 | 52 | 94.54 | 55 | |
| | Contaminated | 0 | 0 | 0 | 0 | 0 | |
| | Dirty | 0 | 0 | 0 | 0 | 0 | |
| Duration of surgery, h | <1 | 3 | 5.45 | 52 | 94.54 | 55 | 0.066 |
| | >1 | 0 | 0 | 0 | 0 | 0 | |
| Duration of inpatient before surgery, days | <3 | 3 | 5.45 | 52 | 94.54 | 55 | 0.088 |
| | >3 | 0 | 0 | 0 | 0 | 0 | |
| Preoperative antibiotic use | Yes | 0 | 0 | 0 | 0 | 0 | 0.051 |
| | No | 3 | 5.45 | 52 | 9.54 | 55 | |
| ASA score | I | 0 | 0 | 0 | 0 | 0 | 0.143 |
| | II | 3 | 5.45 | 52 | 9.54 | 55 | |
| | III | 0 | 0 | 0 | 0 | 0 | |
| | IV | 0 | 0 | 0 | 0 | 0 | |
| Comorbid, DM | Yes | 2 | 3.63 | 9 | 16.36 | 11 | 0.243 |
| | No | 1 | 1.81 | 43 | 78.18 | 44 | |
| Gender | Male | 1 | 1.81 | 49 | 89.09 | 50 | 0.000 |
| | Female | 2 | 3.63 | 3 | 5.45 | 5 | |

ASA score: I = normal healthy patient, II = patient with mild systemic disease, III = patient with severe systemic disease, IV = patient with severe systemic disease that is a threat to life.

# Discussion

Assessment of the quality of antibiotic use was carried out to determine the accuracy of prophylactic antibiotic use. The quality of antibiotic use was carried out by using the Gyssens method because this method was more specific by evaluating every important parameter associated with the use of antibiotics which included: indication, effectiveness, safety, price, and spectrum. In addition, the duration of treatment, dosage, interval, and duration of administration were also evaluated [2].

A total of 13 antibiotics were inappropriate in time of administration with details as follows: seven uses of cefuroxime, three uses of ceftriaxone, one use of amoxicillin-clavulanic acid, and three uses of ampicillin. The use of antibiotics in category-I (inappropriate in time of administration) was 20.31%. The choices of these half-life antibiotics from cefuroxime, ceftriaxone, amoxicillin-clavulanic acid, and ampicillin were 1.5; 8.2; 1 – 1, and 1 h, respectively. These antibiotics were categorized as inappropriate in time of administration because they were given more than 1 h before the incision. Based on the guidelines for the use of antibiotics, prophylactic antibiotics should be given at less than 30 min before the incision and ideally at induction [7]. Prophylactic antibiotic administration must be appropriate to ensure the achievement of high or optimal levels in serum or tissue at the time of incision, thus these levels must be maintained during the operation. If the surgical procedure is longer than the half-life of the antibiotic given, the antibiotic administration can be repeated during the operation [8]. Meanwhile, the American Urological Association guidelines state that

prophylactic antibiotics in urological surgery can be given up to 1 h before the incision [9].

In this study, as many as 79.68% of prophylactic antibiotic use in urological surgery patients were in category-0 (appropriate-rational), while 20.31% were included in category-I (inappropriate administration time) [2].

Observation of the incidence of SSI was carried out on nine patients who underwent open surgery through skin incisions. During the study, there was no incidence of SSI. Meanwhile, a study conducted in one teaching hospital in Brazil showed that the incidence rate of SSI was 22.96% in urological surgery with a risk factor for comorbid DM, which showed a significant relationship with the incidence of SSI [10]. A study conducted at a hospital in Riau showed that there were two incidences of SSI from a total of 84 patients who underwent open Urinary Tract Stone surgery [11].

Of the 55 surgeries performed through urethral incisions, there were three incidents of UTI. A clinician diagnosed the UTI criteria after a complete examination and urine check. Case finding of UTI was obtained when the patient returned to control on the seventh day after discharged from hospital. From the analysis, it was found that the risk factors for age and catheter use had a significant relationship with the incidence of UTI (Table 3).

The proportion of incidence of UTI was more in female than in male, which were two patients to one patient. Female get more UTI because the female urethra is shorter than the male urethra so that bacteria can easily reach them [12]. In addition, there is less colonization around the urethra due to the absence of a place such as the vagina in female. Meanwhile, for the risk factors for using catheters, all UTI incidents occurred in patients who used catheters. Catheter-associated UTI can be influenced by various factors (size, duration catheter used, and catheter quality) [13]. Asbone, Rosa, and Ulfa [14] in a hospital in Yogyakarta show an incidence rate of UTI, which is 33.49%, due to the use of catheters and germs that cause UTI, which are *Escherichia coli*.

This study indeed has limitation, which is the small number of open surgeries through incisions in the skin, so that it cannot be further discussed about the incidence of SSI and the risk factors that influence it.

## Conclusions

In this study, as many as 79.68% of prophylactic antibiotic use in urological surgery patients are in category-0 (appropriate and rational), while 20.31% are included in category-I (inappropriate administration time) and no incidence of surgical wound infection is found.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.

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Ahmad Dzulfikri Nurhan, Maria Apriliani Gani, Aniek Setiya Budiatin, Siswandono Siswodihardjo and Junaidi Khotib*

# Molecular docking studies of *Nigella sativa* L and *Curcuma xanthorrhiza Roxb* secondary metabolites against histamine *N*-methyltransferase with their ADMET prediction

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**Abstract**

**Objectives:** Histamine *N*-methyltransferase (HNMT) is an enzyme that plays a crucial role in the inactivation of histamine in central nervous system, kidneys and bronchi. Inhibition of HNMT is known to have a potential role in treating attention-deficit hyperactivity disorder, memory impairment, mental illness and neurodegenerative illnesses. Therefore, to find potential compounds that could be developed as novel HNMT inhibitors, this study conducted an *in silico* study of the secondary metabolites of *Nigella sativa* L and *Curcuma xanthorrhiza Roxb*.

**Methods:** In this study, we conducted a molecular docking study of 36 secondary metabolites of *N. sativa* L and 26 secondary metabolites of *C. xanthorrhiza Roxb* using an *in silico* approach targeting HNMT protein (PDB ID: 2AOT) using AutoDockVina software. The prediction of ADMET characteristics was done using the pkCSM Online Tool.

**Results:** This study obtained one metabolite from *N. sativa* L (longifolene) and seven metabolites from *C. xanthorrhiza Roxb* {(+)-beta-atlantone, humulene epoxide, (–)-beta-curcumene, (E)-caryophyllene, germacrone, (R)-(–)-xanthorrhizol, and (–)-beta-caryophyllene epoxide} which were predicted to have potential to be developed as HNMT inhibitors.

**Conclusions:** This study found several secondary metabolites of *N. sativa* L and *C. xanthorrhiza Roxb* which had activity as HNMT inhibitors. This research can likewise be utilized as a basis for further research, both *in vitro*, *in vivo*, and clinical trials related to the development of secondary metabolites from *N. sativa* L and *C. xanthorrhiza Roxb* as novel HNMT inhibitor compounds.

**Keywords:** *Curcuma xanthorrhiza*; mental illness; HNMT inhibitor; in silico studies; *Nigella sativa*.

***Corresponding author: Junaidi Khotib**, Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, Phone: +6281331840710, E-mail: junaidi-k@ff.unair.ac.id
**Ahmad Dzulfikri Nurhan, Maria Apriliani Gani and Aniek Setiya Budiatin,** Department of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia
**Siswandono Siswodihardjo,** Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia

## Introduction

Histamine is a chemical mediator produced by neurons, mast cells and circulating basophils through mediation by IgE. Histamine is synthesized from its precursor (L-histidine) by cytoplasmatic catalyst L-histidine decarboxylase (HDC) in central nervous system (CNS), at that point put away in vesicles and afterward delivered from axon terminals through a quick turnover component to work as a neurotransmitter [1, 2]. The principal role of histamine is mediated through interaction with four different G-protein coupled receptors (GPCRs), the $H_1$, $H_2$, $H_3$, and $H_4$ receptors, which have special ramifications for various pathological processes such as allergy and anaphylaxis. In addition, histamine also plays a role in gastric acid secretion and neurotransmission in CNS and peripheral nervous system (PNS) [3, 4].

It has been currently recognized that histamine is inactivated through two primary metabolic pathways, the ring methylations mechanism by histamine-*N*-methyltransferase (HNMT) which brings about the transformation of histamine to *N*-methylhistamine; and through oxidative deamination by diamine oxidase so it becomes imidazole acetic acid [5, 6]. Interestingly, only HNMT is considered the most promising therapeutic target, especially regarding selective regulation of histaminergic neurotransmission in the CNS [7].

Moreover, a few studies have suggested that increased histaminergic neurotransmission in the CNS can be beneficial in the treatment of various diseases such as:

attention-deficit hyperactivity disorder [8], memory impairment and neurodegenerative illnesses [7]. Interestingly, the increase in histaminergic neurotransmission in the CNS is known to be achieved by the intercession of HNMT inhibitors through their role in inhibiting HNMT, which is a histamine metabolizing enzyme. This has implications for disruption of histamine metabolism, resulting in an increase in histamine [9]. Therefore, the development of compounds that have activity as HNMT inhibitors will be of great benefit, particularly in the treatment of histamine-mediated diseases of the CNS.

Several studies have tried to develop a compound that is proposed as an HNMT inhibitor. Metoprine, a 2,4-diaminopyridine derivative, is referred to have acted as an HNMT inhibitor. Previous studies utilizing metoprine demonstrate that activation of the histaminergic system in the CNS influences diverse brain functions, including attenuation of methamphetamine-induced behavioral disorders, increased cognitive function and antiepileptic effects. However, metoprine was additionally found to inhibit mammalian dihydrofolate reductase and decrease cellular folate metabolism, bringing about debilitated cell development [7, 10]. Interestingly, another investigation has prevailing with regards to developing SKF91488 as an HNMT inhibitor that specifically inhibits the enzymatic activity. Unfortunately, SKF91488 has poor blood–brain barrier (BBB) permeability [7]. Consequently, exploration and development of novel HNMT inhibitor compounds with high specificity and adequate BBB permeability are still needed.

This study investigates the metabolites of *Nigella sativa* L and *Curcuma xanthorrhiza Roxb* which have can possibly be developed as HNMT inhibitors. These two plants were selected based on some evidences that extracts containing secondary metabolites from the two plants have neuropharmacological effects against neurodegenerative illnesses, such as Alzheimer's, depression, encephalomyelitis, epilepsy, ischemia, Parkinson's, and traumatic brain injury [11, 12]. However, which secondary metabolites have the most potential to cause these effects and the mechanisms underlying them are not yet fully understood, so it is interesting to investigate further in this study, especially regarding their potential as an HNMT inhibitor.

# Materials and methods

### Ligand determination and preparation

In this study, the ligands utilized were secondary metabolites of *N. sativa* L and *C. xanthorrhiza Roxb*. The secondary metabolites of *N. sativa* L and *C. xanthorrhiza Roxb* were acquired through a search on the KNApSAcK Family database http://www.knapsackfamily.com/, by entering the keywords "*N. sativa*" and "*C. xanthorrhiza*", respectively on July 23, 2020. Furthermore, the three-dimensional ligand structure was formed and optimized using ChemDraw software and Chem3D version 15.0 (PerkinElmer®). Then, the ligands were prepared by removing water molecules, protonated and gasteiger charge was added using AutoDockTools-1.5.6 software.

### Protein determination and virtual elucidation

Histamine *N*-methyl transferase (HNMT) protein subatomic structure was downloaded from the Protein Data Bank (https://www.rcsb.org/). We selected the HNMT protein with PDB ID: 2AOT, which contained the reference ligand *N*-[2-(benzhydryloxy)ethyl]-*N*,*N*-dimethylamine. Then, the protein was prepared using AutoDockTools-1.5.6 software.

### Validation of molecular docking study

Validation was performed by re-docking the reference ligand to the suitable pocket binding site on the HNMT protein multiple times. The protein structure and the pocket binding site were then visualized using DoGSiteScorer (http://dogsite.zbh.uni-hamburg.de). The validity of the molecular docking study on the histamine *N*-methyl transferase protein was assessed based on the root mean square deviation (RMSD) determined by comparing the reference ligand before and after docking by means of PyMOL version 2.3.4. Re-docking was accepted if RMSD <2 Å. The grid box used was 40; 40; 40 Å center at *X*=52,797; *Y*=(−) 12,625; *Z*=30,172.

### Molecular docking study

Molecular docking was performed using AutoDockVina. The best docking results were investigated by comparing the structure of the docked molecules (the ligands under investigation) with the crystal structure of the reference ligand, each at the binding site. The result was introduced as binding energy. The most minimal energies indicated the best binding poses between the ligand functional groups and the amino acid residues of the protein. Next, the potential ligand–protein complex (binding energy better than the reference ligand) was visualized utilizing the Discovery Studio Visualizer v17.2.016349 software.

### Prediction of ADME and compound toxicity

Prediction of pharmacokinetic properties as well as absorption, distribution, metabolism, excretion (ADME) and toxicity of secondary metabolites of *N. sativa* L and *C. xanthorrhiza Roxb* which were potential as HNMT inhibitors was carried out using pkCSM (http://biosig.unimelb.edu.au/pkcsm/) Online Tool. To begin with, these metabolites were made into 2D atomic structures using ChemDraw and replicated to Chem3D to make 3D structures. Second, all secondary metabolites were translated into SMILES design using the SMILES Translator Online Help (http://cactus.nci.nih.gov/translate/). In the SMILES design, the test compounds were processed using pkCSM Online Tool to predict ADME and the toxicity of these compounds.

# Results

Molecular docking studies were validated by redocking the reference ligand with HNMT protein. The results indicated that the reference ligand occupied a pocket binding site on the HNMT protein reliably. This was proved by acquired mean RMSD estimation of 1.852 ± 0.019 Å (<2 Å) after three replications. The binding energy value between replications was additionally the equivalent, i.e. −8.7 kcal/mol. Visualization of the pocket binding site and the interaction between the reference ligand and HNMT protein can be seen in Figure 1.

## Potential histamine *N*-methyl transferase inhibitor from *N. sativa* L and *C. xanthorrhiza Roxb*

From KNApSAcK family information base, we acquired 36 secondary metabolites of *N. sativa* L and 26 secondary metabolites of *C. xanthorrhiza Roxb*. Furthermore, these metabolites were prepared for docking analysis at the same pocket binding sites as the reference ligand. The docking analysis obtained showed that as many as six secondary metabolites of *N. sativa* L and nine secondary metabolites of *C. xanthorrhiza Roxb* were predicted to have good interactions with HNMT protein (Figures 2 and 3, respectively).

## ADMET prediction of potential histamine *N*-methyltransferase inhibitor from secondary metabolites of *N. sativa* L and *C. xanthorrhiza Roxb*

Predictions of physicochemical attributes of secondary metabolites *N. sativa* L and *C. xanthorrhiza Roxb* which may be potential as histamine N-methyl transferase inhibitors can be seen in Table 1, while the predictions of the characteristics of absorption, distribution, metabolism, excretion, and toxicity (ADMET) of these metabolites can be found in Table 2.

# Discussion

With a molecular docking study, we found that as many as six out of 36 secondary metabolites of *N. sativa* L had potential to be developed as HNMT inhibitors (PDB ID: 2AOT). This was because these metabolites had great interactions with HNMT protein with a demonstrate that the yielded binding energy was lower than the binding energy of the reference ligand (≤−8.7 kcal/mol). The binding energy value scope of these potential metabolites to HNMT went from −10.9 to −9.3 kcal/mol. Besides, this investigation likewise found that upwards nine out of 26 secondary metabolites of *C. xanthorrhiza Roxb* had potential to be developed as HNMT inhibitors with a binding energy value range against HNMT of −10.3 to −9.1 kcal/mol. All these potential secondary metabolites can form hydrogen bonding interactions and/or hydrophobic bond interactions with HNMT proteins which have implications for the binding energy value and the strength of the interactions formed (Figures 2 and 3).

With an assortment of databases, we acquired numerous molecules that could become appropriate ligands for a receptor. Notwithstanding, regardless of how great the ligand coordinate with the receptor, candidate molecules will be difficult to develop their usefulness if they are toxic and having poor ADME characteristics [13, 14]. Investigation of more than 2000 drugs acquired from the World Drugs Index (WDI) information base lead to the determination that a compound has great absorption or

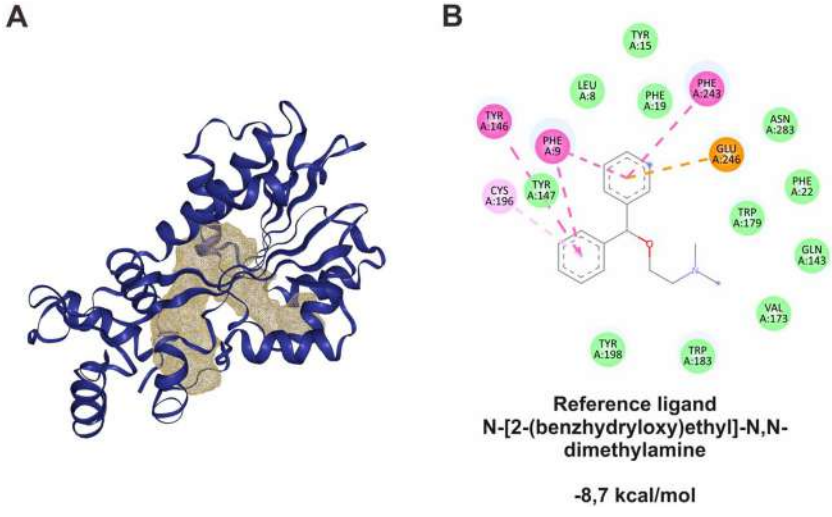


**Figure 1:** (A) Cartoon representation of histamine *N*-methyltransferase protein and its pocket binding site; (B) interaction between reference ligand and histamine *N*-methyltransferase protein.

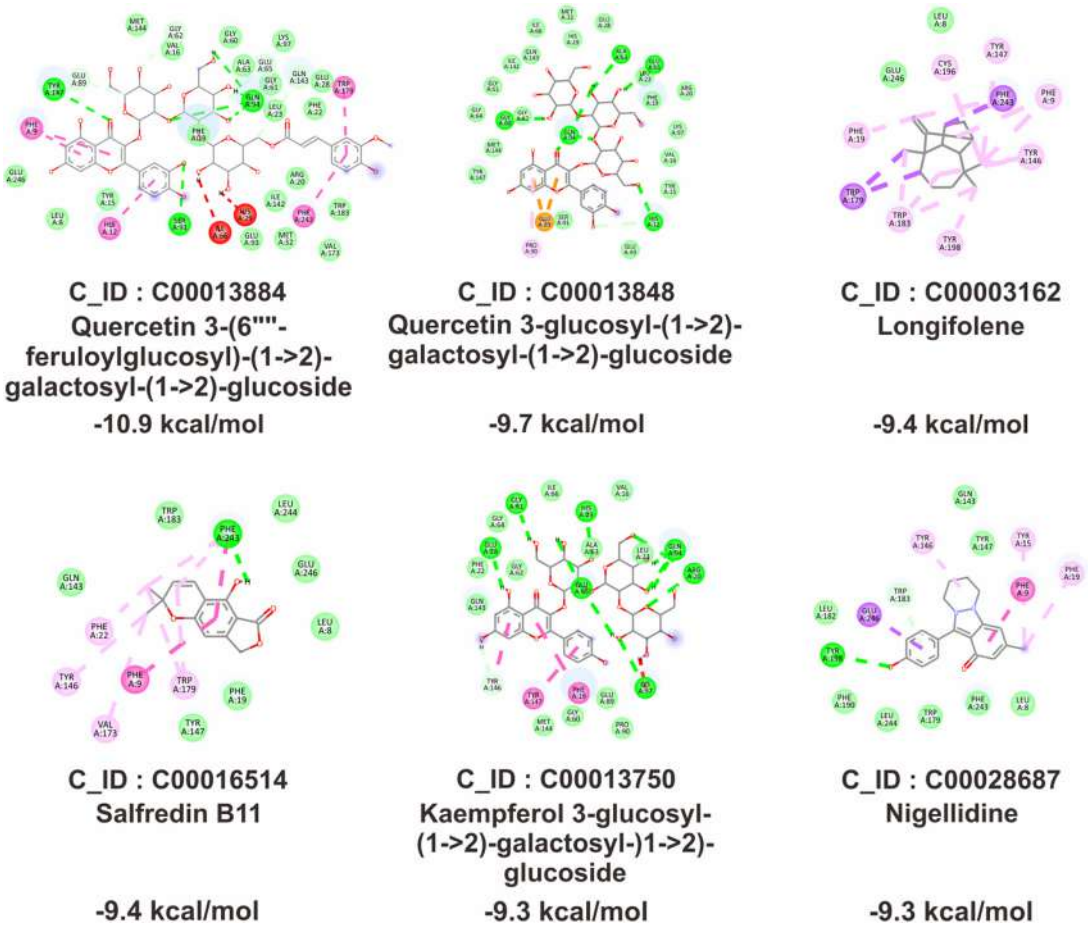


**Figure 2:** Interaction of potential metabolites from *N. sativa L* with histamine *N*-methyltransferase protein.

permeability characteristics if it meets a minimum three of the accompanying four standards: MW ≤500; calculated octanol/water partition coefficient ≤+5; has ≤5 HBD expressed as the number of O–H and N–H groups; and has ≤10 HBA which are expressed as the number of N and O atoms. The above standard is otherwise called the Lipinski Rules of Five [28].

An *in silico* study of physicochemical attributes of the six potential *N. sativa* L metabolites to be developed as HNMT inhibitors showed that three of those six compounds complied with the Lipinski Rules of Five. These compounds include: longifolene; salfredin B11 and nigellidine. Interestingly, the physicochemical characteristics of the nine potential secondary metabolites present in *C. xanthorrhiza Roxb* all met the Lipinski Rules of Five [15]. These various compounds included: demethoxycurcumin; curcumin; (+)-beta-atlantone; humulene epoxide; (+)-beta-curcumene; (E)-caryophyllene; gemacrone; (R)-(–)-xanthorrhizol; and (–)-beta-caryophyllene epoxide. In this manner, we can predict that as many as three secondary metabolites from *N. sativa* L and nine secondary metabolites from *C. xanthorrhiza Roxb* can be easily absorbed and have good permeability (Table 1).

Skin permeability is a significant viewpoint to improve drug adequacy, particularly in transdermal dosage forms. A molecule will barely penetrate the skin if the log $K_p$ surpasses –2.5 cm/h [16, 17]. Table 2 shows that skin permeability values of potential secondary metabolites found in *N. sativa* L vary, ranging from –3.276 to –1.740 cm/h. Likewise, potential metabolites of *C. xanthorrhiza Roxb* with skin permeability values had a range from –3.062 to –1.221 cm/h. Subsequently, it can be predicted that some of these potential secondary metabolites have good skin penetration, while others do not.

Monolayer Caco-2 cells are broadly utilized as an *in vitro* model of human intestinal mucosa to predict absorption of orally administered drugs by estimating the log of permeability coefficient (log Papp; log cm/s). In particular, in the prescient model using pkCSM, the high permeability of Caco-2 is portrayed as a log Papp prediction value of >0.90 cm/s [17]. In this investigation, three of the six potential secondary metabolites found in *N. sativa* L and nine

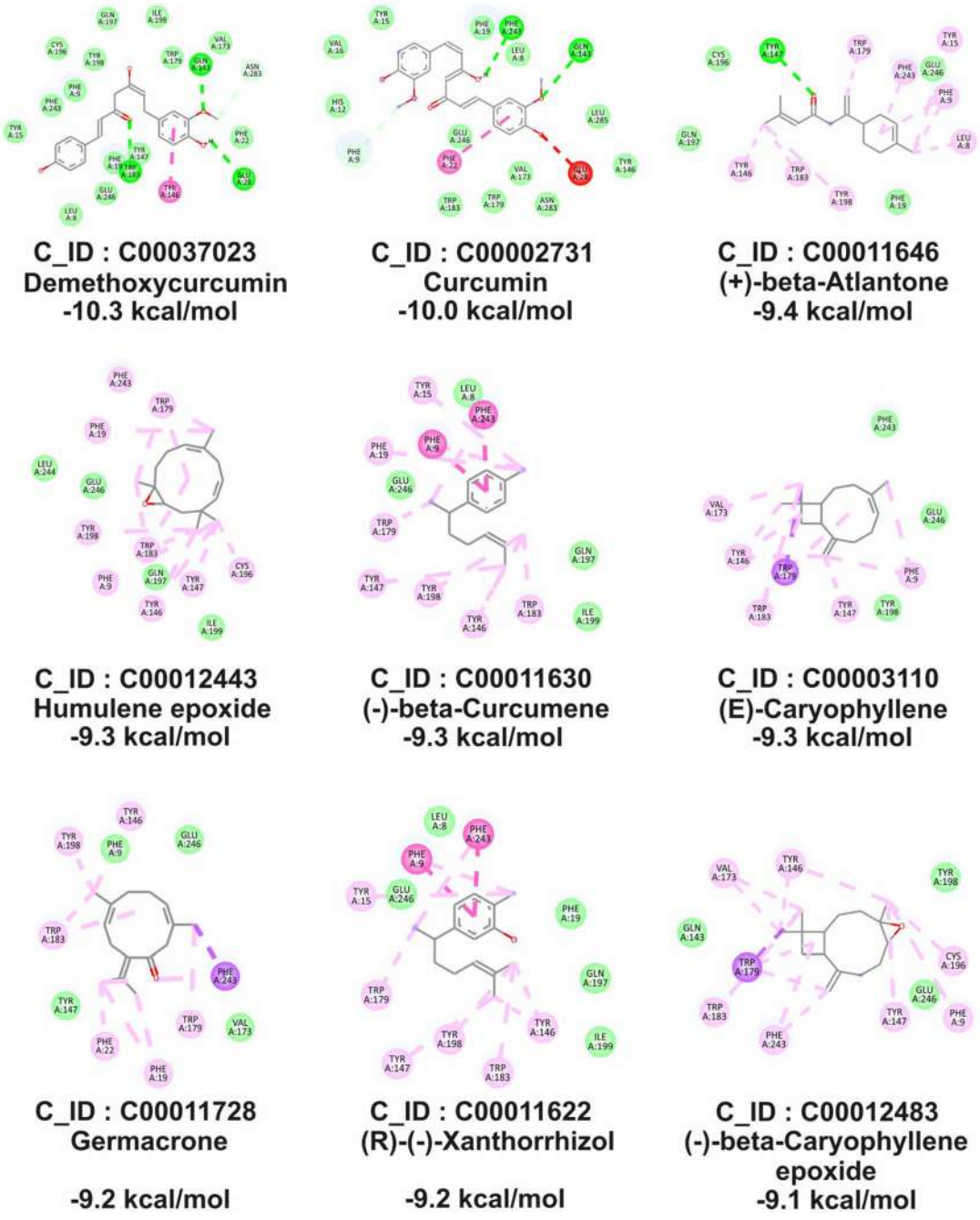


**Figure 3:** Interaction of potential metabolites from *C. xanthorrhiza Roxb* with histamine *N*-methyltransferase protein.

potential secondary metabolites from *C. xanthorrhiza Roxb* were predicted to have good Caco-2 permeability (log Papp>0.90 cm/s) (Table 2). Data synergy showed that these secondary metabolites were the same as those that met the Lipinski Rules of Five which were predicted to be easily absorbed and had high permeability.

Regarding volume distribution (VD) prediction, the higher the VD value, the more prominent the amount of drug concentrated to the tissue compared to plasma. This investigation utilized a model made from the steady-state volume distribution (VDss) assessment, which was then expressed in log L/kg. This prescient model uncovered that the distribution rate of a molecule was considered high if the VDss value was more than 2.81 L/kg (log VDss>0.45) and was considered low if the VDss value was less than 0.71 L/kg (log VDss<−0.15) [17, 18]. Therefore, the predictive results for *N. sativa* L's potential metabolites as HNMT inhibitors had a log VDss range of −0.163 to 0.781, where

**Table 1:** Prediction of *in silico* values of physicochemical properties of potential histamine *N*-methyl transferase inhibitor from *N. sativa L* and *C. xanthorrhiza Roxb* using pkCSM online tool.

| C_ID | MW | Log P | Torsion | HBA | HBD | PSA, $A^2$ | Water solubility |
|---|---|---|---|---|---|---|---|
| **Potential secondary metabolites from *N. sativa* L** | | | | | | | |
| C00013884 | 964.832 | −2.9120 | 14 | 25 | 14 | 381.704 | −2.892 |
| C00013848 | 788.661 | −4.8905 | 10 | 22 | 14 | 307.489 | −2.891 |
| C00003162 | 204.357 | 4.4150 | 0 | 0 | 0 | 94.141 | −5.668 |
| C00016514 | 232.235 | 2.2468 | 0 | 4 | 1 | 98.468 | −2.725 |
| C00013750 | 772.662 | −4.5961 | 10 | 21 | 13 | 302.694 | −2.878 |
| C00028687 | 294.354 | 3.2294 | 1 | 4 | 1 | 128.116 | −4.401 |
| **Potential secondary metabolites from *C. xanthorrhiza Roxb*** | | | | | | | |
| C00037023 | 338.359 | 3.8440 | 6 | 5 | 3 | 144.997 | −3.949 |
| C00002731 | 368.385 | 3.8526 | 7 | 6 | 3 | 156.475 | −4.162 |
| C00011646 | 218.340 | 4.2144 | 4 | 1 | 0 | 98.935 | −4.356 |
| C00012443 | 220.356 | 4.2466 | 0 | 1 | 0 | 99.571 | −4.139 |
| C00011630 | 204.357 | 5.0354 | 4 | 0 | 0 | 94.774 | −6.145 |
| C00003110 | 204.357 | 4.7252 | 0 | 0 | 0 | 94.458 | −5.515 |
| C00011728 | 218.340 | 4.2585 | 0 | 1 | 0 | 98.935 | −4.385 |
| C00011622 | 218.340 | 4.5505 | 4 | 1 | 1 | 98.821 | −4.429 |
| C00012483 | 220.356 | 3.9364 | 0 | 1 | 0 | 99.255 | −4.376 |

C_ID, chemical identity; MW, molecular weight; Log P, logarithm of octanol/water partition coefficient; Torsion, bond between rotating atoms (rotatable bond); HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; PSA, polar surface activity.

**Table 2:** ADMET properties of potential histamine *N*-methyltransferase inhibitor from *N. sativa* and *C. xanthorrhiza* by pkCSM Online Tool.

| C_ID | Absorption | | | Distribution | | Metabolism | | Excretion | Toxicity | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Intestinal absorption | Skin permeability | Caco-2 permeability | VDss | BBB permeability | CYP2D6 inhibitor | CYP3A4 inhibitor | Total clearance | Ames toxicity | Hepatotoxicity |
| **Potential secondary metabolites from *N. sativa*** | | | | | | | | | | |
| C00013884 | 0 | −2.735 | −1.342 | −0.123 | −3.262 | No | No | −0.791 | No | No |
| C00013848 | 0 | −2.735 | −1.203 | −0.161 | −2.849 | No | No | −0.030 | No | No |
| C00003162 | 95.767 | −1.740 | 1.405 | 0.781 | 0.805 | No | No | 0.901 | No | No |
| C00016514 | 93.601 | −3.174 | 1.254 | 0.075 | 0.120 | No | No | 0.412 | Yes | No |
| C00013750 | 0 | −2.735 | −1.055 | −0.163 | −2.626 | No | No | 0.182 | No | No |
| C00028687 | 96.020 | −3.276 | 1.290 | 0.375 | −0.163 | No | No | 0.537 | No | Yes |
| **Potential secondary metabolites from *C. xanthorrhiza*** | | | | | | | | | | |
| C00037023 | 89.180 | −2.756 | 1.281 | −0.087 | −0.857 | No | Yes | 0.139 | No | No |
| C00002731 | 83.572 | −2.750 | 0.286 | −0.047 | −1.057 | No | Yes | 0.137 | No | No |
| C00011646 | 96.583 | −1.735 | 1.299 | 0.373 | 0.632 | No | No | 1.454 | No | No |
| C00012443 | 95.525 | −2.982 | 1.418 | 0.462 | 0.667 | No | No | 1.065 | No | No |
| C00011630 | 94.339 | −1.221 | 1.408 | 0.638 | 0.780 | No | No | 1.440 | No | No |
| C00003110 | 94.827 | −1.601 | 1.428 | 0.645 | 0.740 | No | No | 1.088 | No | No |
| C00011728 | 95.708 | −1.953 | 1.542 | 0.308 | 0.523 | No | No | 1.416 | No | No |
| C00011622 | 92.167 | −1.500 | 1.462 | 0.663 | 0.589 | No | No | 1.224 | No | No |
| C00012483 | 95.889 | −3.062 | 1.583 | 0.585 | 0.660 | No | No | 0.905 | No | No |

C_ID, chemical identity; Caco-2, human colon carcinoma cell line; VDss, volume of distribution at steady state; CYP2D6, cytochrome P450 2D6; CYP3A4, cytochrome P450 3A4.

three metabolites had log VDss<−0.015 {i.e.: Quercetin 3-(6′′′-feruloylglucosyl)-(1−>2)-galactosyl-(−>2)-glucoside, Quercetin 3-glucosyl-(1−>2)-galactosyl-(1−>2)-glucoside, and Kaempferol 3-glucosyl-(1−>2)-galactosyl-(1−>2)-glucoside}, whereas two of the potential metabolites derived from *C. xanthorrhiza Roxb* had log VDss values <−0.015 {i.e.: Demethoxycurcumin and Curcumin} (Table 2). Thus, it can be predicted that three of the six potential metabolites derived from *N. sativa* L as well as seven of the nine potential metabolites derived from *C. xanthorrhiza Roxb* can be distributed evenly and provide a relatively equal blood plasma level.

The role of blood–brain barrier (BBB) has been broadly recognized in protecting the brain from exogenous compounds. The capacity of drugs to penetrate into the brain is an significant boundary in order to reduce side effects and harmfulness or to increase the efficacy of drugs whose pharmacological activity is aimed at the brain. The permeability of the BBB *in vivo* is calculated as the logarithmic ratio of brain-to-plasma drug concentration (log BB). Drug compounds are predicted to be able to cross the BBB if they have a log BB value >0.3 [17, 19]. Furthermore, if a drug compound has a log BB value <–1, it can be predicted that the compound will barely reach the brain. Table 2 shows that the BBB permeability value of potential metabolites found in *N. sativa* L varies with a range between –3.262 and 0.805. Likewise, the potential metabolites of *C. xanthorrhiza Roxb* had skin permeability values ranging from –1.057 to 0.780. Thus, it can be predicted that some of these potential metabolites are able to penetrate the brain well, while others are not.

Cytochrome P450 is an significant detoxifying enzyme in the body, found mainly in the liver. This compound oxidizes xenobiotics to encourage their discharge cycle. Inhibitors of this enzyme are known to affect drug metabolism which has suggestions for changes in the pharmacokinetic attributes of medications. Along these lines, it is critical to assess whether a compound to be administered is an inhibitor of cytochrome P450. Two main isoforms that play a crucial role in drug metabolism are cytochrome P2D6 (CYP2D6) and cytochrome P3A4 (CYP3A4) [17, 20]. In this study all potential metabolites of *N. sativa* L were not inhibitors of the CYP2D6 or CYP3A4 enzymes. However, the potential metabolites of *C. xanthorrhiza Roxb* were found to be two compounds that are predicted to inhibit CYP3A4 enzymes (namely: demethoxycurcumin and curcumin) (Table 2). This finding is interesting for further investigation, how much is the inhibitory effect of the two potential metabolites of *C. xanthorrhiza Roxb* on CYP3A4 enzyme.

Steady-state conditions can be accomplished when the bioavailability of the drug is available in sufficient concentrations with suitable dosing. This relies upon drug clearance which can be resolved from total hepatic clearance and renal clearance (total clearance). The higher the total clearance value of a compound, the faster the excretion process [21]. In this study, total log clearance value of the potential secondary metabolite *N. sativa* L ranged from –0.791 to 0.901, while the total log clearance value of the potential secondary metabolite *C. xanthorrhiza Roxb* ranged from 0.137 to 1.454 (Table 2). By these values, the rate of excretion of these metabolites can be predicted.

Determination of the toxicity of a compound can be possible by utilizing Ames toxicity test. This test is a strategy for predicting the mutagenic capability of a compound using bacteria. A positive test demonstrates that the compound is mutagenic and can be carcinogenic. In this study, we found one potential metabolite of *N. sativa* L that is predicted to have a mutagenic effect, the salfredin B11. Furthermore, another crucial parameter of toxicity is hepatotoxicity to estimate the safety level of a drug against the liver [22]. This test also found one potential metabolite of *N. sativa* L which was predicted to be hepatotoxic, namely the nigellidine. Interestingly, all potential metabolites of *C. xanthorrhiza Roxb* were predicted to be neither mutagenic nor hepatotoxic.

## Conclusions

In conclusion, the present results demonstrated that a metabolite from *N. sativa* L, the longifolene, and seven metabolites from *C. xanthorrhiza Roxb*, i.e. (+)-beta-atlantone, humulene epoxide, (–)-beta-curcumene, (E)-caryophyllene, germacrone, (R)-(–)-xanthorrhizol and (–)-beta-caryophyllene epoxide were found to have good potential to be developed as a drug that functions as an HNMT inhibitor. This is based on the results of molecular docking studies and the predictive value of the ADMET characteristics of these metabolites. The data obtained from this study contributed to the development of novel HNMT inhibitor compounds with high specificity and adequate BBB permeability. This research can likewise be utilized as a basis for further research, both *in vitro*, *in vivo*, and clinical trials related to the development of secondary metabolites from *N. sativa* L and *C. xanthorrhiza Roxb* as novel HNMT inhibitor compounds.

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Burhan Ma'arif, Hilwa Fitri, Nisfatul Lailatus Saidah, Luqman Alfani Najib, Achmad Hamdan Yuwafi, Ria Ramadhani Dwi Atmaja, Fidia Rizkiah Inayatillah, Meilina Ratna Dianti*, Hening Laswati and Mangestuti Agil

# Prediction of compounds with antiosteoporosis activity in *Chrysophyllum cainito* L. leaves through *in silico* approach

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**Abstract**

**Objectives:** Estrogen deficiency causes various health problems in postmenopausal women, including osteoporosis. Phytoestrogen emerged as a potential alternative of estrogen with minimum side effects. The aims of this study were to analyze the metabolite profiling results of various extract of *Chyrsophyllum cainito* L. leaves, which contain phytoestrogen, through *in silico* study against 3OLS protein, an X-ray protein of ERβ, so it can predict the types of the phytoestrogen contents which have antiosteoporosis property.

**Methods:** *In silico* analysis was carried out for the compounds from the metabolite profiling data of *C. cainito* leaves from our previous study. The structure compounds from metabolite profiling results of various extract of *C. cainito* leaves were prepared with Avogadro 1.0.1 software, molecular docking was done using PyRx 0.8 software, and Biovia Discovery Studio Visualizer 2016 software was used to visualize the structure of compounds against 3OLS protein. The physicochemical characteristics of the compounds were analyzed using the SwissADME web tool.

**Results:** From *in silico* studies, it was known that there were total 11 compounds in *C. cainito* leaves that predicted as phytoestrogens which have ERβ agonist properties against 3OLS protein. The ERβ agonist was a compound that has parameters similar to 17β-estradiol in its interaction with 3OLS protein, which has a pharmacophore distance of 10.862 Å, and binding to amino acids His 475 and Glu 305 or Arg 346 at receptor-ligand docking simulation.

**Conclusions:** *C. cainito* leaves contain 11 compounds that are predicted to be phytoestrogens with ERβ agonist properties, which is responsible for antiosteoporosis activity.

**Keywords:** antiosteoporosis; *Chrysophyllum cainito* L.; *in silico*; phytoestrogen.

## Introduction

Estrogen deficiency causes various health problems in postmenopausal women, including osteoporosis [1, 2]. Osteoporosis is recognized as a serious health problem with about 200 million people being affected worldwide [3–5]. Over 40% of women and 20% of men with osteoporosis likely to have osteoporosis fractures during their lifespan. The percentage of mortality with osteoporosis fractures ranges from 15 to 30%, a rate similar to breast cancer and stroke [4, 6].

Estrogen deficiency-induced osteoporosis can generally be treated with hormone replacement therapy (HRT) [7]. However, regular use of HRT can increase the risk of breast cancer and cardiovascular disorders, so needed for safer alternative therapies [8]. Phytoestrogen emerged as a potential alternative for estrogen deficiency with minimum side effects. Phytoestrogens are a group of compounds derived from plants that have a structure similar to estrogen or can replace the function of estrogen in maintaining homeostasis in the brain, both in conjunction with estrogen receptors (ER-dependent) or not (ER-independent) [9], thus it is an alternative treatment for estrogen deficiency-induced osteoporosis [10].

*Chyrsophyllum cainito* L. (Figure 1), is one type of plant that is widely grown in East Java, Indonesia [11], and suspected contain phytoestrogen compounds [12]. In previous

***Corresponding author: Meilina Ratna Dianti**, M.Kep., Ns., Department of Pharmacy, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia, Phone: +6282221006667, E-mail: meilina@farmasi.uin-malang.ac.id
**Burhan Ma'arif, Hilwa Fitri, Nisfatul Lailatus Saidah, Luqman Alfani Najib, Achmad Hamdan Yuwafi, Ria Ramadhani Dwi Atmaja and Fidia Rizkiah Inayatillah,** Department of Pharmacy, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia, E-mail: burhan.maarif@farmasi.uin-malang.ac.id (B. Ma'arif)
**Hening Laswati,** Department of Physical Medicine and Rehabilitation, Universitas Airlangga, Surabaya, Indonesia
**Mangestuti Agil,** Department of Pharmacognosy and Phytochemistry, Universitas Airlangga, Surabaya, Indonesia

**Figure 1:** *Chyrsophyllum cainito* L [12].

research, 96% ethanol extract of *C. cainito* had an activity to increase osteoblast cell number in the trabecular vertebrae bone of dexamethasone-induced male mice. This activity occurs due to the presence of phytoestrogen compounds in the 96% ethanol extract of *C. cainito* [13]. In other previous research, *C. cainito* leaves was extracted with a serial exhaustive of extraction, which involves successive extraction with solvents of increasing polarity from a nonpolar to a polar solvent, such as n-hexane, ethyl acetate, and methanol, to ensure that a wide polarity range of compound could be extracted. In total there were detected 31 compounds in n-hexane extract, 50 compounds in ethyl acetate extract, and 35 compounds in methanol extract [12].

This study aimed to analyze the metabolite profiling results of various extract of *C. cainito* leaves through *in silico* study against 3OLS protein, an X-ray protein of estrogen receptor beta (ERβ). ERβ was chosen because it spread widely in bone cells, both osteoblasts and osteoclasts, and affect the bone remodeling process when it activated with the bond with 17β-estradiol in the body [9]. Whereas the 3OLS protein was chosen because this X-ray protein of ERβ has a classification as a receptor that binds to the 17β-estradiol as native ligand, which is used as positive control, as well as has good resolution. So it can predict the types of the phytoestrogens contents which have antiosteoporosis property. The *in silico* approach has several advantages, including short processing time, inexpensive, and can clearly explain the mechanisms that might occur when a natural substance enters the body [14, 15]. This research can also be used as a theoretical basis for further research on antiosteoporosis treatment.

# Materials and methods

## Materials

The materials used were compounds from the metabolite profiling results of various extract of *C. cainito* leaves using ultra performance liquid chromatography–quadrupole time of flight–mass spectrometry (UPLC-QToF-MS/MS) from our previous research [12], and X-ray protein of ERβ (PDB ID: 3OLS) from protein data bank (PDB) (https://www.rcsb.org).

## Methods

The first process was to download the 3OLS protein and perform preparation to separate its native ligand (17β-estradiol) from the 3OLS protein, using the Biovia Discovery Studio Visualizer 2016. The preparation of compound structures from the metabolite profiling results of n-hexane, ethyl acetate, and methanol extract of *C. cainito* leaves was done using Avogadro 1.0.1 software, for energy optimization and produces a stable 3D structure. The molecular docking of the compounds structure was done using PyRx 0.8 software and AutoDock Vina method, and the Biovia Discovery Studio Visualizer 2016 software was used to visualize the structure of compounds against 3OLS protein, so that can predict the compounds that act as phytoestrogens with ERβ agonist property based on its interaction with 3OLS protein. Furthermore, compounds that predicted as ERβ agonists were analyzed using the SwissADME web tool to know its physicochemical properties.

# Results

The compounds in various extract of *C. cainito* leaves were analyzed with molecular docking using the PyRx 0.8 software and AutoDock Vina as a docking simulator. From the redocking test of native ligand in 3OLS protein, the root mean standard deviation (RMSD) value was <2 Å. The RMSD value <2 Å indicates that the docking protocol was better and can be used for docking process of the compounds in 3OLS protein [16, 17].

The next step after molecular docking process was visualizing the bonds between native ligand and compounds against 3OLS protein using the Biovia Discovery Visualizer 2016 software. From the visualization data, it can be seen that the compounds that classified as an ERβ agonist if it has several parameters similar to the native ligand. This includes a pharmacophore distance that was similar to the native ligand which is approximately 10.862 Å, and has one pharmacophore group that binds to the amino acid His 475, and another pharmacophore group that binds to the amino acids Glu 305 or Arg 346 (Figure 2). From the analysis with Discovery Studio Visualizer 2016, it was found that there were total 11 compounds from 116

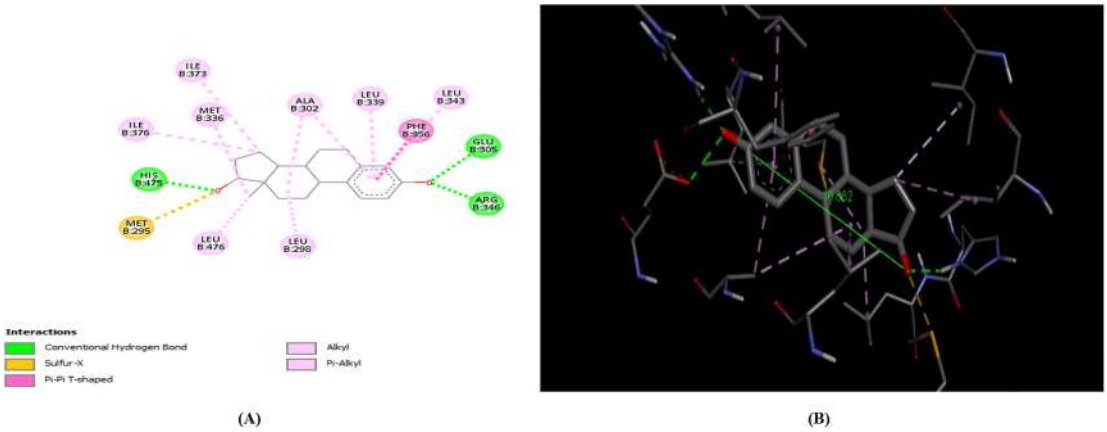


**Figure 2:** Native ligand (17β-estradiol) position after docking with 3OLS: (A) 2D, (B) 3D.

total compounds in *C. cainito* leaves that predicted as phytoestrogens which have ERβ agonist properties (Table 1).

The ERβ agonist compounds were screened using SwissADME web tool to identify the physicochemical properties of compounds. After being analyzed, it were known that all the ERβ agonist compounds possess the ability to penetrate the cell membrane that indicated by topological polar surface area (TPSA) value <140 $Å^2$. In addition, all the compounds also met the criteria of Lipinski's rule of 5, so that those compounds have biological activities designed for administration by the oral route (Table 1).

## Discussion

Estrogen is a hormone that plays an important role in the body, especially for growth and differentiation and other functions in several tissues [18, 19]. Estrogen has an important role in maintaining bone remodeling homeostasis by inhibiting excessive osteoclastogenesis and induce osteoblastogenesis [8]. When women enter the postmenopausal period, there will be estrogen deficiency that leads to osteoporosis, along with an increased risk for fractures, particularly in the hip, vertebrae, and wrist [20–22]. Phytoestrogen then emerged as a potential alternative for estrogen deficiency with minimum side effects [23–25].

The prediction of phytoestrogen compounds found in a plant can be carried out with *in silico* approach. It assists in simple predictions by correlating the structure of physicochemical properties with application analysis regarding its potential as medicine [26]. The *in silico* process begins with the preparation of native ligand from the 3OLS protein, and then preparation of compounds from various extracts of *C. cainito* leaves from previous study [12]. The preparation was carried out using the Avogadro 1.0.1 software to optimize force field from compounds structure so that the most stable structure of the compounds was obtained for further process [27, 28].

The molecular docking process was carried out using PyRx 0.8 with the Autodock Vina method. At this process, molecular docking was performed between each compound and the 3OLS protein, and then the binding affinity value was obtained. The reason for choosing Autodock Vina was that this method had the ability to perform analysis quickly and easily adjustable ligand pose mapping known as an autogrid. Autogrid was able to map the ligand pose during the docking process to minimize data bias [29]. At this stage, each compound's binding affinity value to 3OLS protein was obtained, which indicates the degree of affinity of the compound when it binds to the 3OLS protein. The concept of binding affinity is used to describe the energy in the complex bonding of a compound with the receptor, the more negative the binding affinity value indicates, the more stable the conformation of the compound with the receptor [30, 31].

The molecular docking results were then analyzed using Biovia Discovery Studio Visualizer 2016 to see the mapping of pharmacophore distance and amino acid bindings in the 3OLS protein of each compound. ERβ agonist compounds were indicated by the similarity of pharmacophore distances and types of amino acid bonds with native ligand in 3OLS protein. Pharmacophore distance is the minimum distance required by molecular atoms to bind to the receptor and produce activity. The similarity of the pharmacophore distance of the pharmacophore groups in the native ligand can be used as a reference to predict other compounds with the same

**Table 1:** Compounds from various extract of *C. cainito* leaves which act as ERβ agonist.

| No | Compounds | % Area | RMSD average, Å | Pharmacophore distance, Å | Amino acid | Binding affinity (kcal/mol) | GI absorption | TPSA (Å$^2$) | Lipinski's rule of 5 |
|---|---|---|---|---|---|---|---|---|---|
| **Native ligand** | | | | | | | | | |
| 1 | 17β-estradiol | – | 0.000 | 10.862 | His 475<br>Glu 305<br>Arg 346 | −10.5 | High | 40.46 | Yes |
| ***n*-Hexane extract** | | | | | | | | | |
| 2 | Cetylamine | 19.949 | 0.000 | 9.060 | His 475<br>Glu 305 | −5,8 | High | 26.02 | Yes |
| 3 | Safingol | 1.006 | 2.764 | 10.021 | His 475<br>Glu 305 | −4.6 | High | 66.48 | Yes |
| 4 | Bolandiol | 22.674 | 0.000 | 10.998 | His 475<br>Glu 305 | −10.8 | High | 40.46 | Yes |
| **Ethyl acetate extract** | | | | | | | | | |
| 5 | 11-Aminoundecanoic acid | 14.042 | 2.350 | 10.462 | His 475<br>Glu 305<br>Arg 346 | −5.5 | High | 63.32 | Yes |
| 6 | 2,2'-(Tridecylimino)diethanol | 15.470 | 1.795 | 10.401 | His 475<br>Arg 346 | −5.3 | High | 43.7 | Yes |
| 7 | N-(2-Hydroxyethyl)-N-(2-hydroxy octadecyl)-β-alanine | 0.943 | 3.938 | 9.064 | His 475<br>Glu 305 | −0.3 | High | 81.00 | Yes |
| 8 | 2-Methoxy-N-[2-(1-piperidinyl sulfonyl)ethyl] ethanesulfonamide | 0.076 | 3.821 | 8.622 | His 475<br>Glu 305 | −6.0 | High | 109.54 | Yes |
| 9 | Metaxalone | 0.017 | 4.669 | 10.107 | His 475<br>Arg 346 | −6.8 | High | 47.56 | Yes |
| 10 | Nandrolone | 1.325 | 1.880 | 10.920 | His 475<br>Glu 305<br>Arg 346 | −9.1 | High | 37.3 | Yes |
| **Methanol extract** | | | | | | | | | |
| 11 | 1,1'-(Octylimino)di(2-propanol) | 2.096 | 4.179 | 9.783 | His 475<br>Glu 305<br>Arg 346 | −5.7 | High | 43.70 | Yes |
| 12 | Lauryldiethanolamine | 26.209 | 2.688 | 10.522 | His 475<br>Glu 305 | −5.6 | High | 43.70 | Yes |

pharmacological effects [32]. The compound was said to an ERβ agonist if it has a pharmacophore distance of about 10.862 Å. The pharmacophore suitability score can be seen as a limit of 10%, or the tolerance scale is considered to be one different in value from its native ligand [26]. However, the pharmacophore distance that slightly exceeds the tolerance limit can still be predicted to have similar activity by considering other parameters, such as amino acid bindings. The compounds predicted to have agonist interactions are binding to amino acid His 475, and with the amino acid Glu 305 or Arg 346. The bound amino acid is relatively the same as the native ligand. This shows the same pattern of interactions when a compound binds at least the same two amino acids. However, the more similarities in the bound amino acids and type of bond, the higher the activity similarity is predicted [33, 34].

The compounds predicted by ERβ agonists were analyzed using the SwissADME web tool to predict their physicochemical properties based on the TPSA value and Lipinski's rule of 5. TPSA is a value that describes the ability of a compound to penetrate the membrane. The compounds are categorized as being able to penetrate the cell membrane if it has a TPSA value < 140 Å$^2$ [35]. From the physicochemical analysis, there were 11 compounds that have TPSA value < 140 Å$^2$. These 11 compounds showed a high GI absorption value [Table 1], which can also be seen in the Boiled Egg diagram [Figure 3]. The compounds in the white area in Boiled Egg diagram shows good GI absorption, while the yellow area shows the ability to penetrate blood brain barrier (BBB). From these data, it shows that the 11 compounds have good potential when developed into oral drugs. The Lipinski's rule of five is used to predict

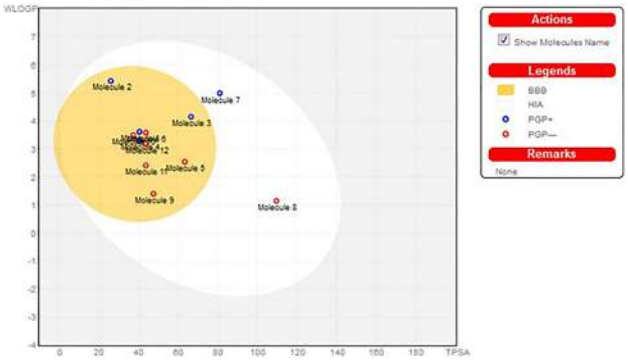


**Figure 3:** Boiled egg diagram of 11 compounds that predict ERβ agonists.

the resemblance of drugs to molecules with a specific biological activity designed for oral administration, those are if molecular weight of compound lower than 500 Da, number of donor hydrogen bonds less than 5, number of acceptor hydrogen bonds less than 10, and the log p lower than 5 [36]. The results of physicochemical analysis also indicate that the 11 compounds meet the criteria of the Lipinski's rule of 5, so that they have the potential to be developed as an oral drug [37, 38].

## Conclusions

Based on the visualization of molecular docking between compounds from *C. cainito* leaves with 3OLS protein and comparison with native ligand using Biovia Discovery Visualizer 2016 software, it was known that 11 compounds were predicted to be phytoestrogens with ERβ agonist in *C. cainito* leaves. The 11 compounds also have the potential to be developed as an oral drug based on their physicochemical properties.

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**Competing interests:** Author state no conflict of interest.
**Informed consent:** Author state no informed consent because there was no individuals included in this study.
**Ethical approval:** Not applicable.

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Honey Dzikri Marhaeny, Aty Widyawaruyanti, Tri Widiandani, Achmad Fuad Hafid and Tutik Sri Wahyuni*

# Phyllanthin and hypophyllanthin, the isolated compounds of *Phyllanthus niruri* inhibit protein receptor of corona virus (COVID-19) through *in silico* approach

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**Abstract**

**Objectives:** *Phyllanthus niruri* has been known as an immunomodulator and also reported to possess an antiviral activity against several RNA viruses, such as hepatitis B virus and hepatitis C virus by inhibiting viral entry and replication. Since the current situation of Coronavirus Disease 2019 (COVID-19) which infected among the world and caused severe disease and high morbidity, it urgently needed to find new agents against COVID-19. Therefore, *in silico* screening against COVID-19 receptors is carried out as an initial stage of drug discovery by evaluating the activity of phyllanthin and hypophyllanthin, an isolated from *Phyllanthus niruri*, in inhibiting spike glycoprotein (6LZG) and main protease (5R7Y) which play as target receptors of COVID-19.

**Methods:** Molegro Virtual Docker 6.0 used to determine the best binding energy through the rerank score which shows the total energy bonds calculation.

**Results:** Phyllanthin and hypophyllanthin demonstrated to possess greater binding affinity toward the COVID-19 inhibition sites than their native ligand. The rerank score of phyllanthin and hypophyllanthin are lower than the native ligands 6LZG and 5R7Y. This result indicated that phyllanthin and hypophyllanthin have a stronger interaction than the native ligands both in spike glycoprotein (entry inhibitor) and main protease (translation and replication inhibitor).

**Conclusions:** In conclusion, phyllanthin and hypophyllanthin are predicted to have strong activity against COVID-19 through inhibiting spike glycoprotein and main protease under *in silico* study. Further research is needed to support the development of *P. niruri* as inhibitor agents of COVID-19 through bioassay studies.

**Keywords:** COVID-19; hypophyllanthin; *in silico*; phyllanthin; *Phyllanthus niruri*.

## Introduction

The outbreak of Coronavirus Disease 2019 (COVID-19) is a respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a positive-sense single-stranded RNA viral (+ssRNA) belongs to the betacoronavirus species. The first cases of COVID-19 were reported in Wuhan, Hubei Province, China, toward the end of December 2019. The initial patient's diagnosis is the pneumonia of an unknown etiology after visiting seafood and wet animal wholesale market in Wuhan, which is thought to be the origin of COVID-19 [1]. Globally, the total confirmed cases of COVID-19 as of September 29th, 2020 were 33,206,004 cases which are 999,239 people from 211 countries, including Indonesia, were reported to death [2].

Nowadays, there are no FDA-approved drugs or vaccines that specifically effective in overcoming this pandemic. However, in early October 2020, WHO has released a draft list of COVID-19 vaccine candidates that are still undergoing trials including 42 in clinical trials and 151 in pre-clinical trials [3]. The COVID-19 vaccine candidates are developed using different technologies which are RNA, DNA, protein, and viral vectored vaccines. This vaccine has the potential to be widely

***Corresponding author: Tutik Sri Wahyuni,** Department of Pharmaceutical Science, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute Tropical Disease, University of Airlangga, Surabaya, Indonesia, E-mail: tutik-s-w@ff.unair.ac.id
**Honey Dzikri Marhaeny and Tri Widiandani,** Department of Pharmaceutical Science, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia
**Aty Widyawaruyanti and Achmad Fuad Hafid,** Department of Pharmaceutical Science, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute Tropical Disease, University of Airlangga, Surabaya, Indonesia

used under an Emergency Use Authorization (EUA) [4]. Meanwhile, the current drug of COVID-19 is based on other similar viruses, such as Interferon (IFN)-α, Ribavirin, and Remdesivir [5]. Other drugs that are also used are antimalarial drugs (chloroquine and hydroxychloroquine) that are given only as EUA because of the many adverse effects and interaction with other drugs [6].

Natural products, especially derived from medicinal plants, are a good starting point for drug discoveries inspiration through producing a variety of compounds with unique structural and chemical diversity – such as alkaloids, flavonoids, phenols, chalcones, coumarins, lignans, polyketides, alkanes, alkenes, simple aromatics, peptides, terpenes, and steroids – as drug metabolites. These properties are reported to produce physiological and pharmacological effects including antiviral by interacting with a lot of proteins, enzymes, and other biological molecules [7, 8].

*Phyllanthus niruri* is an annual herb from the Euphorbiaceae family. This medicinal plant is commonly spread in tropics throughout the world and has been used as anti-inflammatory, hepatoprotective, and antiviral. *P. niruri* is also known as immunomodulatory. The phytochemicals of *P. niruri* have been investigated, which are alkaloids, flavonoids, lignans, simple lactones, phenolics, tannins, steroids, and triterpenes [9]. Lignans, especially phyllanthin (a bitter constituent) and hypophyllanthin (a nonbitter constituent) have been identified as the essential bioactive compounds in this herb [10]. Phyllanthin and hypophyllanthin obtained from *P. niruri* were revealed to play an important role in enhancing humoral and cell-mediated immune responses, such as T-lymphocyte and TNF-α, to the pathogens [10, 11]. Furthermore, it is also reported to possess antiviral activity against several RNA viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) by inhibiting viral entry and replication [12–15]. The ethanolic extract of *P. niruri* was reported to inhibit HCV with an $IC_{50}$ value of 4.14 μg/mL without expressing cytotoxicity [14]. It was also reported to inhibit HBV viral entry with an $IC_{50}$ value of 170.5 μg/mL and significantly reduce extracellular DNA levels without expressing cytotoxicity [15].

Since the current situation of this pandemic which infected the world and caused severe disease and has a high morbidity, it urgently needed to find new agents against COVID-19. Therefore, *in silico* screening against COVID-19 receptors is carried out as an initial stage of drug discovery. This research aimed to evaluate the activity of phyllanthin and hypophyllanthin, compounds of *P. niruri*, in inhibiting protein receptors of COVID-19.

# Materials and methods

## Protein structure preparation

The spike glycoprotein (6LZG) and main protease (5R7Y) of COVID-19 were prepared by downloading via the RCSB Protein Data Bank Database website (www.rcsb.org). The receptor of 6LZG is involved in inhibiting viral entry while 5R7Y viral replication and transcription. Those proteins structures were used as a receptors model in this study. In general, the protein structure requires a repairment due to loss of water molecules or lack of amide groups. However, the problem is resolved automatically through a computer program. Furthermore, the native ligand of and 5R7Y receptors – respectively – NAG_601 [B] and JFM_1001 [A].

## Ligand preparation

The 2D and 3D structures of tested compounds, phyllanthin, and hypophyllanthin, were prepared using ChemBioOffice 15.0 and its energy was minimized using MMFF94.

## Drug-likeness evaluation and the toxicity properties prediction

The pharmacological significance of phyllanthin and hypophyllanthin is determined by its drug-likeness evaluation and toxicity properties prediction. The drug-likeness evaluation was examined using the DruLiTo program based on Lipinski's rule [16]. Meanwhile, the toxicity properties were analyzed using the pkCSM server (http://biosig.unimelb.edu.au/pkcsm/prediction) for predicting AMES toxicity and hepatotoxicity of the ligands.

## Validation of docking protocol

The validation of docking protocol was performed by re-docking the native ligand to protein in the active site. The parameters for docking analysis are acceptable when the value of root mean square deviation (RMSD) is less than 2.0 Å [17]. The RMSD of the 6LZG receptor was 0.56 Å and 5R7Y was 1.41 Å, which is considered very good poses of the receptors with native ligand and demonstrated a valid behavior of docking protocol.

## Docking analysis

The docking analysis was performed using Molegro Virtual Docker 6.0 to determine the best binding energy through the rerank score which shows the total energy bonds calculation. The rerank score of those compounds was compared with its native ligand of receptors where the lowest energies indicate a strong and stable bond against the receptor.

# Results

## Drug-likeness evaluation and the toxicity properties (AMES toxicity and hepatotoxicity) prediction of phyllanthin and hypophyllanthin

The results of DruLiTo program shows phyllanthin and hypophyllanthin satisfy the rule of five by Lipinski which are the molecular weight (MW) of less than 500 Da, LogP value of less than five, the numbers of hydrogen bond donor (HBD) of less than five, and the numbers of hydrogen bond acceptor (HBA) less than 10 [16]. The value of MW, HBD, and HBA are related to the compounds' ability to penetrate the membrane cell while LogP is generally related to the absorption rate of the compounds. These results indicated phyllanthin and hypophyllanthin have good solubility and oral absorption (Table 1).

AMES toxicity is used to assess the mutagenic potential of compounds and hepatotoxicity is used to know the compounds ability to induce liver injury [18, 19]. The results of the pkCSM server show phyllanthin and hypophyllanthin predicted to have no toxic effect on the AMES toxicity and hepatotoxicity model which indicated the safety of both compounds would be good (Table 1).

## Docking analysis of phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin (Figure 1), an isolated compound from *P. niruri*, are selected as the potential compounds against spike glycoprotein (6LZG) and main protease (5R7Y) which play as the target receptors of COVID-19. The interaction between ligand and receptor was determined based on the rerank score values. The results revealed a strong interaction between phyllanthin and hypophyllanthin with 6LZG (Figure 2) and 5R7Y receptors (Figure 3). The rerank score of the 6LZG receptor showed −83.05040 kcal/mol for phyllanthin, −76.98840 kcal/mol for hypophyllanthin, and −61.68640 kcal/mol for the native ligand of NAG_601 [B]. Meanwhile, the rerank score with the receptor of 5R7Y showed −90.65187 kcal/mol for phyllanthin, −89.20210 kcal/mol for hypophyllanthin, and −67.36807 kcal/mol for the native ligand of JFM_1001 [A]. This result indicated that phyllanthin and hypophyllanthin have a stronger interaction than the native ligands both in 6LZG and 5R7Y receptors.

Intermolecular interactions between ligands and amino acid residues of 6LZG and 5R7Y receptors observed the hydrogen bonding and Steric-Van der Walls interaction. NAG_601 [B] shows interaction with several residues of 6LZG which forms hydrogen bonds with Asp 364, Cys 336, and Val 362 while it shows hydrophobic bonds with Ala 363, Cys 336, Phe 338, and Val 367 (Figure 2A). Phyllanthin shows interaction with several residues of the 6LZG receptor which forms hydrogen bonds with Asp 364 and Gly 339 while it shows hydrophobic bonds with Asp 364, Cys 336, Phe 338, and Val 362 (Figure 2B). Hypophyllanthin shows interaction with several residues of 6LZG which forms a hydrogen bond with Asn 343 while it shows hydrophobic bonds Asp 364, Cys 336, Phe 342, Phe 374, and Val 362 (Figure 2C). As well, several residues of the 5R7Y receptor also represent interaction with ligands. JFM_1001 [A] forms a hydrogen bond only with His 41 while it shows hydrophobic bond only with Met 165 (Figure 3A). Phyllanthin forms hydrogen bonds with His 41, Thr 24, and Thr 25 and hydrophobic bonds with Arg 188, Cys 44, Gln 189, and Thr 24 (Figure 3B). Hypophyllanthin forms hydrogen bonds with Ser 46 and Thr 25 while it shows hydrophobic bonds with Arg 188, Cys 44, Gln 189, His 41, and Met 49 (Figure 3C).

# Discussion

The current novel coronavirus outbreak is a global health crisis that occurs almost throughout the world. The therapeutic use of natural products, especially in herbal preparations from medicinal plants, has been used for generations by 80% of the world's population [20]. An earlier study has clearly proven that phytotherapy is able to prevent infection and has a potential antiviral agent for

**Table 1:** Drug-likeness evaluation and the toxicity properties prediction of the ligands.

| | Drug-likeness activity | | | | Toxicity properties | |
|---|---|---|---|---|---|---|
| | MW, Da | LogP | HBD | HBA | AMES toxicity | Hepatotoxicity |
| Lipinski | ≤500 | ≤5 | ≤5 | ≤10 | – | – |
| Phyllanthin | 418.24 | 2.488 | 0 | 6 | No | No |
| Hypophyllanthin | 430.20 | 2.078 | 0 | 7 | No | No |

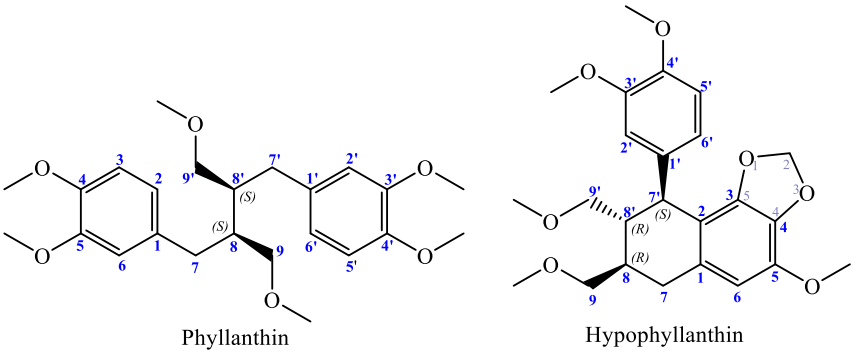


**Figure 1:** Molecular structure of phyllanthin and hypophyllanthin.

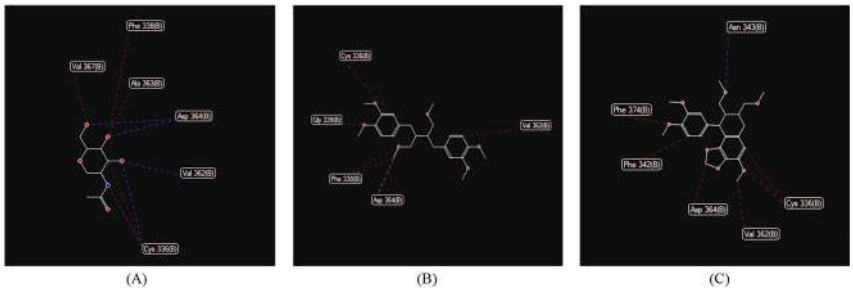


**Figure 2:** The interaction between amino acid residues of 6LZG receptor and (A) NAG_601 [B], (B) phyllanthin, and (C) hypophyllanthin. Hydrogen bond interaction showed in dashed blue-line and Steric-Van der Walls bond interaction showed in dashed red-line.

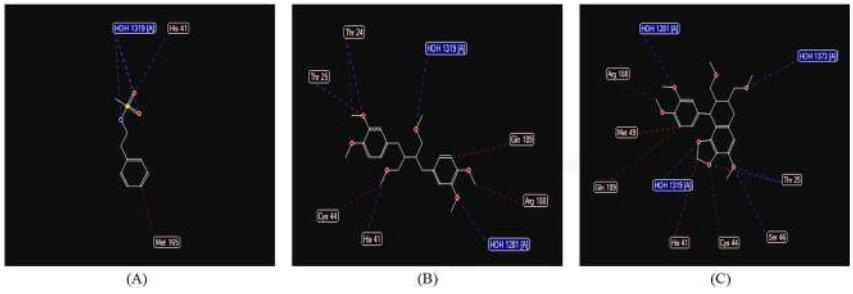


**Figure 3:** The interaction between amino acid residues of 5R7Y receptor with (A) JFM_1001 [A], (B) phyllanthin, and (C) hypophyllanthin. Hydrogen bond interaction showed in dashed blue-line and Steric-Van der Walls bond interaction showed in dashed red-line.

COVID-19 [21]. The pathogenicity of SARS-CoV-2 is not much different from SARS-CoV. The SARS-CoV-2 genetic sequence shows ≥70% similarity to SARS-CoV on the host cell receptor which infects humans, i.e., angiotensin-converting enzyme 2 (ACE2), which plays an essential role in the viral invasion process [22]. However, SARS-CoV-2 was reported to have a higher virulence level due to its better sequence identity of spike (S) glycoprotein which shows 10–20-fold higher affinity instead of SARS-CoV when it binds to the ACE2 receptor [22, 23].

The potential target infections of SARS-CoV-2 are lung alveolar, heart, and small intestine due to high expression of the ACE2 receptor. In the lungs, alveolar type II (AECII) epithelial cells express 83% ACE2 thus revealing that AECII serves as a reservoir for viral invasion as it facilitates the replication of coronavirus in the lungs [24]. SARS-CoV-2 infects the alveolar epithelial cells and binds to the host receptors on target cells inducing endocytosis of the entire part of the viral, including capsid proteins, into the cell. This process is followed by the fusion of host and viral membrane, thus enabling penetration of the viral genome into the cytoplasm of the host. Furthermore, SARS-CoV-2 replicates its genetic material and synthesizes the other needed proteins within the cell to form virions which are released on the cell surface [25, 26]. This replication stage inflicts cytokine storm syndrome (hypercytokinaemia) and lung tissue damage. Hypercytokinaemia is a severe uncontrolled immune response due to the overproduction of pro-inflammatory cytokines (IL-2, IL-7, IL-10, GCSF, IP-10, MCP-1, MIP1A, and TNF-α). Hypercytokinaemia inflicts Acute Respiratory Distress Syndrome (ARDS) and multiple organ failure through decreasing the immune function in patients with SARS-CoV-2 infection [22].

Our present study evaluated the potential activity of phyllanthin and hypophyllanthin, isolated compounds of *P. niruri*, in inhibiting the crucial targets of COVID-19 which is spike glycoprotein (6LZG) and main protease (5R7Y). The receptor of 6LZG is a crystal structure of the SARS-CoV-2 C-terminal domain (SARS-CoV-2-CTD) spike (S) protein in complex with human ACE2 (hACE2), which

exposes a hACE2-binding mode identical overall to the observed for SARS-CoV [27]. Thus, the 6LZG receptor is involved in inhibiting viral entry. Meanwhile, the 5R7Y receptor is a crystal structure of 3C-like protease ($3CL^{pro}$) in complex with Z45617795 that is involved in inhibiting viral translation and replication [28, 29]. Based on docking analysis, we represent phyllanthin and hypophyllanthin give the lower rerank scores instead of the native ligand which indicated the stronger interaction among both receptors.

Phyllanthin and hypophyllanthin are the bioactive compounds classified in the lignan group [30]. This class of compounds is reported to have several strong modes of antiviral activity due to the capability along with the structures to bind the enzymes and also the interesting effect on nucleic acids [31]. In addition, lignans are also reported to have antioxidant activity [32].

Phyllanthin is a dibenzylbutane-type lignan with two simple phenylpropanoids bounded by C-8 and C-8′. The phenylpropanoid structure of phyllanthin is a catechol derivative [33, 34]. Catechol derivatives have different antiviral activity depend on the position and structure of the functional groups attached to the aromatic skeleton. These derivatives can produce reactive oxygen species (ROS) to activate nuclear factor-κB (NF-κB) as a promising antiviral route [35]. Furthermore, this reaction is also supported by the strong antioxidant properties of phyllanthin [36]. The interaction of phyllanthin at the 6LZG receptor (Figure 2B) shows hydrophobic bonds with Cys 336 at C-1 and -6, Asp 364 and Phe 338 at 9-$OCH_3$, and Val 362 at C-5′ while it shows hydrogen bonds with Asp 364 at 9-$OCH_3$ and Gly 339 at 4-$OCH_3$. At the 5R7Y receptor, phyllanthin (Figure 3B) showed hydrophobic bonding with Arg 188 at 4′-OCH3, Cys 44 at 9-$OCH_3$, and Gln 189 at C-5′ while it shows hydrogen bonds with Thr 24 and Thr 25 at 5-$OCH_3$ and His 41 at 9-$OCH_3$.

Meanwhile, hypophyllanthin is an arylnaphthalene-type lignan consisting of arylnaphthalene and 1,3-dioxolane ring. Arylnaphthalene lignans have better antiviral activity than other naphthalenic derivatives with an inhibitory effect on reverse transcriptase (RT) at the same site as transcriptase inhibitors in general [31]. Moreover, arylnaphthalene lignans also show antioxidant activity due to inhibition action against NO production [32]. The structure of 1,3-dioxolane and its derivatives are recognized as an important moiety in the construction of many pharmacologically active molecules including antivirals [37, 38]. The interaction of hypophyllanthin at the 6LZG receptor (Figure 2C) shows hydrophobic bonds with Asp 364 at C-2 in the 1,3-dioxolane ring, Cys 336 at C-5 and -6 and 5-$OCH_3$, Phe 342 at C-5′, Phe 374 at 4′-$OCH_3$, and Val 362 at 5-$OCH_3$ while it shows hydrogen bond Asn 343 at 9′-$OCH_3$. At the 5R7Y receptor, hypophyllanthin (Figure 3C) shows hydrophobic bonds with Arg 188 at 4′-$OCH_3$, Cys 44 at 3-dioxolane, Gln 189 and Met 49 at C-5′, and His 41 at C-2 in the 1,3-dioxolane ring while it shows hydrogen bonds with Ser 46 at 5-$OCH_3$ and Thr 25 at 3-dioxolane and 5-$OCH_3$.

Thus, phyllanthin and hypophyllanthin showed great antiviral activity against the 6LZG receptor by mimicking the interaction of native ligands-protein at the same binding site which is Asp 364, Cys 336, Phe 338, and Val 362 for phyllanthin and Asp 364, Cys 336, and Val 362 for hypophyllanthin. As well, the potential antiviral activity of them, phyllanthin and hypophyllanthin, against 5R7Y receptor due to its interaction with specific amino acid residues of the main protease at the allosteric site which is His 41 on pharmacophores of both ligands.

Moreover, we also investigated the oral bioavailability – including the ability to penetrate biological membranes and absorption properties – of phyllanthin and hypophyllanthin to evaluate the drug-likeness by Lipinski's rule. The parameters of Lipinski's rule are MW≤500 Da, LogP≤5, HBD≤5, and HBA≤10 which suggested a good oral bioavailability [16]. Decreasing of MW compounds thrust an increase of the permeation rate in the lipid bilayer, indicating the lower MW compounds were more likely to be orally active due to their easy ability to absorbed, diffused, and transported than those with higher MW. Lipophilicity is a physicochemical property which generally related to the rate of absorption and asserted in log P where values less than five are more likely to be bioavailable orally. Furthermore, the value of HBD and HBA in a compound also affect the penetration ability of molecules into the membrane bilayer. Large amounts of HBD and HBA can reduce the permeability of compounds into the lipophilic milieu [39].

The prediction of the toxicity properties of these compounds through AMES toxicity and hepatotoxicity model also observed to estimate its safety as a drug candidate. The toxicity of AMES is used to assess the mutagenic potential of compounds which may cause carcinogenic effects whereas hepatotoxicity is associated with the impaired normal function of the liver [18, 39]. Based on the results (Table 1), both ligands represent a good feasibility of oral bioavailability by satisfying Lipinski's rule and predicted to safe because have no toxic effect on the toxicity test. Hence, phyllanthin and hypophyllanthin can be utilized to develop as drug candidates for COVID-19.

## Conclusions

Our study concludes that phyllanthin and hypophyllanthin are predicted to have strong antiviral activity through inhibiting the entry step (6LZG) and the post entry including translation and replication (5R7Y) of COVID-19 life-cycle based on *in silico* study. These results suggest that *P. niruri* may a promising plant to be a potential candidate as an antiviral agent for COVID-19. Further research is needed to support the development of *P. niruri* as inhibitor agents of COVID-19 through bioassay studies.

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Lidya Tumewu, Fendi Yoga Wardana, Hilkatul Ilmi, Adita Ayu Permanasari, Achmad Fuad Hafid and Aty Widyawaruyanti*

# *Cratoxylum sumatranum* stem bark exhibited antimalarial activity by Lactate Dehydrogenase (LDH) assay

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**Abstract**

**Objectives:** The antimalarial drug resistance is an obstacle in the effort to overcome malaria. The new alternative antimalarial drug became in great attention of urgent need. Current antimalarial drugs were derived from plants. Therefore, the plant is considering a potential source of new drugs. *Cratoxylum sumatranum* belongs to the Hypericaceae family contain xanthones and phenolic compounds, which was reported for their antimalarial activities. This study aims to determine the antimalarial activities of *C. sumatranum* extracts and fractions.

**Methods:** *Cratoxylum sumatranum* stem bark (BP14-SB) collected from Balikpapan Botanical Garden in East Kalimantan, Indonesia, was extracted gradually with n-hexane, dichloromethane, and methanol by ultrasonic-assisted extraction method. All extracts were tested against *Plasmodium falciparum* 3D7 by lactate dehydrogenase (LDH) assay and followed by $IC_{50}$ determination. The most active extract was further separated and tested for their antimalarial activities.

**Results:** The results showed that dichloromethane stem bark extract (BP14-SB-D) had the strongest inhibition of parasite growth with the $IC_{50}$ value of 0.44 ± 0.05 μg/mL and moderately toxic with the $CC_{50}$ value of 29.09 ± 0.05 μg/mL. Further fractionation of BP14-SB-D by open column chromatography using silica gel and gradient hexane–ethyl acetate obtained 12 fractions. LDH assay for these 12 fractions of BP14-SB-D showed that Fraction-6 ($IC_{50}$ value of 0.19 ± 0.03 μg/mL) was performed the strongest inhibition of parasite growth, compared to other fractions. TLC identification showed that BP14-SB-D contains xanthone.

**Conclusions:** The dichloromethane extract of *C. sumatranum* stem bark (BP14-SB-D) and Fraction-6 from this extract exhibited antimalarial activity and the potential to be developed an antimalarial substance.

**Keywords:** antimalarial; *Cratoxylum sumatranum* stem bark; LDH assay.

## Introduction

Malaria is an infectious disease caused by Plasmodium parasites. It was transmitted to humans through the bite of an infective *Anopheles* mosquito. The World malaria report 2019 was declared 228 million cases of malaria happen in 2018, an estimated 405,000 deaths caused by malaria globally [1]. One of the greatest challenges to implement malaria control was antimalarial drug resistance. It has become a significant factor in the occurrence and severity of epidemics in some regions [2]. The limited number of effective antimalarial drugs and the spread of drug resistance was encouraged the search for alternative antimalarial drugs [3].

The natural substances derived from plants are potentially good sources for antimalarial drugs. Quinine and artemisinin were being an outstanding examples of antimalarial drugs from plants [4]. The Cratoxylum is a tropical plant belongs to the Hypericaceae family, which has been reported for their antimalarial compounds. The active compounds identified as 5-O-methylcelebisantone, celebisantonite, β-mangostin, and cochinchinone C were isolated from the roots of

***Corresponding author: Aty Widyawaruyanti**, Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, 60115, Indonesia; and Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, 60115, Indonesia, Phone: +62 315933150, E-mail: aty-w@ff.unair.ac.id
**Lidya Tumewu,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Doctoral Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Fendi Yoga Wardana, Hilkatul Ilmi and Adita Ayu Permanasari,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
**Achmad Fuad Hafid,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

*Cratoxylum cochinchinense*. They exhibited antimalarial activity against *Plasmodium falciparum* with an $IC_{50}$ value less than 7 µg/mL [5]. Vismione B, which isolated from *Cratoxylum maingayi* and *C. cochinchinense*, also showed antimalarial activity against *P. falciparum* with an $IC_{50}$ value 0.66 µg/mL [6]. Most of their antimalarial compounds were belong to the xanthone group.

*Cratoxylum sumatranum* is one species of the genus Cratoxylum which potential to be studied. *C. sumatranum* has various biological actions, including antimicrobial, antioxidant, and cytotoxic properties. Several compounds have been isolated from *C. sumatranum* which identified as cratosumatranone A-B; pruniflorone N; neriifolone B; isocudraniaxanthone B; 10-O-methylmacluraxanthone; macluraxanthone; 5-O-methyl-2-deprenylrhediaxanthone B; pancixanthone B; pruniflorone M; 1,5-dihydroxy-8-methoxyxanthone; 1,5-dihydroxy-6,7-dimethoxyxanthone; 1,3,6-trihydroxy-7-methoxyxanthone; 1,3,5,6-tetrahydroxyxanthone; 1,2,8-trihydroxyxanthone; 2,8-dihydroxy-1-methoxyxanthone; 1,7-dihydroxyxanthone; trapezifolixanthone, and cratoxyarborenone A-F [7, 8]. Wardana et al. (2020) also reported two compounds isolated from *C. sumatranum*, namely cochinchinoxanthone and cochinchinone D. Cochinchinoxanthone showed the greatest level of anti amebic activity both in a cell-based and enzymatic assay, yielding $IC_{50}$ values of 4.57 and 12.17 µg/mL, respectively [9]. We assumed that *C. sumatranum* active substances will produce similar antimalarial activity with the previous reported study based on the chemotaxonomy concept. Therefore, this study aims to determine the antimalarial activities of *C. sumatranum* extracts and fractions.

# Materials and methods

## Plant material

The stem bark of *C. sumatranum* (BP14-SB) was collected from Balikpapan Botanical Garden in East Kalimantan, Indonesia. Plant determination was conducted by a licensed botanist at Purwodadi Botanical Garden, East Java. A voucher specimen (No. 0074/IPH.06/HM/XII/2015) of this raw material was stored at the Herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

## Extraction and fractionation

The stem bark of *C. sumatranum* (BP14-SB) as much as 400 g was extracted at room temperature by an ultrasonic-assisted method using 2 L of n-hexane as a solvent. After n-hexane extract filtration, the residue was further extracted by the same method using 2 L of dichloromethane as a solvent. Dichloromethane extract was then filtrated. The solvent's removal by rotary evaporator was afforded yellow-brown color of n-hexane extract (3.2 g) and a dichloromethane extract (4 g), respectively. The most active extract was further fractionated by open column chromatography using gradient solvents of hexane–ethyl acetate, ethyl acetate, chloroform–methanol, and methanol. All fractions were identified for TLC profiles and tested for their antimalarial activity.

## Phytochemical screening

Dried extracts were diluted in methanol. The phytochemical screening was performed by thin layer chromatography (TLC) method using RP-18 as a stationary phase and chloroform–methanol (9.5/0.5 v/v) as a mobile phase. The phytochemical screening was identified by using 10% sulfuric acid, sulfuric acid anisaldehyde, $FeCl_3$, 10% KOH in methanol, and Dragendorf as spray reagents. The spots observation was conducted under UV 254 and 366 nm.

## *P. falciparum* culture

*P. falciparum* strain 3D7 was obtained from the Malaria Laboratory at The Center of Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. Parasites were cultured in 2% hematocrit type O human red blood cell (RBC) in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 25 mL MHEPES buffer, 2 g/L sodium hydrogen bicarbonate, 50 µg/mL hypoxanthine, 10 µg/mL gentamicin sulfate, and 0.5% (w/v) Albumax II (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) under 5% $O_2$ and 5% $CO_2$ at 37 °C. Human RBCs were received from the Indonesian Red Cross Society.

## *P. falciparum* growth inhibition assay

The antimalarial assay was conducted using lactate dehydrogenase (LDH) method [10, 11]. Parasites at the ring stage and 0.3% parasitemia were placed in a 96-well plate as much as 100 µL per well. Initially, extracts and fractions of *C. sumatranum* were tested against *P. falciparum* 3D7 to observe their inhibition activities at a final concentration of 4 µg/mL. Each sample was assayed in triplicate. After 72 h of incubation, parasite growth was determined by diaphorase-coupled lactate dehydrogenase (LDH) assay. Each well's absorbance was measured at 650 nm using a Multiskan sky-high microplate spectrophotometer (Thermo fisher scientific). The inhibition rate was calculated with the absorbance of uninfected wells defined as 100% inhibition. Hit sample was defined as those inhibiting more than 50% activity at a 4 µg/mL concentration. Z′-factor, S/N, S/B, and SW were calculated as previously reported. The $IC_{50}$ of hit sample was determined under the same assay condition for the screening, with the addition of the sample in serial dilution (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 µg/mL). Each dilution was assayed in triplicate, and $IC_{50}$ values were calculated by applying four parameters logistic regression curve to the dose-response data using GraphPad Prism 7.0 software (GraphPad Co. Ltd., San Diego, CA, USA). An extract was considered highly active with an $IC_{50}$<5 µg/mL, promising extracts with an $IC_{50}$ value of 6–15 µg/mL, moderate activity with an $IC_{50}$ value of 16–30 µg/mL, low activity with an $IC_{50}$ value of 31–50 µg/mL, and inactive extracts if the $IC_{50}$ value more than 50 µg/mL [12].

### *In vitro* cytotoxicity assay in Huh7it

The cytotoxicity of samples was performed following the enzymatic MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) described by Wahyuni [13]. In brief, Huh7it cells in 96-well plates were treated with serial dilutions of samples and control for 48 h. The Dulbeco's Modified Eagle Medium (GIBCO Invitrogen, USA) medium was replaced with MTT reagent containing that medium and incubated for 4 h. The MTT solution was removed and 100 µL/well of DMSO 100% was then added for dissolution. The absorbance at 560 nm was measured using a GloMax reader. The percentages of cell viability were calculated by comparison to the control, and 50% cytotoxicity concentration ($CC_{50}$) values were calculated using GraphPad Prism 7.0 software (GraphPad Co. Ltd., San Diego, CA, USA). An extract was considered very toxic with $CC_{50}$ value of 10 µg/mL, moderately toxic with $CC_{50}$ value of 11–30 µg/mL, slightly toxic with $CC_{50}$ value of 31–50 µg/mL, and potentially nontoxic with $CC_{50}$>50 µg/mL [14].

## Results

The stem bark of *C. sumatranum* (BP14-SB) was extracted gradually using n-hexane, dichloromethane, and methanol by ultrasonic-assisted extraction method described in the materials and methods section, and examined for antimalarial activities against *P. falciparum* 3D7 strain. The results revealed that the dichloromethane extract of *C. sumatranum* stem bark (BP14-SB-D) had the strongest inhibition of parasite growth with the $IC_{50}$ value of 0.44 ± 0.05 µg/mL and moderately toxic with the $CC_{50}$ value of 29.09 ± 0.05 µg/mL (Table 1). The selectivity index (SI) of BP14-SB-D was ≥2, indicated the extract is a good candidate for the treatment of malaria. The phytochemical screening showed that BP14-SB-D contains flavonoids, xanthones, polyphenols, terpenoids, and anthraquinone groups (Table 2).

Further fractionation of BP14-SB-D by open column chromatography using silica gel and gradient hexane–ethyl acetate obtained 12 fractions. Screening antimalarial activity showed that eight fractions had inhibitions against *P. falciparum* 3D7 more than 50% at 4 µg/mL (Figure 1). Quality control parameters, including Z′-factor, S/B, S/N, and CV (%), were also calculated with values of 0.94, 2.88, 120.33, and 1.74, respectively, all were indicating the high quality and performance of the screening. All hits are determined for the $IC_{50}$ value (Table 3). LDH assay for these 12 fractions of BP14-SB-D showed that

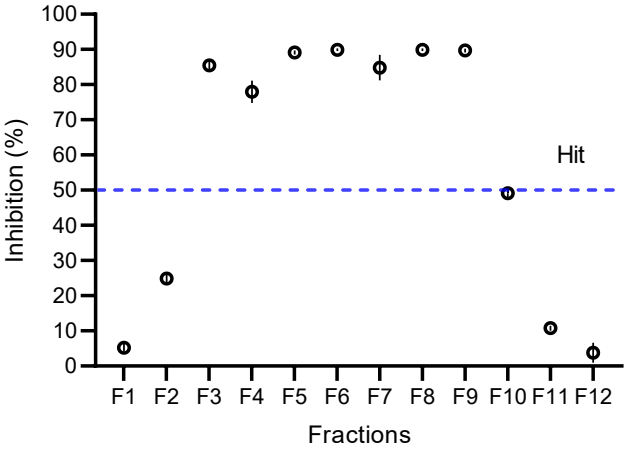


**Figure 1:** Inhibition percentages of all fractions against *P. falciparum* 3D7 at a concentration of 4 µg/mL.

**Table 1:** Antimalarial activity ($IC_{50}$), cytotoxicity concentration ($CC_{50}$), and selectivity index (SI) of extracts from *C. sumatranum* stem bark.

| Sample | $IC_{50}$, µg/mL | $CC_{50}$, µg/mL | SI |
|---|---|---|---|
| n-hexane (BP14-SB-H) | 0.57 ± 0.06 | 28.26 | 49.57 |
| Dichloromethane (BP14-SB-D) | 0.44 ± 0.05 | 29.69 | 67.47 |
| Methanol (BP14-SB-M) | NA | NT | – |

NA, not active; NT, not tested.

**Table 3:** Antimalarial activity ($IC_{50}$) of active fractions from a dichloromethane extract (BP14-SB-D) of *C. sumatranum* stem bark.

| Sample | $IC_{50}$ µg/mL |
|---|---|
| Fraction-3 | 6.37 ± 0.03 |
| Fraction-4 | 2.73 ± 0.03 |
| Fraction-5 | 1.21 ± 0.03 |
| Fraction-6 | 0.19 ± 0.03 |
| Fraction-7 | 1.94 ± 0.04 |
| Fraction-8 | 0.21 ± 0.09 |
| Fraction-9 | 0.41 ± 0.09 |

**Table 2:** Phytochemical constituents of n-hexane, dichloromethane, and methanol extracts of *C. sumatranum* stem bark.

| Sample | Phytochemical constituents | | | | |
|---|---|---|---|---|---|
| | Flavanoid | Terpenoid | Phenolic | Antraquinone | Alkaloid |
| n-hexane (BP14-SB-H) | + | + | + | + | – |
| Dichloromethane (BP14-SB-D) | + | + | + | + | – |
| Methanol (BP14-SB-M) | + | – | – | – | – |

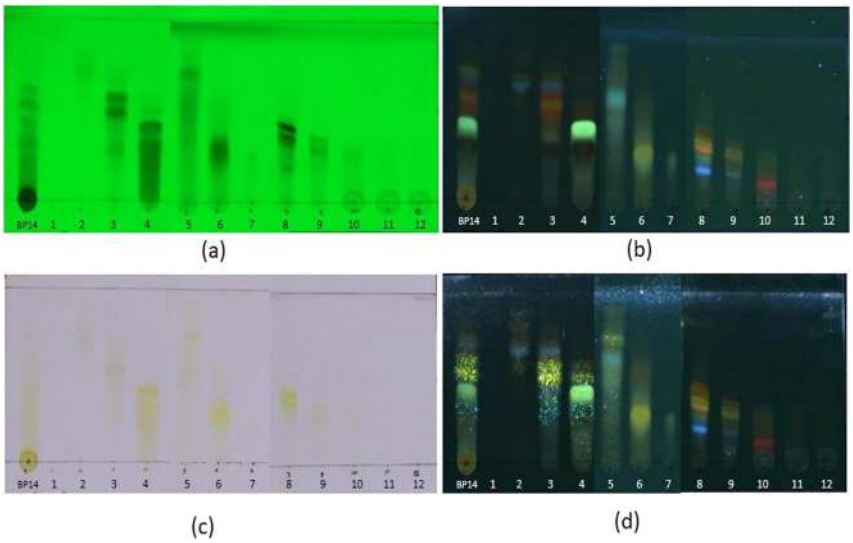


**Figure 2:** TLC profile of dichloromethane extract (BP14-SB-D) and fractions (F1-F12) using silica gel as a stationary phase and chloroform–methanol (95/5 v/v) as a mobile phase. The TLC spots were observed under UV 254 nm (a), UV 366 nm (b), sprayed with $H_2SO_4$ 10% and heated 105 °C for 5 min (c), UV 366 nm after sprayed with $H_2SO_4$ 10% and heated 105 °C for 5 min (d).

Fraction-6 had the strongest inhibition of the parasite growth with an $IC_{50}$ value of 0.19 ± 0.03 µg/mL, compared to other fractions. Phytochemical screening showed that Fraction-6 contains xanthone. The TLC profile shows a dominant brownish–yellow spot at the UV wavelength of 366 nm with *Rf* = 0.25. These spots indicate that a compound is a xanthone group (Figure 2).

## Discussion

*C. sumatranum* belongs to the Hypericaceae family, which is a wild plant and in abundance in East Kalimantan secondary forest, Sumatra, and Java [15]. This plant is also widely distributed in Asia-Tropical, Burma, Indochina, Thailand, Malaysia, and Philippines [16, 17]. *C. sumatranum* is used as a traditional medicine in Indonesia. The stems were used as an energy drink or aphrodisiac; the bark is used to treat abdominal pain, leaves for burns, and both leaves and stems for fever [15]. In the present study, BP14-SB was subjected to extraction in different polarities of solvents, and antimalarial activities of the extracts were examined against *P. falciparum* 3D7 strain. The results revealed that dichloromethane extract (BP14-SB-D) possessed the most potent activity, suggesting that a semipolar compound extracted by dichloromethane was involved in the antimalarial activity. The toxicity assay result showed that BP14-SB-D was moderately toxic with the $CC_{50}$ value of 29.09 ± 0.05 µg/mL. However, BP14-SB-D had good selectivity indices (>2), suggesting good therapeutic potentials for malaria treatment [3].

We further fractionated BP14-SB-D to obtain 12 fractions. These fractions were tested against *P. falciparum* at a concentration of 4 µg/mL. The result showed that seven fractions were inhibited more than 50% of *P. falciparum* growth (Figure 1). A further antimalarial assay was conducted to determine the $IC_{50}$ value of active fractions. Fraction-6 showed the strongest inhibition of the parasite growth with an $IC_{50}$ value of 0.19 ± 0.03 µg/mL (Table 3). Based on the antimalarial activity criteria, fraction-6 was classified as a highly active antimalarial substance as well. Fraction-6 had a lower $IC_{50}$ value compared to dichloromethane extract, meaning that Fraction-6 more active than its crude extract. This result was interesting, considering many cases are known where the crude extract is more efficiently active than fractions or purified compounds from the extract [18]. The effectivity of fraction-6 was possibly due to synergism of active compounds present in the fraction more than in the extract.

TLC profile of fraction-6 indicated xanthone as a major content. Several studies have been isolated xanthone from *C. sumatranum*. Sumatranaxanthone A, a new xanthone, was isolated from the root bark of *C. sumatranum* [19]. Six new xanthones, cratoxyarborenones A-F, were isolated from the leaves, twigs, and stem bark of *C. sumatranum* [8]. Previous study, we have been isolated two xanthones from *C. sumatranum* stem bark, namely cochinchinoxanthone and cochinchinone D [9]. Two novel anthraquinobenzophenones, cratoxyarborequinones A (7) and B (8) were also isolated from *C. sumatranum* [8].

The antimalarial activity of fraction-6 was probably due to its xanthone content. This result was following a previously reported study. Numerous antimalarial compounds were reported between 2010 and 2017. Among the 477 isolated compounds with $IC_{50}$ ≤3 µM reported, 17.4% are polyphenols, including xanthones, bioflavonoids, prenylated flavonoids, coumarin, and lactones [20]. The xanthones and their derivatives have been well known as potential antimalarial activity against *P. falciparum* [21–23]. Inhibition of the heme polymerization process was proposed as a

mechanism action of xanthone on *P. falciparum* growth inhibition [23]. Decreased antimalarial activity of xanthones with a hydroxyl group in the peri-position was reported. The intramolecular hydrogen bonding with the carbonyl, which results in reduced affinity for heme, explained it. Meanwhile, xanthones' potency was increased by paired hydroxyls attached to the lower half of xanthones [24]. This study suggested fraction-6 as a potent antimalarial substance. Meanwhile, the active compounds were not identified yet. The further study regarding isolation and identification of active compounds was need to be addressed.

## Conclusions

This study reported the extract and fraction from *C. sumatranum* stem bark were exhibited antimalarial activities. The dichloromethane extract of *C. sumatranum* stem bark (BP14-SB-D) and Fraction-6 from this extract was exhibited antimalarial activity and potential to be developed as antimalarial substances.

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Kartika Dyah Palupi*, Muhammad Ilyas and Andria Agusta

# Endophytic fungi inhabiting *Physalis angulata* L. plant: diversity, antioxidant, and antibacterial activities of their ethyl acetate extracts

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**Abstract**

**Objectives:** Endophytic fungi are an essential source of biologically active compounds. They have the ability to synthesize secondary metabolites which are the same or have a high degree of similarity to their host plants. In this study, we aimed to explore the biodiversity and the bioactivities of active metabolites produced by 14 endophytic fungi isolated from the medicinal plant *Physalis angulata* L. (PA).

**Methods:** Fourteen endophytic fungi were isolated from the flowers, stems, leaves, and fruit husks of PA. The endophytic fungi were cultured and incubated in the PDB medium at room temperature. After three weeks, the cultures were extracted using ethyl acetate and dried using a rotary evaporator. The antioxidant activity was evaluated against DPPH while antibacterial activity was evaluated against *Escherichia coli* and *Staphylococcus aureus* using microdilution technique. TLC analysis was also done to profile the active compounds within the extract.

**Results:** Hyphomycetes fungus isolated from the flower of PA exhibited a moderate antioxidant activity with an antioxidant index value of 0.59 ($IC_{50}$ = 52.43 μg/mL). Six isolates have strong antibacterial activity against *E. coli* and *S. aureus* with minimum inhibitory concentration (MIC) value ranging from 8–64 μg/mL. These endophytic fungi are one Hyphomycetes fungus isolated from the flower, one *Fusarium* sp. isolated from the stem, and four *Colletotrichum* sp. isolated from leaf and fruit husk of PA.

**Conclusions:** Endophytic fungi isolated from PA are potential novel sources of active metabolites especially for antibacterial compounds.

**Keywords:** endophytic fungi; *Escherichia coli*; free radical scavenger; *Physalis angulata* L.; *Staphylococcus aureus*.

## Introduction

Natural products have been holding an essential role in the drug discovery field. Almost 40% of the FDA-approved new molecular entities are originated from natural products and their derivatives [1]. Natural compounds are known to have better interactions with their target and have better pharmacokinetic as well as pharmacodynamic profiles compared to synthetic compounds [2]. Therefore, drug discovery and development from natural products are still promising.

Natural compounds can be obtained from plants, animals, and microorganisms. Endophytic fungi are microfungi that reside inside living plant tissue without generating harmful diseases or producing substances that are detrimental to the host plant [3]. The symbiotic relationship between fungal endophytes and the host plants have been attracting much attention in the last two decades. Fungal endophytes involve in the plant defense mechanism and also have the ability to generate the same or similar bioactive secondary metabolites as those synthesized by the host plant [4]. Furthermore, an increasing number of novel lead compounds isolated from endophytic fungi with a wide variety of pharmacological activities have been reported [5]. Hence, endophytic fungi become an essential source for potential bioactive compounds.

*Physalis angulata* L. is a traditional medicinal plant that widely used in Central and South America as well as in Asia. This plant produces potential bioactive compounds such as terpenoids, withanolides, and physalins [6]. Like other plants, PA hosts endophytic fungi inside its leaves, fruits, flowers, and stems. However, the information regarding the endophytic fungi from this plant is still very limited. In this study, we explored the bioactivities of secondary metabolites produced by 14 endophytic fungi obtained from PA.

***Corresponding author: Kartika Dyah Palupi**, Research Center for Chemistry, Indonesian Institute of Sciences, Kawasan PUSPIPTEK, Tangerang Selatan, Banten 15314, Indonesia, Phone: +62 217560929, E-mail: kart009@lipi.go.id. https://orcid.org/0000-0002-5999-122X
**Muhammad Ilyas,** Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Jawa Barat, Indonesia
**Andria Agusta,** Research Center for Chemistry, Indonesian Institute of Sciences, Kawasan PUSPIPTEK, Tangerang Selatan, Banten, Indonesia. https://orcid.org/0000-0002-9226-6265

# Materials and methods

### Culture of endophytic fungi from *P. angulata* L. (EFPA)

Endophytic fungi were isolated from the flowers, stems, leaves, and fruit husks of PA (Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia) with surface sterilization as described before [7]. EFPA were cultured on cornmeal malt agar (BD Difco, Le Pont-de-Claix, France) supplemented with 0.05 g/L chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and re-cultured on potato dextrose agar (PDA) (BD Difco, Le Pont-de-Claix, France) until single isolate was obtained. All of the isolated fungi were deposited at Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences. The isolates were then inoculated to 200 mL potato dextrose broth (PDB) in a 500 mL Erlenmeyer flask and fermented for 3 weeks under a dark and static condition at room temperature.

### Identification of EFPA

The identification of endophytic fungi was performed by observing their morphological characters. Macroscopic characterization includes the observation of surface color and reverses color of the colony, shape, texture, and exudate. Microscopic characterization was performed under a light microscope with one drop of 1% lactophenol blue staining. Pigmentations of hyphae, septate, spore, clamp connection, and other reproductive structures were observed.

### Extraction of secondary metabolites

After 3 weeks fermentation, fungi biomass and growth media were extracted with ethyl acetate three times. The solvent was removed from the extract using a rotary evaporator (IKA, Germany) and further dried with nitrogen gas.

### Determination of antioxidant activity

The antioxidant activity was determined by DPPH microdilution assay with two-fold serial dilution. Briefly, two-fold dilution series (100 µL) of stock samples dissolved in methanol (Merck, Darmstadt, Germany) were prepared in a flat-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark). Subsequently, 100 µL of 61.5 µg/mL DPPH solution in methanol was added to the wells and homogenized. After 90 min of incubation under a dark condition at room temperature, the absorbances were read using spectrophotometer Multiskan GO (Thermo Scientific, Waltham, USA) at 517 nm. The assay, the calculation of $IC_{50}$, as well as the AAI value was conducted as described before [8].

### Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) values were determined using *in vitro* broth microdilution assay (Figure 1). Bacterial strains (*Escherichia coli* InaCC B4 and *Staphylococcus aureus* InaCC B5) were obtained from the Indonesian Culture Collection (InaCC). All extracts were prepared in DMSO with the final concentration of DMSO in each well ≤2.5%. Two-fold dilution series (100 µL) of stock samples dissolved in Mueller-Hinton Broth (MHB) (BD Difco, Le Pont-de-Claix, France) were prepared in a sterile flat-bottom 96-well microtiter plate (Nunc, Roskilde, Denmark). Subsequently, 100 µL ($10^6$ CFU/mL) bacterial suspension were added to the wells. After 20 h of incubation at 37 °C, 10 µL of 4 mg/mL Iodonitrotetrazolium p-violet (INT) (Sigma–Aldrich, Steinheim, Germany) was added. MIC was defined visually as the lowest concentration with the absence of purple color indicating the absence of bacterial growth. MIC determinations were performed in triplicate with final concentrations of the extract were in the range of 1–256 µg/mL. Chloramphenicol was used as a positive control.

### TLC-direct bioautography

TLC-direct bioautography (TLC-DB) was used to profile the active antibacterial part of the extract. Onto a silica gel $GF_{254}$ TLC glass plate (Merck, Darmstadt, Germany), 10 µL extract (2 mg/mL) was loaded. TLC plate was developed using $CH_2Cl_2$:MeOH (10:1) and visualized under ultraviolet light (254 and 366 nm). Subsequently, the TLC plate was immersed into bacterial suspension in MHB and incubated for 18 h at 37 °C under a humid condition. Bacterial growth inhibition activity was visualized by spraying INT (4 mg/mL) onto the TLC plate and observed as white spots against a purple background.

### Chemical profiling with TLC

Onto a silica gel $GF_{254}$ TLC aluminum plate (Merck, Darmstadt, Germany), 10 µL extract (2 mg/mL) was loaded. TLC plate was developed using dichloromethane: methanol (10:1) and visualized under ultraviolet light (254 and 366 nm) as well as using spray reagent cerium (IV)-sulfuric acid, *p*-anisaldehyde-sulfuric acid, Liebermann–Burchard, Dragendorff (Merck, Darmstadt, Germany), $AlCl_3$, and boric acid-citric acid.

# Results

## EFPA isolates

A total of 14 endophytic fungi was isolated from the flowers, leaves, stems, and fruit husks of PA (Table 1). According to macroscopic and microscopic characterization (Figure 2), the identified endophytic fungi consist of one Hyphomycetes isolate, three *Fusarium* sp. isolates, nine *Colletotrichum* sp. isolates, and one *Xylaria* sp. isolate.

## Antioxidant activity of EFPA ethyl acetate extracts

The radical scavenging activity measurements of EFPA ethyl acetate extracts were performed against free radical DPPH. The antioxidant activity of each extract was

**Table 1:** Endophytic fungi associated with *P. angulata* L. plant and their bioactivities.

| No. | Plant parts | Isolate code | Fungal taxa | Antioxidant activity | | Antibacterial activity MIC, µg/mL | |
|---|---|---|---|---|---|---|---|
| | | | | IC50, µg/mL | AAI | *S. aureus* | *E. coli* |
| 1 | Flower | BgPa-1 | Hypomycetes | 55.91 ± 3.35 | 0.55 ± 0.032 | 64 | 32 |
| 2 | Stem | BtPa-1 | *Fusarium* sp. | 290.73 ± 2.78 | 0.11 ± 0.001 | 128 | 64 |
| 3 | | BtPa-2 | *Colletotrichum* sp. | >512 | <0.06 | >256 | >256 |
| 4 | | BtPa-3 | *Colletotrichum* sp. | >512 | <0.06 | >256 | >256 |
| 5 | | BtPa-4 | *Colletotrichum* sp. | 122.57 ± 3.21 | 0.25 ± 0.007 | >256 | >256 |
| 6 | | BtPa-5 | *Colletotrichum* sp. | >512 | <0.06 | >256 | >256 |
| 7 | Leaf | DnPa-1 | *Xylaria* sp. | 129.26 ± 4.12 | 0.24 ± 0.008 | >256 | >256 |
| 8 | | DnPa-2 | *Colletotrichum* sp. | >512 | <0.06 | >256 | >256 |
| 9 | | DnPa-3 | *Colletotrichum* sp. | >512 | <0.06 | 64 | 16 |
| 10 | Fruit husk | SbPa-1 | *Colletotrichum* sp. | >512 | <0.06 | 32 | 16 |
| 11 | | SbPa-2 | *Fusarium* sp. | 220.67 ± 3.90 | 0.14 ± 0.002 | 256 | 256 |
| 12 | | SbPa-3 | *Colletotrichum* sp. | >512 | <0.06 | 32 | 8 |
| 13 | | SbPa-4 | *Colletotrichum* sp. | >512 | <0.06 | 32 | 16 |
| 14 | | SbPa-5 | *Fusarium* sp. | 158.75 ± 1.96 | 0.19 ± 0.002 | >256 | >256 |

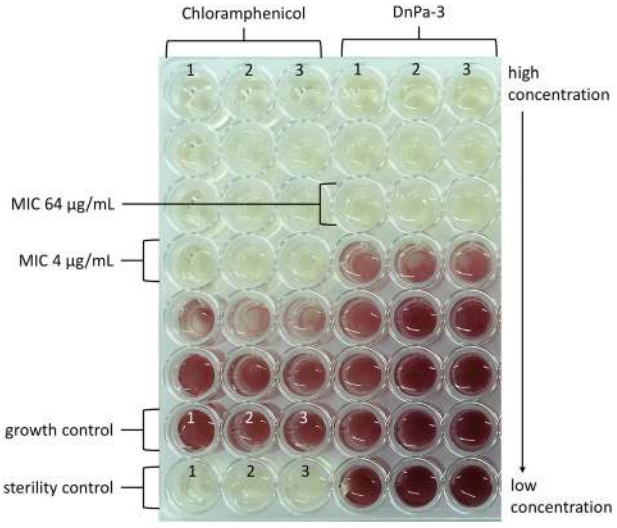


**Figure 1:** MIC determinations of chloramphenicol (positive control) and DnPa-3 endophytic fungus extract against *S. aureus*.

classified based on its antioxidant activity index (AAI) as described by Scherer and Godoy [9]. AAI value <0.5 classified as poor, 0.5 – ≤1 classified as moderate, >1 and ≤2 classified as strong, and >2 classified very strong. In this study, only BgPa-1 exhibited moderate antioxidant activity (AAI = 0.59) while other isolates had weak antioxidant activities (Table 1).

## Antibacterial activity of EFPA ethyl acetate extracts

The antibacterial activities of EFPA ethyl acetate extracts were tested against *S. aureus* and *E. coli*. The strength of antibacterial activity of EFPA was classified as good (MIC = <100 µg/mL), moderate (MIC = 100–500 µg/mL), weak (MIC = 500–1,000 µg/mL), and inactive (MIC = >1,000 µg/mL) [10]. BgPa-1, SbPa-1, SbPa-3, SbPa-4,

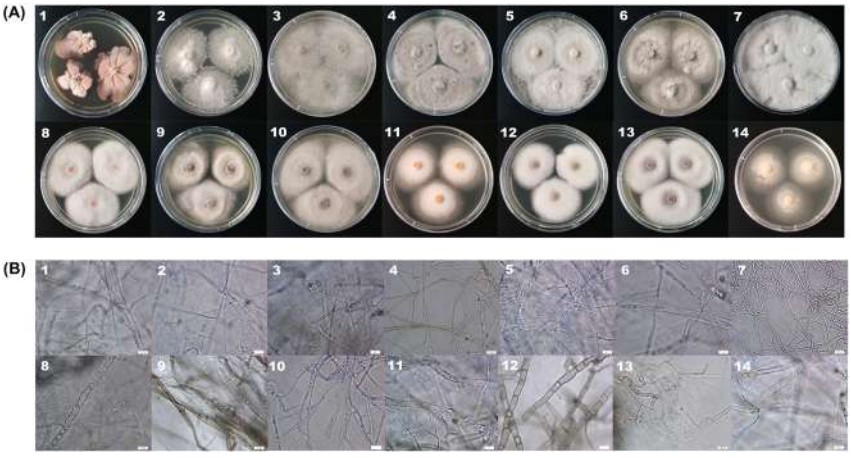


**Figure 2:** (A) The macroscopic view of 14 EFPA from Cibinong, West Java Province on PDA medium, 7–10 days incubation at room temperature. (B) The microscopic view of EFPA mycelia. The list of endophytic fungi inhabiting *Physalis angulata* L. (1–14) is presented in Table 1.

and DnPa-3 had good antibacterial activities against *S. aureus* and *E. coli* while BtPa-1 displayed good antibacterial activity against *E. coli* but moderate activity against *S. aureus* (Table 1). Chloramphenicol was tested as a positive control with MIC value 4 µg/mL against both of the bacterial strains.

## TLC profiling of active antibacterial composition within the extracts

TLC-DB was used to pinpoint the active antibacterial compounds within the extract. The active parts of the extract were indicated as clear zones against a purple background (Figure 3). The compounds within BgPa-1 that responsible for its antibacterial effect against *S. aureus* were located around Rf 0.6–0.8. While for DnPa-3, SbPa-1, SbPa-3 and SbPa-4 extracts, the compounds that were responsible for their antibacterial activity against *S. aureus* were located around Rf 0.5–0.6.

On the other hand, for the *E. coli*, the compounds located around Rf 0.5–0.6 were also the main suggested compounds responsible for the antibacterial activities of BgPa-1, BtPa-1, DnPa-3, SbPa-1, SbPa-3, and SbPa-4 extracts. However, the compounds below Rf 0.5 within BgPa-1, SbPa-1, SbPa-3, and SbPa-4 extracts were also exhibited antibacterial activity against *E. coli* which displayed as clear bands (Figure 3).

For further analysis of the responsible compounds, TLC profiling was done (Figure 4). Cerium (IV)-sulfuric acid was used as a universal spray regent. Liebermann-Burchard was used to visualized steroids and triterpenoids. Steroids or

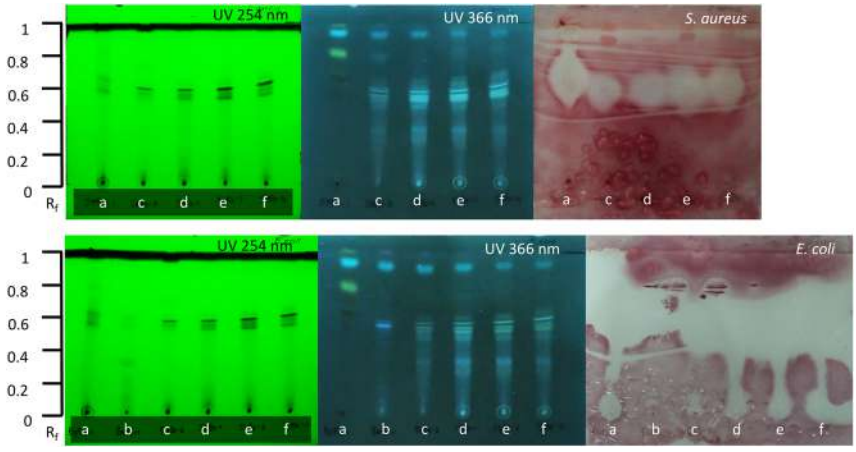


**Figure 3:** Antibacterial TLC-DB profile of EFPA against *S. aureus* and *E. coli*. Mobile phase = dichloromethane: methanol (10:1). a = BgPa-1, b = BtPa-1, c = DnPa-3, d = SbPa-1, e = SbPa-3, and f = SbPa-4.

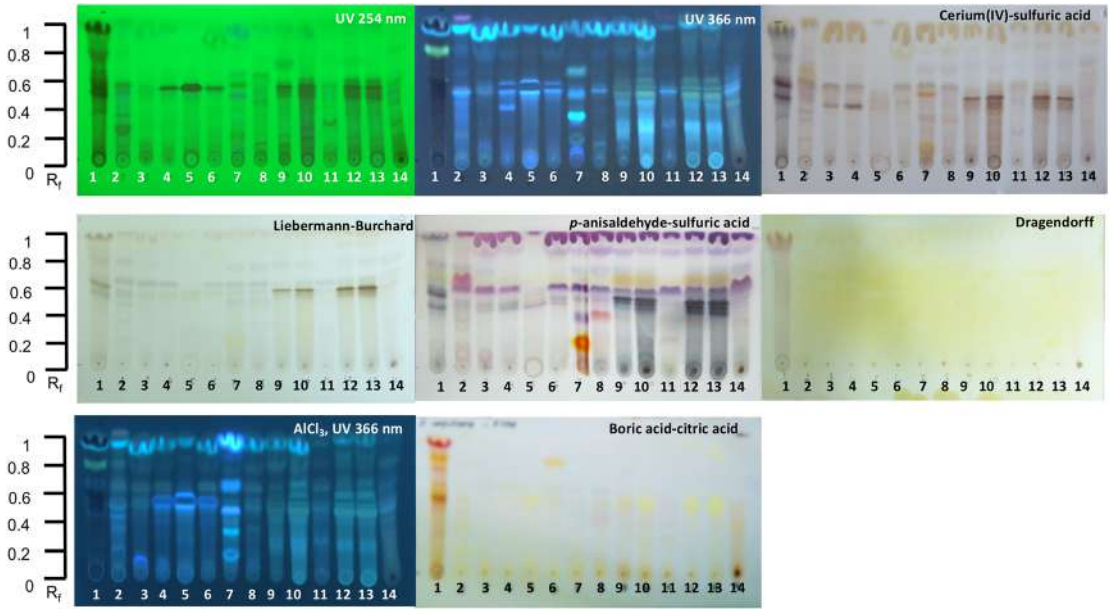


**Figure 4:** TLC profile of EFPA secondary metabolites. The TLC spots were visualized using UV lights, cerium (IV)-sulfuric acid, Liebermann-Burchard, *p*-anisaldehyde-sulfuric acid, Dragendorff, $AlCl_3$, and boric acid-citric acid spray reagent. The list of EFPA extracts (1–14) is presented in Table 1. Mobile phase = dichloromethane: methanol (10:1).

terpenoids will appear as blue, green, or brown spots with Liebermann-Burchard [11]. *P*-anisaldehyde-sulfuric acid is also a universal spray reagent but it could produce specific colors for terpenoids and steroids [12]. Monoterpenes detected as blue spots, triterpenes as purple spots, while steroids appeared as gray spots [13]. The detection of flavonoid was performed using $AlCl_3$ [14] and boric acid-citric acid reagent. Flavonoids formed yellow spots under UV 366 nm with $AlCl_3$ reagent and also yellow spots under normal light using boric acid-citric acid reagent [15]. The active spots within BgPa-1 (code number:1 from Table 1), BtPa-1 (2) DnPa-3 (9), SbPa-1 (10), SbPa-3 (12), and SbPa-4 (13) extracts around Rf 0.5–0.8 were positive for steroid, terpenoid and flavonoid detections (Figures 3 and 4). Analysis of alkaloids using Dragendorff reagent showed negative results.

## Discussion

The isolation of endophytic fungi from different plant parts of *P. angulata* L. resulted in 14 isolates from diverse fungal taxa. In this study, the stems and fruit husks of PA harbored a higher number of isolates compared to the leaves and flowers. Studies reported that, usually, the colonization or infection rate and the diversity of endophytic fungi were higher in the stem than in the leaves [16–19]. The age of the host plant was also suggested to influence the richness and diversity of the isolated endophytic fungi. Younger plant tissue harbored a lower diversity of isolated endophytic fungi but produced a higher isolation rate [20]. Higher diversity of endophytic fungi in older plants is caused by the alteration in the tissue structure of older plants, allowing the invasion of diverse endophytic fungi [21]. Furthermore, as the plant gets older, the occurrence of endophytic fungi infections spreading through wind or rainfall are also higher [21].

The EFPA species composition isolated in this study also have some differences compared to a recent study [22]. Environmental factors such as annual rainfall [23], geographical location, and the vegetation of a certain region also play a major role in the distribution and colonization of endophytic fungi [24]. The distribution of endophytic fungi from the same region is usually very similar [25], however, the population structures of endophytic fungi from different regions usually display a very low degree of similarity even if the host plants are from the same species [24, 26]. In addition, a study also showed that canopy cover can also affect the colonization of endophytic fungi [27].

Endophytic fungi are potential sources for bioactive metabolites. They can produce active metabolites that are the same or similar to their host plant and also produce metabolites that protect the host plants from pathogenic microorganism and harmful conditions [28–30]. In this study, we evaluated the antioxidant and antibacterial activities of EFPA ethyl acetate extracts. Only one isolate (BgPa-1) displayed moderate antioxidant activity while the other isolate displayed weak antioxidant activities. In contrast, six isolates (BgPa-1, BtPa-1, DnPa-3, SbPa-1, SbPa-3, and SbPa-4) had good antibacterial activities against *E. coli* and five of them also had good antibacterial activities against *S. aureus* indicating a broad antibacterial spectrum.

The MIC values indicated that *E. coli* was more susceptible to these six extracts than *S. aureus*. Usually, Gram-negative bacteria are more resistant to antibacterial agents because they have an impermeable cell wall. From the TLC-DB profile, the main parts within the extracts that responsible for the antibacterial activity against these two bacterial strains were similar. However, there were other parts or compounds within the extracts that could inhibit the growth of *E. coli* but could not inhibit the growth of *S. aureus*. The mixture of these active compounds may create an additive or synergistic effect and as a result, produce a better antibacterial activity against *E. coli*.

In this study, the TLC profiling of the compounds within the extracts revealed diverse metabolites produced by EFPA. These metabolites were detected as spots with different Rfs on the TLC plates under UV lights and with spray reagents. Groups of endophytic fungi with similar TLC profiles were also spotted. The *Colletotrichum* sp. BtPa-3 (4) and BtPa-5 (6), isolated from the stem of PA, possessed similar TLC profiles as well as similar antioxidant and antibacterial activities. These similarities were also found in four active *Colletotrichum* sp. isolates, DnPa-3 (9), SbPa-1 (10), SbPa-3 (12), and SbPa-4 (13) which were isolated from the leaf and the fruit husk of PA. On the other hand, BtPa-2 (3), BtPa-4 (5), and DnPa-2 (8) that also identified as *Colletotrichum* sp. displayed different TLC profiles from these previous two groups. The isolated *Colletotrichum* sp. with similar TLC profiles and bioactivities from this study, may belong to the same species of *Colletotrichum*. Furthermore, for the three *Fusarium* sp. isolated in this study, the TLC analysis exhibited different profiles for BtPa-1 (2) which was isolated from the stem of PA but similar profiles for SbPa-2 (11) and SbPa-5 (14) which were isolated from the fruit husk of PA. BtPa-1 (2) also displayed higher antioxidant and antibacterial potencies than the other two *Fusarium* sp. These results further suggested that the same species of endophytic fungi could reside in a different part of a plant and that metabolite profile could also be a useful tool in the grouping or distinguishing the close related endophytic fungi. However, further study using molecular analysis for precise identification of endophytic fungi is still needed to confirm the TLC profiling (chemotaxonomy) suggestion.

TLC profiling analysis showed that the main antibacterial parts within the extract were positive for steroids, terpenoids and flavonoids detection. Previous studies have reported that *P. angulata* L. produce terpenoids, steroids, and flavonoids such as physalins, withanolides, and myricetin glycoside [6, 31–33] which displayed potential anticancer and antimicrobial activity [6, 32–34]. Besides its pure compounds, *P. angulata* L. as an extract also exhibited potent antibacterial and antioxidant activities [35, 36]. In addition, terpenoids, steroids, and flavonoids isolated from endophytic fungi also have displayed potential antibacterial activities [37–39]. As endophytic fungi have the ability to synthesis the same or similar active compounds that originated from host plants, active microbial compounds synthesized by the host plant may also be produced by the EFPA isolates. However, as the TLC profiling method cannot identify the exact antibacterial compounds within EFPA extracts, further compound elucidation analysis still needs to be performed in the future studies.

## Conclusions

Six extracts of EFPA exhibited good antibacterial activity against *S. aureus* and *E. coli*. These extracts are potential to be studied further as potential novel sources for antibacterial agents.

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Retno Widyowati*, Neny Purwitasari, Rice Disi Oktarina, Wiwied Ekasari, Saarah Khairunnisa and Hsin-I. Chang

# Exploration of several plants from Baung Forest on bone formation cell models

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**Abstract**

**Objectives:** Osteoporosis is an ailment described by a skeletal degradation of bone skeletal dominating to increases the chance of fracture. In order to find out the bone formation agents from Baung Forest plants, this research analyzed the effects of 96% ethanol extract of several plants from Baung Forest on antioxidant activity and the effect of osteoblast differentiation-related to the bone formation on the most potent extract.
**Methods:** The antioxidant effect and osteoblast differentiation of 96% ethanol extracts were evaluated by measuring DPPH scavenging and alkaline phosphatase in *p*-nitrophenyl phosphate effects by the enzyme-linked immunosorbent assay (ELISA) reader method, respectively.
**Results:** The 96% ethanol extract of *Elaeocarpus serratus* L. from Baung Forest had the strongest DPPH radical scavenging as anti oxidant (82.17%) and stimulated osteoblast differentiation (116%). Then, this extract had been fractionated based on polarity to become hexane, ethyl acetate, butanol, and aqueous fractions. All the fractions stimulated their alkaline phosphatase (ALP) activity to 138.11 ± 9.72%, 108 ± 5.05%, 148.56 ± 8.47, and 144.58 ± 1.04, respectively.
**Conclusions:** The extract and fractions of *E. serratus* L. can successfully inhibit DPPH radical scavenging value and increase ALP activities as markers of osteoblast functions.

**Keywords:** 96% ethanol extract; alkaline phosphatase; bone formation; DPPH scavenging; *Elaeocarpus serratus*.

***Corresponding author: Retno Widyowati**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 81615886978, E-mail: rr-retno-w@ff.unair.ac.id. https://orcid.org/0000-0002-6166-1289
**Neny Purwitasari, Rice Disi Oktarina, Wiwied Ekasari and Saarah Khairunnisa,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Hsin-I. Chang,** Department of Biochemical Science and Technology, National Chiayi University, Chiayi, Taiwan, P. R. China

## Introduction

Osteoporosis is an ailment described by a skeletal degradation of bone skeletal dominating to increases the chance of fracture and being a quiet ailment in many complex situations [1]. This ailment can occur because a disproportion of bone resorption relative to bone formation products in effectiveness bone equilibrium at the tissue. During growth, bone formation surpasses bone resorption, resulting in bone elaboration [2]. It is a prominent matter of elderly and estimated to increase with rising age and life span. At 1992, the 200 million global population were expected to endure from osteoporosis [3]. Then in 2000, statistical data from the International Osteoporosis Foundation represented that 1 out of 3 women over 50 years old and 1 out of 5 men will endure osteoporosis fractures for the spend of their lives [4]. This problem too occurs in Indonesia, which has reached a level of caution because the amount of osteoporosis sufferer has increased from the latest data (>19.7%). The amount of elderly in Indonesia is estimated to increase by 14% during of 1990–2025, while in the 2000–2015 period, menopausal women donated to an intensify of osteoporosis sufferers by 8.5 million [5]. WHO estimates that in 2050 the number of fracture sufferers will increase by 2 times in women and 3 times in men [6, 7].

In this study, we have found out bone formation agents from Baung Forest plants. Baung Forest is a nature tourism park with an area of 195.5 ha [8]. This forest has its natural biodiversity, beauty, and geology. In the forest, there are various types of plants that are commonly used by local residents for health therapy. The 36 plant extracts from this forest were screened for antioxidant activity (DPPH inhibition values) and the most potent extract was analyzed for its effect on osteoblast proliferation and differentiation by evaluating alkaline phosphatase (ALP). Oxidative stress in bone cells results in the production of reactive oxygen species (ROS) from lipoxygenase and oxidase [9]. ROS can affect bone cells through decreased production of bone matrix protein (characterized by decreased ALP value) [10]. ALP is an identified biochemical marker of bone formation on the osteoblast plasma membrane reflecting osteoblastic activity on bone remodeling process [11] and plays an important role in osteoid formation and bone mineralization [12].

# Materials and methods

### Cell culture and reagents

Reagent chemicals, such as Alkaline Phosphatase Colorimetric Assay Kit, Acid Phosphatase Leukocyte Kit, and all other chemicals, were acquired by Sigma-Aldrich Co. (St Louis, MO, USA). All cell culture materials and solvents were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and analytical grade (J.T. Baker, USA). Mouse osteoblast-like cells (7F2) were obtained from Department of Biochemical Sciences & Technology, National Chiayi University, Taiwan, and refined in Dulbecco's Modified Eagle's Medium (DMEM). They were farther strengthened by 10% v/v Fetal Bovine Serum (FBS), 100 μg/mL streptomycin, and 100 units/mL penicillin. Cells were incubated in a dabbled incubator with 5% $CO_2$ at 37 °C.

### Materials

The plants were collected in middle July 2018 in Baung Forest, Indonesia, and voucher samples were stored at Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Airlangga, Indonesia. The plants were identified by the Plant Conservation Institution, Purwodadi Botanical Garden.

### Extraction

Fresh plants obtained from Baung Forest, Purwodadi, were cleaned and washed with clean running water, then dried under indirect sun to dry. After drying, the particle sizes were reduced by grinding until a powder was obtained. A total of 100–200 g of plant powders were extracted with 96% ethanol-aqueous (100 mL × 3) by maceration method. Each of 96% ethanol solution was evaporated using a rotary evaporator to get each of 96% ethanol extract (*E*) (Table 1). The potent extract was sequentially fractioned with hexane, ethyl acetate, butanol, and aqueous to provide hexane-soluble (EH), ethyl acetate-soluble (EE), butanol-soluble (EB), and aqueous-soluble (EA) fractions.

### DPPH measurement

The antioxidant activity of 96% ethanol extracts was defined by di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. The 0.25 mM DPPH solution was processed using DPPH solution in methanol. The 100 μg/mL of 96% ethanol extracts was mixed with 0.25 mM DPPH reagent in equal amounts (100 μL) in 96 well plates. Blank solution was the mixture of sample solvent (ethanol, 100 μL) and methanol (100 μL). DPPH reagent (100 μL) was mixed with methanol (100 μL) to serve as control. The reaction mixtures were shaken gently in the dark for 15–30 min at 25 °C. After the incubation, the absorbance was evaluated at 517 nm using a Tecan, infinite M200 microplate reader. The measurements were performed in triplicates. The DPPH radical scavenging was counted by equation [13, 14].

$$\text{DPPH radical scavenging effect} = \frac{(1 - \text{sample groups absorbance} - \text{blank absorbance})}{\text{Control group absorbance}} \times 100\%$$

### Cell viability assay

The 7F2 cells were plated for cell growth studies at a density of $10^4$ cells/well in 96-well plates. DMEM medium composing 100 units/mL penicillin, 10% FBS, and 100 μg/mL streptomycin was used to restore the cell. After 24 h, the E extract, EH, EE, EB, and EA fractions of *Elaeocarpus serratus* L. from Baung Forest were incubated at various concentrations for another 24 h at 37 °C. The cell supernatants were subsequently extracted, after 200 μL 3-(4,5-dimethylthiazol-2-yl)- and 100 μL of 2,5-diphenyltetrazolium bromide (MTT) reagent (100 μg/mL) were incubated during 4 h. Similarly, to dissolve the formazan crystals, 100 μL of dimethyl sulfoxide (DMSO) was added. The absorbance was ruminated at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were performed in triplicate, with the relative cell viability (%) declared as a portion relative to the unprocessed control cells [15, 16].

### Differentiation of cellular alkaline phosphatase activity (ALP)

The 7F2 osteoblast-like cells were plated in 24-well plates at $10^4$ in DMEM containing 5 mM β-glycerol phosphate (β-GP), 10% FBS, and 50 μg/mL of ascorbic acid (2GF medium) with or without E extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest for 4 and 7 days incubation period at 37 °C in a 5% $CO_2$ atmosphere. Phosphate buffered saline (PBS) was applied to clean the supernatants. After that, a percentage of the v/v triton solution was inserted and incubated for 10 min at 37 °C. After incubation, the cell lysates were examined for ALP by adding 200 μL of *p*-nitrophenyl phosphate (PNPP) and diethanolamine buffer into each well for a period of 30 min and at room temperature. The 50 μL/well stop solution was inserted to stop the reaction while ELISA reader at 405 nm was applied to measure the absorbance [15, 16].

### Statistical analysis

The experiments were performed for three times using similar methods. It was then expressed as means ± standard deviations. The one-way ANOVA and LSD test were used to illustrate data analysis. The differences proved to be statistically significant at $p<0.05$.

# Results

## The effect of 96% extracts from Baung Forest plants on DPPH radical scavenging

During our project in order to discover antiosteoporotic delegates from natural sources [16–20], we screened 36 plants from Baung Forest on antioxidant by measuring DPPH scavenging. Oxidative stress produces a breakage of cellular owing to membranes structural change, lipid oxidation, and oxidation of nucleic acids and proteins. The breakage may expand to the organs and become systemic [21]. Many

**Table 1:** Baung forest plants collection and their DPPH radical scavenger effect (%).

| No. | Name of plant | Indonesian name | Family name | Part of plant | *E* (% yields) | DPPH at 100 µg/mL, % |
|---|---|---|---|---|---|---|
| 1 | *Ixora nigricans* | Jejarum | Rubiaceae | Leaves | 7.52 | 28.06 ± 2.19 |
| 2 | *Brucea javanica* | Buah makasar | Simaraubaceae | Leaves | 7.87 | 17.34 ± 5.56 |
| 3 | *Mitrephora polypyrena* | Janglot, kalak | Annonaceae | Leaves | 6.14 | 62.47 ± 1.92 |
| 4 | *Hypoestes phyllostachya* | Polkadot | Acanthaceae | Leaves | 8.76 | 19.60 ± 7.60 |
| 5 | *Eranthemum nervosum* | – | Acanthaceae | Aerial part | 8.07 | 22.22 ± 1.65 |
| 6 | *Protium javanicum* | Trenggulum | Burseraceae | Aerial part | 8.88 | 67.86 ± 3.30 |
| 7 | *Urena lobata* | Pulutan | Malvaceae | Leaves | 4.68 | 37.46 ± 8.24 |
| 8 | *Blumea lacera* | Sembung kuwuk | Asteraceae | Leaves | 11.66 | 11.87 ± 7.95 |
| 9 | *Allophylus serratus* | – | Sapindaceae | Leaves | 6.67 | 50.15 ± 5.61 |
| 10 | *Melicope latifolia* | Parijoto | Rutaceae | Leaves | 14.61 | 46.35 ± 2.42 |
| 11 | *Plumbago zaelanica* | Daun encok | Plumbaginaceae | Leaves | 4.46 | 29.73 ± 1.91 |
| 12 | *Parameria leivigata* | Kayu rapet | Apocynaceae | Leaves | 7.49 | 26.90 ± 3.85 |
| 13 | *Elaeocarpus serratus* | Genitri | Elaeocarpaceae | Leaves | 12.32 | 82.17 ± 2.95 |
| 14 | *Reulia tuberosa* | Pletekan | Acanthaceae | Leaves | 7.45 | 36.39 ± 5.72 |
| 15 | *Dracaena elliptica* | Drakaena | Asparagaceae | Leaves | 9.85 | 65.71 ± 3.30 |
| 16 | *Garuga floribunda* | Kilangit | Burseraceae | Leaves | 8.36 | 70.95 ± 3.37 |
| 17 | *Sida acuta* | Sidaguri | Malvaceae | Aerial part | 6.58 | 13.59 ± 4.82 |
| 18 | *Plumeria acuatifolia* | Kemboja | Apocynaceae | Leaves | 7.52 | 47.66 ± 9.66 |
| 19 | *Memecylon myrsinoides* | Baho | Melastomataceae | Leaves | 7.09 | 81.02 ± 1.17 |
| 20 | *Solanum torvum* | Takokak | Solanaceae | Leaves | 5.40 | 36.01 ± 4.88 |
| 21 | *Solanum verbascifolium* | Terong teter | Solanaceae | Leaves | 7.27 | 30.91 ± 5.14 |
| 22 | *Lantana camara* | Saliara | Verbenaceae | Aerial part | 6.82 | 15.45 ± 4.65 |
| 23 | *Polyscias nodosa* | Tirotasi | Araliaceae | Leaves | 10.70 | – |
| 24 | *Harrisonia perforata* | Rui | Rutaceae | Aerial part | 8.62 | 49.45 ± 4.18 |
| 25 | *Hibiscus surattensis* | Waru | Malvaceae | Leaves | 8.58 | 75.38 ± 1.92 |
| 26 | *Lantana camara* | Saliara | Verbenaceae | Flos | 11.29 | 18.94 ± 4.69 |
| 27 | *Melanolepis multiglandulosa* | Daun kapur | Euphorbiaceae | Leaves | 6.72 | 13.52 ± 3.72 |
| 28 | *Rauvolfia tetraphylla* | Pule pandak | Apocynaceae | Leaves | 11.16 | 32.06 ± 3.33 |
| 29 | *Gloriosa superba* | Kembang sungsang | Liliaceae | Leaves | 9.26 | 14.68 ± 1.92 |
| 30 | *Centrosema pubescens* | Centro | Fabaceae | Flos | 4.96 | – |
| 31 | *Centrosema pubescens* | Centro | Fabaceae | Aerial part | 7.94 | 15.35 ± 2.93 |
| 32 | *Voacanga glandiflora* | Kalantong | Apocynaceae | Leaves | 9.20 | 24.32 ± 1.63 |
| 33 | *Phaleria octandra* | Mut | Thymelaeaceae | Leaves | 8.96 | 6.09 ± 1.25 |
| 34 | *Melia azedarach* | Mindi kecil | Meliaceae | Leaves | 5.52 | 16.94 ± 2.32 |
| 35 | *Hypoestes phyllostachya* | Polkadot | Acanthaceae | Leaves | 6.08 | 71.47 ± 3.55 |
| 36 | *Aglaia lawii* | – | Meliaceae | Leaves | 11.99 | 30.12 ± 1.11 |

ailments have been related to oxidative stress and inserting bone diseases (osteoporosis). Antioxidants reduce acceleration of bone damage thru encoragement of tumor necrosis factor alpha (TNFα) [22]. Based on the screening result, the 96% ethanol extract of *E. serratus* L. (13), *Memecylon myrsinoides* (19), *Hibiscus surattensis* (25), and *Hypoestes phyllostachya* (35) from Baung Forest showed high DPPH radical scavenging (82.17 ± 2.95, 81.02 ± 1.17, 75.38 ± 1.92 and 71.47 ± 3.55%, respectively) (Table 1). Therefore, the most potent plant as an antioxidant is *E. serratus* L. In Indonesia, the leaves of this plant are used traditionally to treat arthritis [23] and in India, it is used as Ayurveda of anti osteoporosis [24] and arthritis [25]. Then, the % DPPH radical scavenging toward this plant at different concentration were explored.

## The effect of 96% ethanol extract of *E. serratus* L. leaves on DPPH radical scavenging

In this research, we analyzed the effects of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest leaves toward antioxidant related to bone turnover. Several researches reported on the pharmacological effects of plant extract (Elaeocarpaceae family) from several countries

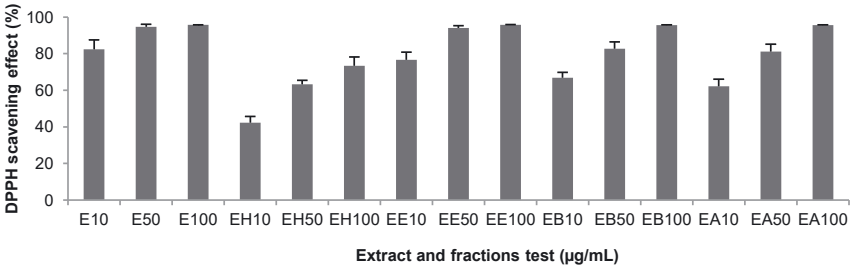


**Figure 1:** Antioxidant activity using DPPH method of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *E. serratus* leaves. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL.

[23, 24, 26–29], but there have been no reports on 96% ethanol extract of *E. serratus* L. from Baung Forest, Indonesia.

DPPH is steady nitrogen that focuses on free radical that can receive hydrogen radical or electron to finish a steady diamagnetic molecule. DPPH radicals respond with appropriate reducing agents as a yield of which the electrons get couple off becoming the corresponding hydrazine. Thus, the antioxidant activity of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest with several concentrations (10, 50, and 100 µg/mL) was detected by DPPH scavenging assay in a range of concentration. Based on the result, the *E* extract, EH, EE, EB, and EA fractions had $IC_{50}$ value of 23.27, 42.47, 19.93, 30.12, and 34.90, respectively (Figure 1).

### The ALP stimulation effect of 7F2 osteoblasts of 96% ethanol of *E. serratus* L

The viability results of *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest in 7F2 osteoblastic cell lines was carried out using MTT test. The viability cells of their extract and fractions increased in dose-related, in which they showed that high concentration of extract and fractions were not toxic (Figure 2) and elevated cellular uptake. Then, ALP experiments were proceed.

The ALP stimulation of 7F2 osteoblast cells using *E* extract, EH, EE, EB, and EA fractions of *E. serratus* L. from Baung Forest was incubated for 4 and 7 days. The results of samples on increasing ALP assay in the 7F2 osteoblasts againsted to the 2GF group on EH, EB, and EA fractions for 4 days (Figure 3). After 7 days, the EB, EA, and EH fractions stimulated their ALP activity to 148.56 ± 8.47, 144.58 ± 1.04, and 138.11 ± 9.72%, respectively (Figure 3).

## Discussion

Geographically, the Baung Purwodadi forest area is located between 7°49′9″–7°47′23″ south latitude and 112°16′23″–112° 17′17″ east longitude with the topography in general being bumpy to hilly, the altitude of this area ranges from between 200 and 501 masl, red–yellow mediterranean soil types and latosols, soil derived from old quarter rock with the main material in the form of metamorphic sediment, climate type D rainfall with a value of $Q$ = 81.82%, the average annual amount of 2.654, 10 mm/year with an average number of rainy days of 141.05 days [30]. In the forest there are plant communities. Potential flora in the TWA Gunung Baung area, including *Brucea javanica*, *Urena lobata*, *Plumbago zaelanica*, *Parameria leivigata*, *Garuga floribunda*, *Plumeria acuatifolia*, *Lantana camara*, *Rauvolfia tetraphylla*, *Gloriosa superba*, *Melia azedarach* and others (Table 1). These plants are used by the local community for treatment such as lowering sugar levels, fever, inflammation, high blood pressure, treating stomach aches, relieving joint pain, headaches, worming, and urination.

The use of these plants as traditional medicine is only based on inheritance from ancestors without knowing the

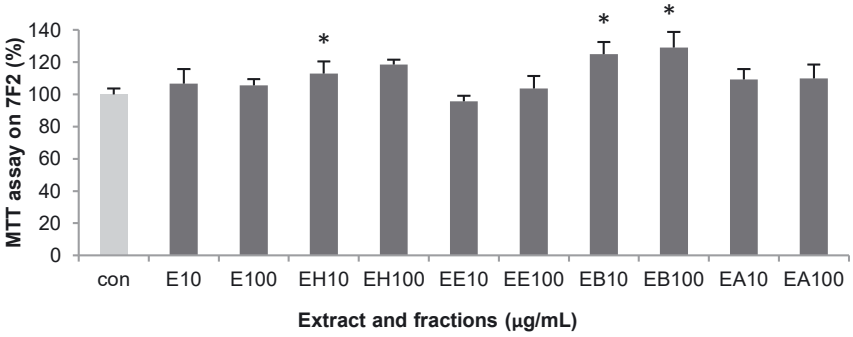


**Figure 2:** The MTT test of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *Elaeocarpus serratus* leaves on 7F2 osteoblast cells. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL.

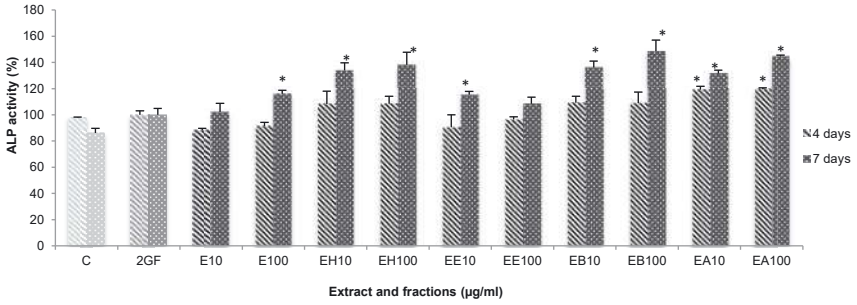


**Figure 3:** The ALP activity of 96% ethanol extract, hexane, ethyl acetate, butanol, and aqueous fractions of *Elaeocarpus serratus* leaves for 4 and 7 days incubation. The number of 10, 50, and 100 following extract (*E*) and fractions (EH, EE, EB, and EA) indicate their concentrations at 10, 50, and 100 µg/mL. The sign * means $p<0.05$–2GF.

chemical content that plays a role in treatment [31]. Therefore, to determine the exact chemical content for treatment, it is necessary to explore plants, especially forest plants that have a large enough potential. The initial screening was antioxidant potential because the assay is simple and easy for large quantities. Oxidative stress occurs as a result of overproduction of ROS which is not balanced, which can cause bone disruption. The altered redox state is also associated with the bone remodeling process which enables the continuous regeneration of bone through the coordinated action of bone cells. Changes in ROS and/or the antioxidant system involve in the pathogenesis of bone loss. ROS induces apoptosis (death) of osteoblasts and osteocytes, this encourages osteoclastogenesis and inhibits mineralization and osteogenesis [32]. Based on DPPH Radical Scavenging result on several plants in the Baung forest, *E. serratus* L. has the highest potential in trapping DPPH radical scavening (82.17 ± 2.95). Therefore, it continues the exploration of this plant to determine their ability to increase bone density.

Natural plants have performed a pivotal part in pharmaceutical drugs and dietary supplement developments for the therapy and precaution of ailment [33]. One of them is *E. serratus* L. from Baung Forest which belongs to the Elaeocarpaceae family. Traditionally, it is used to treat migraine, stress, anxiety, depression, lack of concentration, palpitation, nerve pain, epilepsy, asthma, hypertension, liver diseases [23], arthritis [34], Ayurveda of anti osteoporosis [24], and Ayurveda of osteoarthritis [25]. Several studies have shown that this plant is pharmacologically active and can be functioned as the treatment of arthritis [35], antimicrobial [36], anti inflammatory, analgesic, pesticide, nematoside, antioxidant [25], antibacterial, diarrhea, and dysentery [37]. The leaves contain flavonoids, carotenoids [34, 38], fatty acid [26], myricitrin, and mearnsetin derivatives [39]. Myricitrin has the greatest antioxidant activity in this plant [39]. It was also proved in this study that 96% ethanol extract of *E. serratus* L. leaves had a radical scavening DPPH value of 82.17 ± 2.95% (Table 1). This is the greatest value of its activity compared to other plant extracts from Baung Forest. Based on Figure 2, almost all fractions has the ability to trap free radical >50% at concentration of 10–100 µg/mL but the hexane fraction (EH) at 10 µg/mL cannot trap DPPH radicals by up to 50%. The greater percentage value of trapping, the better antioksidan activity in DPPH radical scavening [40]. Consequently, we explored this extract for bone formation activity.

Several studies have associated antioxidants with bone metabolism. Lower plasma antioxidants can be found in elderly women or women with osteoporosis. Oxidative stress in estrogen deficiency of postmenopausal osteoporosis has been linked to the activation of NADPH oxidase and/or alleviated synthesis of antioxidant enzymes and glutathione (GSH) levels [21, 26]. This antioxidant leads the acceleration of bone loss through activation of tumor necrosis factor alpha (TNFα) [22]. Converting in the redox state is also linked to the process of bone remodeling that permits continuous bone regeneration thru coordinated action of bone cells such as osteoblasts, osteocytes, and osteoclasts. Antioxidants directly contribute to activating osteoblast differentiation in bone formation and mineralization processes.

Based on the results, the 96% ethanol extract of *E. serratus* L. leaves had a strong antioxidant activity and also played a role in the activation of osteoblast differentiation which is directly related to bone formation. Osteoblast differentiation is characterized by measuring levels of ALP. ALP is an important enzyme that is a useful biochemical marker of bone formation [41]. This enzyme plays a role in osteoid formation and mineralization. So that the ALP enzyme and bone mineralization have a significant correlation and become a biochemical marker [42]. Bone growth and healing during bone fracture cause high ALP enzymes in bones. However, if the ALP enzyme appears in excess, it can be an indicator of osteosarcoma to bone metastases [43]. The 96% ethanol extract of *E. serratus* L. leaves stimulated ALP activity in dose of dependent manner (116% of 100 µg/mL). Among the fractions, EB had the strongest ALP activity (148.56 ± 8.47%). It is a potential fraction for activation of bone formation. Ethanol extract from this plant contains fatty acid ester derivatives such as

*n*-dotriacontanol (10.70%), *n*-octadecanol (10.08%), docosanoic acid, 1,2,3-propanetriyl ester (9.07%), *n*-hexadecene (8.52%), bis-(3,5,5-trimethylhexyl) ether (6.30%), ethanone, 1-cyclopentyl- (4.81%), cyclohexane, ethyl- (4.05%), and minor components were hexadecanoic acid methyl ester (0.80%), ricinoleic acid (0.77%), citronellyl isobutyrate (0.69%), and farnesol (0.51%) [26]. Fatty acid has a role in increasing bone formation by stimulated β catenin activity in osteoblast and resulting in increased in osteoblastogenesis [44, 45]. The mechanisms of fatty acid are complex and involve protectins and resolvins, prostaglandins, growth elements, cytokines, and few other molecular signaling routes [45]. This plant also contains carotenoids [38], that have a encourage effect on osteoblastic bone formation *in vitro*, therefrom escalating bone mass. This effects the gene expression of various proteins associated to bone formation [45]. Thus, the 96% ethanol extract of *E. serratus* L. leaves has potential effect to maintain bone health and decrease bone loss.

## Conclusions

The extract and fractions of *E. serratus* L. can successfully inhibit DPPH radical scavenging value and increase ALP activities as markers of osteoblast functions.

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Marsih Wijayanti, Hilkatul Ilmi, Einstenia Kemalahayati, Lidya Tumewu, Fendi Yoga Wardana, Suciati, Achmad Fuad Hafid and Aty Widyawaruyanti*

# *In vitro* antimalarial activity of *Garcinia parvifolia* Miq. Stem extracts and fractions on *Plasmodium falciparum* lactate dehydrogenase (LDH) assay

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**Abstract**

**Objectives:** The rapid spread of antimalarial drug resistance is becoming a problem in the treatment of malaria. The fact was indicated the importance of finding new antimalarial drugs. The genus Garcinia is well known to be a rich source of bioactive prenylated xanthones and triterpenes reported for their antimalarial activity. *Garcinia parvifolia* is one of the Garcinia genera that can be explored for the search of new antimalarial drugs. This study was aimed to determine the antimalarial activities of *G. parvifolia* extracts and fractions.

**Methods:** *Garcinia parvifolia* Miq. stem was collected from Balikpapan Botanical Garden in East Kalimantan, Indonesia, was extracted gradually with n-hexane, dichloromethane, and methanol by ultrasonic assisted method. The most active extract was further separated using the open column chromatography method. All extracts and fractions were tested against *Plasmodium falciparum* 3D7 using lactate dehydrogenase (LDH) assay and followed by $IC_{50}$ determination.

**Results:** The results showed that all extracts inhibit *P. falciparum* growth by LDH assay. The highest inhibition was showed by dichloromethane stem extract (BP12-S-D) with the $IC_{50}$ value of 6.61 ± 0.09 μg/mL. Further fractionation of BP12-S-D has obtained 10 fractions. All of them were identified by TLC, and a brownish-yellow spot (fraction-1) appears after spraying with 10% $H_2SO_4$. Fraction-1 (F1) performed the highest parasite growth inhibition with the $IC_{50}$ value of 6.00 ± 0.03 μg/mL compared with other fractions. This fraction was classified as having a promising activity of antimalarial. The fraction-1 was identified using HPLC, and two major peaks were observed (A and B). The UV–Vis spectra showed the absorption at wavelengths 250 and 278 (A), 243, 281, and 317 nm (B). Based on the profile of TLC, HPLC, and UV–Vis spectra of F1, it was expected that the active compounds are flavonoid (A) and xanthone (B).

**Conclusions:** The fraction-1 of dichloromethane extract of *G. parvifolia* Miq. stem has the highest antimalarial activity. It might be a potential candidate for the new antimalarial drug.

**Keywords:** antimalarial activity; *Garcinia parvifolia* Miq.; lactate dehydrogenase (LDH).

## Introduction

Malaria is a type of infectious disease that mainly occurs in tropical and subtropical regions, and it remains a problem in the world [1–3]. The World Health Organization (WHO) reported that malaria cases in 2018 are estimated to be 228 million (7.9 million or 3.4% of cases in Southeast Asia). Each year, more than 405,000 people die of malaria, particularly children under the age of five and pregnant women [4]. The appearance of drug-resistant *Plasmodium falciparum* since 1960 has made the treatment of malaria increasingly problematic [5–7]. Therefore, discovering new antimalarial drugs is a priority in the health sector [8]. Recently, attention was focused on medicinal plants to provide new antimalarial drugs [9].

Clusiaceae family has 40 genus and more than 1,000 species spread in tropic and subtropic areas [10]. The main genus in the Clusiaceae is Garcinia and Calophyllum [11].

***Corresponding author: Aty Widyawaruyanti**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Center of Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, E-mail: aty-w@ff.unair.ac.id
**Marsih Wijayanti and Einstenia Kemalahayati,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Hilkatul Ilmi, Lidya Tumewu and Fendi Yoga Wardana,** Center of Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
**Suciati and Achmad Fuad Hafid,** Center of Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia; and Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

Clusiaceae are widely spread in Asia, Africa, New Caledonia, and Polynesia [12]. *Garcinia parvifolia* Miq., one species of the Clusiaceae family, is native in tropical, and subtropical countries of South East Asia such as Malaysia, Thailand, Brunei, and Indonesia [13, 14]. This plant is known as *Garcinia dioica* Blume and *Garcinia globulosa* Ridley. The common name for it is Cherry mangosteen, Kandis, and yellow Kandis [15].

*Garcinia parvifolia* has various biological actions, such as antioxidants [13], antimicrobials [7, 11, 16–18], antiplatelet [19], antiplasmodial [7, 17, 20–22], and larvicide [23]. The fruit and young leaf are sometimes eaten as a vegetable [10]. The *n*-hexane extract of stem bark of *G. parvifolia* Miq. inhibited malarial parasite growth against *P. falciparum* FCR3 (chloroquine-resistance strain) with the $IC_{50}$ value of 4.11 μg/mL [7]. The n-hexane fraction of *G. parvifolia* Miq. stem bark showed antimalarial activity against *Plasmodium berghei* with an $ED_{50}$ value of 74.54 ± 10 mg/kg body weight [21]. Several compounds have been isolated from this plant and whereas identified as flavonoids, triterpenoids, steroids, and xanthones (rubraxanthone, cowinin, and the novel cytotoxic griffiparvixanthone) [11, 24, 25]. Based on these previous studies, *G. parvifolia* is potential as a source of antimalarial drugs. Research on the stems of *G. parvifolia* has not been reported. Therefore, this study was aimed to determine the antimalarial activities of *G. parvifolia* stem extracts and fractions.

# Materials and methods

## Plant material

The stems of *G. parvifolia* were collected from Balikpapan Botanical Garden, East Kalimantan, Indonesia. Identification by a licensed botanist at Purwodadi Botanical Garden, East Java, Indonesia. A voucher specimen number is B-109/IPH.06/AP.01/II/2020. Raw material has been stored at the herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

## Extraction and fractionation

The stem of *G. parvifolia* Miq. was extracted gradually with n-hexane, dichloromethane, and methanol by ultrasound-assisted extraction. The most active extract was further separated using open column chromatography with a gradient of chloroform and methanol (100–0%). Based on thin layer chromatography (TLC) profiles, several fractions with the same profile were combined. All extracts and fractions were identified by TLC profiles using standard methods [26]. All samples were diluted in methanol. TLC was performed using chloroform: methanol (98: 2) as the mobile phase and silica gel 60 $F_{254}$ as the stationary phase. Furthermore, the plates were visualized directly after drying and with the help of UV at 254 and 366 nm in UV TLC visualizer. Ten percent $H_2SO_4$ is used as a spray reagent. Extracts and fractions were tested against *P. falciparum* 3D7 using lactate dehydrogenase (LDH) assay and followed by $IC_{50}$ determination.

Subsequently, the active fraction profile was analyzed using TLC and high-performance liquid chromatography (HPLC) methods. Analytical HPLC system was run on a Shimadzu, LC-06 included two LC-10AD pumps and SCL-10A controller with analytical column Merck RP-18 (4.6 × 250 mm × 5 μm). Fractions were eluted in acetonitrile: water (7:3 v/v) mixture at a flow rate of 0.5 mL/min and injection volume of 40 μL.

## Cultivation of *Plasmodium falciparum*

*Plasmodium falciparum* strain 3D7 (chloroquine-sensitive strain) was obtained from the Center for Natural Product Medicine Research and Development (C-NPMRD), Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. The culture was established using the method by Trager and Jensen with some modifications [27]. Parasites were maintained in fresh human erythrocytes (type O red blood cell) at 2% hematocrit in RPMI 1640 medium (Gibco) containing 10% v/v AlbuMAX™ II (Gibco); 0.05 g hypoxanthine (Sigma); 2 g sodium bicarbonate, and 0.5 mL gentamycin (Sigma). Human RBCs were received from the Indonesian Red Cross, Surabaya, Indonesia.

## *In vitro* antimalarial assay

*In vitro* assay of antimalarial activity was carried out by LDH assay [28, 29]. The lactate dehydrogenase (LDH) method was performed using a 96-well plate (flat bottom). Synchronized ring-stage parasites were obtained by 5% w/v sorbitol [30]. Briefly, the continuous culture of the parasites was maintained in a complete medium. Extracts and fractions of *G. parvifolia* Miq. stem was screened at a concentration 10 μg/mL. One microliter of the sample was added to each well and repeated three times. Then added 99 μL pf parasites (ring-stage). Furthermore, the assay plate was incubated at 37 °C in a gas mixture consisting of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ for 72 h. After 72 h of incubation, the well plate was kept at −30 °C overnight. The antimalarial activity was measured by an LDH assay. Ten milliliters of LDH-buffer (Tris-HCl, sodium L-lactate, Triton X-100, deionized water) was added 2 mg NBT (10 mg/mL, Sigma), 50 μL APAD stock (10 mg/mL, Oriental Yeast Co., Ltd.), 200 μL Diaphorase stock (50 units/mL, Sigma). Mix gently and keep the substrate in the dark. Add 90 μL substrate per well plate. Cover with aluminum foil and place on a flatbed shaker at 400 rpm at room temperature. Incubate for 30 min. The absorbance of each well measured at wavelength 650 nm using a multiscan sky high microplate spectrophotometer (Thermo fisher scientific). The inhibition rate was calculated with the absorbance of uninfected wells defined as 100% inhibition. Hit sample was defined as those inhibiting more than 50% activity at 10 μg/mL. The $IC_{50}$ of the hit sample was determined under the same assay condition for the screening with the addition of sample in serial dilution (0.01; 0.05; 0.1; 0.5; 1; 5; 10 and 50 μg/mL). The $IC_{50}$ values were calculated using GraphPad Prism version 7.0 software.

# Results

The stem of *G. parvifolia* Miq. was extracted gradually using *n*-hexane (BP12-S-H), dichloromethane (BP12-S-D), and methanol (BP12-S-M). This extraction obtained BP12-S-H (1.73 g), BP12-S-D (10.08 g), and BP12-S-M (15.35 g). The antimalarial activity showed that only BP12-S-D inhibited *P. falciparum* growth with the $IC_{50}$ value of 6.61 ± 0.09 µg/mL (Table 1). Quality control parameters, including Z′-factor, S/B, S/N, and CV (%), were also calculated with values of 0.84, 7.42, 266.40, and 22.47 indicating the high quality and performance of the screening.

Future fractionation of BP12-S-D (active extract) by open column chromatography and obtained 10 fractions (F1–F10). Screening antimalarial activity showed that seven fractions had inhibitions against *P. falciparum* 3D7 more than 50% at 10 µg/mL (Figure 1). Therefore, the $IC_{50}$ calculation is carried out on these fractions. The result showed that fraction-1 (F1) had the most active fraction with an $IC_{50}$ value of 6.00 ± 0.03 µg/mL (Table 2). This fraction was classified as active antimalarial based on Chinchilla et al. [31]. The TLC profile shows a dominant brownish-yellow spot at the white light after sprayed with $H_2SO_4$ 10% and heated at 105 °C for 5 min. These spots indicate that a compound is a xanthone group (Figure 2).

**Table 1:** Antimalarial activity ($IC_{50}$) from extracts of *G. parvifolia* stem.

| Sample | $IC_{50}$, µg/mL |
|---|---|
| BP12-S-H | NA |
| BP12-S-D | 6.61 ± 0.09 |
| BP12-S-M | NA |

NA, Not active; Data are reported as mean ± SD from three independent experiments.

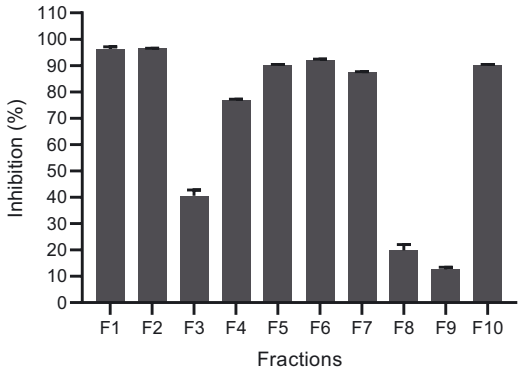


**Figure 1:** Inhibition percentages of all fractions against *P. falciparum* 3D7 at a concentration of 10 µg/mL. Data are reported as mean ± SD from three independent experiments.

**Table 2:** Antimalarial activity ($IC_{50}$) of seven fractions from a dichloromethane extract of *G. parvifolia* stem (BP12-S-D).

| Sample | $IC_{50}$, µg/mL |
|---|---|
| F1 | 6.00 ± 0.03 |
| F2 | 7.14 ± 0.03 |
| F4 | 7.51 ± 0.01 |
| F5 | 6.72 ± 0.02 |
| F6 | 7.49 ± 0.03 |
| F7 | 7.72 ± 0.03 |
| F10 | 7.58 ± 0.03 |

Data are reported as mean ± SD from three independent experiments.

HPLC profile of F1 showed that two major peaks at minute retention times 22.580 (A) and 26.659 (B) (Figure 3). Peak A in The UV–Vis spectra has maximum absorption at 250 and 278 nm (Figure 4). Meanwhile, peak B has maximum absorption 243, 281, and 317 nm (Figure 5).

# Discussion

Medicinal plants are good resources to found a new antimalarial drug candidate. In the present study, we examined the possible antimalarial activity of *G. parvifolia* Miq. extracts and fractions. *G. parvifolia*, which belongs to the family of Clusiaceae, is widely distributed in tropical and subtropical countries of South East Asia such as Malaysia, Brunei, Thailand, and Indonesia [13, 14]. This plant is known to have many pharmacological effects. Infusion is drunk as a post-partum medication by people in Riau Province, Sumatra, Indonesia [32]. This tree's fruit is edible, and the young leaves are sometimes eaten as a vegetable [14].

In this study, *G. parvifolia* stem was subjected to extraction in different polarities of solvents, and antimalarial activities of the extracts were examined against *P. falciparum* 3D7. The results revealed that BP12-S-D possessed the most potent activity with the $IC_{50}$ values of 6.61 ± 0.09 µg/mL (Table 1). We further fractionated the BP12-S-D to obtained 10 fractions (F1–F10). The antimalarial activity showed that fraction 1 (F1) is the most active fraction with the $IC_{50}$ values of 6.00 ± 0.03 µg/mL (Table 2). Based on the antimalarial criteria of Chincilla et al. (2012), BP12-S-D and F1 were classified as active as antimalarials. Basic criteria for antiparasitic drug discovery activities of extracts were classified into four classes according to their $IC_{50}$. Very active (<5 µg/mL), active (>5–50 µg/mL), weakly active (>50–100 µg/mL), and inactive (>100 µg/mL) [31].

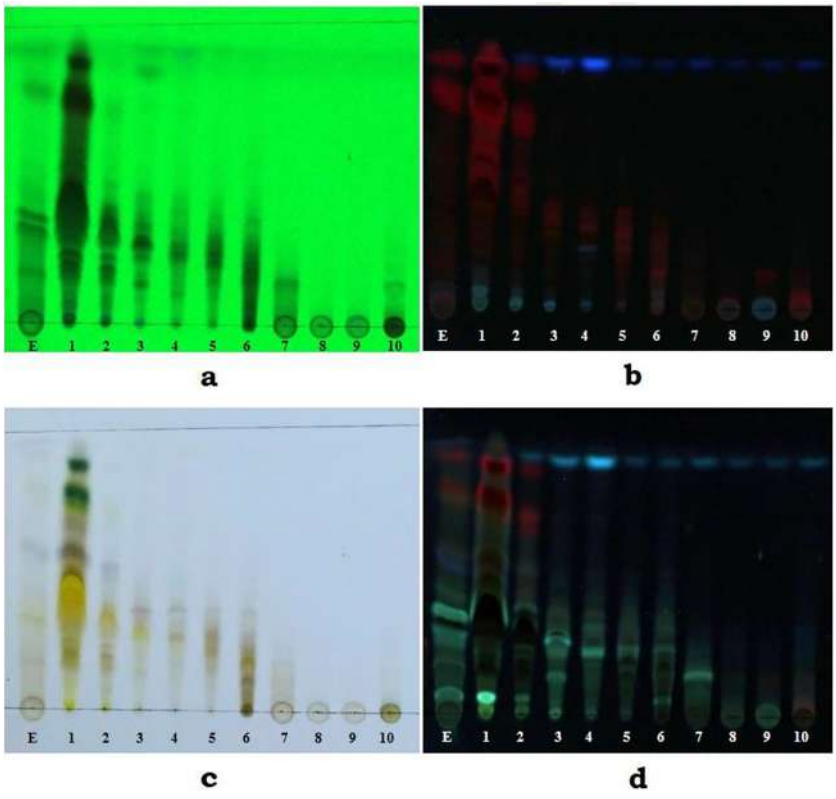


**Figure 2:** TLC profile of dichloromethane extract (BP12-S-D) and fractions (F1–F10) using silica gel as a stationary phase and chloroform: methanol (98:2 v/v) as a mobile phase. The TLC spots were observed under UV 254 nm (a), UV 366 nm (b), white light after sprayed with $H_2SO_4$ 10% and heated 105 °C for 5 min (c), UV 366 nm after sprayed with $H_2SO_4$ 10%, and heated at 105 °C for 5 min (d).

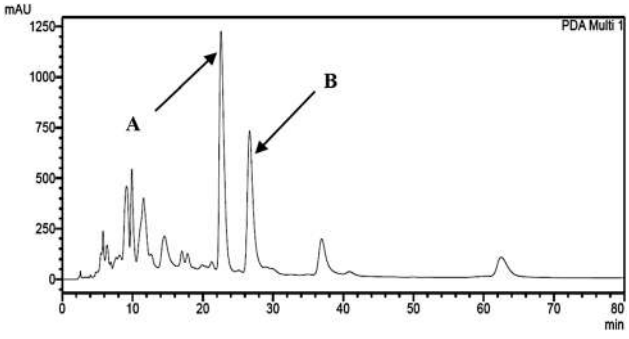


**Figure 3:** HPLC chromatogram of fraction-1 (F1) with acetonitrile: water (7:3 v/v) mixture as a mobile phase at flow rate 0.5 mL/min, injection volume 40 µL and two major peaks were observed as A and B.

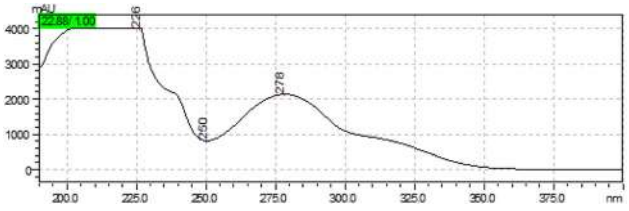


**Figure 4:** UV–Vis spectra of peak A.

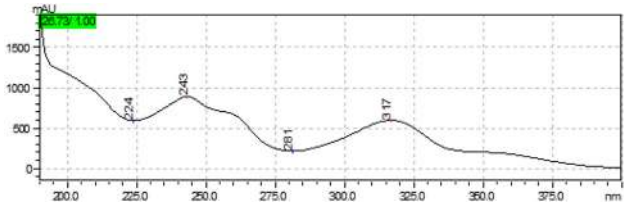


**Figure 5:** UV–Vis spectra of peak B.

HPLC profile of F1 showed that two major peaks at minute retention times 22.580 (A) and 26.659 (B), which is responsible for its antimalarial activity. Peak A in the UV–Vis spectra has maximum absorption at 226, 250, and 278 nm; it is being expected flavonoid [33]. Meanwhile, peak B has maximum absorption 243, 281, and 317 nm, and it is being expected xanthone [26]. In the future, it is necessary to carry out purification and antimalarial assay on the two peaks to determine which compounds are responsible for antimalarials.

Several studies have been isolated xanthone, flavonoids, phenolics, terpenoids, and steroids from *G. parvifolia*. Parvixanthones A-I, nine new xanthones, isolated from dried bark of *G. parvifolia* [34]. Four novel prenylated depsidones had been isolated from the chloroform soluble

fraction of the leaves of *G. parvifolia* [35]. In fact, flavonoids, terpenoids, xanthones isolated from plants had been reported to contain antimalarial properties. 2,3,4,5,6-pentahydroxyxanthone (X5) inhibits the *in vitro* growth of a chloroquine-sensitive and multidrug-resistant strain of *P. falciparum*. This compound has been shown to have antimalarial action by preventing hemozoin formation [36]. Biflavanones (GB-1) were isolated from the ethanol extract of *Garcinia kola* seed inhibit against *P. falciparum* with $IC_{50}$ values of 0.16 μM and *P. berghei* with $ED_{50}$ values of 100 mg/kg BW [37]. Five xanthones (7-o-methylgarcinone, cowanin, cowanol, cowaxanthone and β-mangostin) from the ethanol bark extract of *Garcinia cowa* were found to possess *in vitro* antimalarial activity against *P. falciparum* with $IC_{50}$ values ranging from 1.50 to 3.00 μg/mL [38]. Likhitwitayamuid et al. (1998) has successfully isolated five xanthones (named 1,7-dihydroxyxanthone; 12b-hydroxy-des-D-garcigerrin A; 1-o-methylsymphoxanthone; symphoxanthone and garciniaxanthone) from *Garcinia dulcis*. These xanthones showed an inhibitory effect on the growth of *P. falciparum* with an $IC_{50}$ value of 0.96–3.88 μg/mL [39]. We hope our study could be continued by isolating the active compound and conducting a toxicity assay. The toxicity assay performed is cytotoxicity to determine the effect in a normal cell using the MTT assay method. This assay was carried out to assess the compound's safety. It could be continued for further research, namely the isolation of active compound because the objective of this research was to obtain an active compound as an antimalarial was proven safe.

## Conclusions

This study demonstrated a potential candidate for the new antimalarial drug from dichloromethane extract and fraction of *G. parvifolia* Miq. stem. The fraction-1 (F1) of dichloromethane extract of *G. parvifolia* Miq. stem had the strongest antimalarial activity in LDH assay. F1 showed active antimalarial activity with the $IC_{50}$ value of 6.00 ± 0.03 μg/mL against *P. falciparum* 3D7. The active compounds contained in dichloromethane extract and fraction of *G. parvifolia* Miq. stem are thought to be flavonoids and xanthones.

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**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Informed consent was obtained from all individuals included in this study.

**Ethical approval:** The local Institutional Review Board deemed the study exempt from review.

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Muhammad Sulaiman Zubair*, Siti Qamariyah Khairunisa, Evi Sulastri, Ihwan, Agustinus Widodo, Nasronudin and Ramadanil Pitopang

# Antioxidant and antiviral potency of *Begonia medicinalis* fractions

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**Abstract**

**Objectives:** This study aims to evaluate the antioxidant and antiviral potency of *n*-hexane, ethyl acetate and, water fractions of *Begonia medicinalis* Ardi & D.C.Thomas as well as to identify the chemical constituents.
**Methods:** Assays for antioxidant and antiviral activity (HIV-1) were carried out on MT-4 cells infected with HIV using the DPPH method and the determination of the cytopathic effect. Meanwhile, GC-MS was used to identify the chemical compounds.
**Results:** The determination of antioxidants showed that all fractions possessed potent activity with the $IC_{50}$ ranging from 2.61 to 8.26 μg/mL. From the antiviral activity of MT-4 cells infected by HIV, the *n*-hexane fraction of *B. medicinalis* showed the most potency with the $IC_{50}$ of 0.04 ± 0.05 μg/mL. It has less cytotoxicity (11.08 ± 4.60 μg/mL) affording the high selectivity index of 238.80. Furthermore, GC-MS analysis of n-hexane fraction found the major compound of carboxylic acid derivate with the area percentage of 76.4% and the presence of phenolic compounds (8.38%). Meanwhile, in water fraction, terpenoids were found in a higher concentration (10.05%) than others.
**Conclusions:** Therefore, this study supports the application of *B. medicinalis* as a herbal medicine for antioxidant and antiviral.

**Keywords:** antioxidant; antiviral; *Begonia medicinalis*; GC-MS; HIV-1.

***Corresponding author: Muhammad Sulaiman Zubair**, Department of Pharmacy, Tadulako University, Palu, Indonesia, Phone: +6285242083654, E-mail: sulaiman_zubair80@yahoo.co.id
**Siti Qamariyah Khairunisa,** Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia
**Evi Sulastri, Ihwan and Agustinus Widodo,** Department of Pharmacy, Tadulako University, Palu, Indonesia
**Nasronudin,** Faculty of Medicine, Airlangga University, Surabaya, Indonesia
**Ramadanil Pitopang,** Department of Biology, Tadulako University, Palu, Indonesia

## Introduction

HIV/AIDS became a global pandemic disease that has suffered human life for a long time. In 2019, there were 75.7 million cases of people living with HIV and about 32.7 million died of AIDS. With all the physical rules, religious advice, and medical treatment, life goes on even though HIV/AIDS is everywhere. The United Nations responded by creating a joint program called UNAIDS to handle this crisis around the world [1, 2]. Furthermore, some antiretroviral (ART) drugs have been clinically approved and used for treatment. They were classified into some classes such as nucleoside reverse transcriptase (NRTI's) and protease inhibitors. Each drug proven has led to several future challenges of addressing common issues such as resistance and unwanted side effects. Therefore, it is necessary to search for a new chemical entity to fight against HIV replication and the treatment of AIDS patients.

Antioxidants are substances that can terminate the reaction of free radicals due to the ability of the body's defense system against chronic diseases such as cancer and viral infections. These diseases are always accompanied by the cell/tissue metabolism disorder, which generates the activation of reactive oxygen species (ROS). A study reported that the increased level of ROS in bronchial epithelial cells caused high mortality from pneumonia due to the influenza virus [3]. Therefore, the combinatorial action of the antioxidant and the antivirus is important to prevent the progression of virus replication by ROS. Natural sources have become a place to discover such compounds since they contain secondary metabolites with unique structural conformation and diverse chemical skeleton. Furthermore, several plants were reported to possess the combinatorial effect such as *Euphorbia thymifolia* L. and *Origanum acutide* [4, 5]. The compounds responsible for the activity are mainly flavonoids and phenolic compounds.

As the second-largest bio-megadiversity country in the world, Indonesia has various natural resources that provide tropical plants in the forest to be explored for discovering a new drug. One endemic plant used to treat some diseases such as cancers, fever, cough, and tuberculosis is *Begonia medicinalis* Ardi & D.C.Thomas (local name: *Benalu batu*, family Begoniaceae) (Figure 1). It is a new species of Begonia

from Morowali, Central Sulawesi, Indonesia [6]. Previous studies showed (previously written as *Begonia* sp) the anticancer activity of its methanolic extract against breast and cervical cancer cell lines (T47D and HeLa cells) [7]. A triterpenoid glycoside, 2-*O*-β-glucopyranosyl-cucurbitacin D, and two steroid glycosides i.e. 20,25-dihydroxy-β-sitosterol-3-*O*-β-glucopyranoside and β-sitosterol-3-*O*-β-D-glucopyranoside were reported as main compounds on ethyl acetate fractions. These three compounds possessed anticancer activity by *in vitro* and silico methods [8–10].

Furthermore, antioxidant and antiviral activities of the fractions have been performed by using DPPH and cytopathic effect (CPE) methods. Gas chromatography-mass spectroscopy (GC-MS) analysis was also performed to identify the chemical constituents. This study is the first investigation for antioxidant, antiviral activity, and GC-MS analysis of the plant fractions.

# Materials and methods

## Chemicals

Ethanol (Merck), 0.2 µm nitrocellulose membrane filter (Whatman), Fetal Bovine Serum (Gibco), RPMI-1640 medium (Gibco), Sodium bicarbonate (Merck), Viral-ToxGlo was from Promega (Madison, WI, USA), Dimethyl sulfoxide (Sigma), Sterile water for injection, and aquadest. The human T-cell leukemia cells (MT-4 cells) were from the Institute of Tropical Disease (ITD) laboratory, Airlangga University, Surabaya, Indonesia. MT-4 cells were cultured in RPMI-1640 media, equipped with 10% FBS, and maintained in T25 CCF at 37 °C temperature in a 5% $CO_2$ incubator. HIV was cultured on the MT-4 cells in RPMI-1640 medium completed with FBS 10%. MT-4/HIV cells were kept in CCF T25 at 37 °C in 5% $CO_2$ incubator. HIV isolates were from a seropositive HIV donor labeled by IDU-18 obtained from the ITD laboratory.

## Plant collection, identification, and extraction

*B. medicinalis* was collected from Wawopada village, North Morowali, and identified by Mr. Ardi Wisnu (Botanist) in Bogor Botanical Gardens, Indonesian Science Institute. All part of *B. medicinalis* (not including the root) was washed in running tap water, then cut into 3 cm pieces and dried in temperature room without sunshine. After drying, *B. medicinalis* herb sample was extracted by maceration method using methanol. The extracts are then filtered, evaporated in a rotary evaporator to obtain the viscous extract. After that, liquid-liquid partition was applied using consecutive solvent from nonpolar to polar solvents i.e. *n*-hexane, ethyl acetate, and water. Each obtained filtrate was rotary evaporated until achieved the viscous *n*-hexane, ethyl acetate, and water fractions.

## Antioxidant activity

The radical scavenging activity of *B. medicinalis n*-hexane, ethyl acetate, and water fractions against the DPPH radical was determined as our previous study [11]. About 10 mg extract was dissolved on 10 mL ethanol p.a (1,000 µg/mL) and then diluted to reach the concentration series of 7.8125, 15.625, 31.25, 62.5, and 125 µg/mL. About 0.1 mM DPPH was added to each sample solution with a series of concentrations of 7.8125, 15.625, 31.25, 62.5, and 125 µg/mL) (1:1). After 30 min incubation, the absorbance of each mixtures was measured at 515 nm on spectrophotometry UV–Vis. The blank solution (ethanol and DPPH) and ascorbic acid with the series concentration of 1.95, 3.90, 7.8125, 15.625, and 31.25 µg/mL as positive control were also prepared and measured at the same wavelength. The experiment was carried out in triplicates. The percentage of inhibition was calculated and further analyzed to determine the $IC_{50}$

## In vitro antiviral activity test

The *in vitro* antiviral activity was performed by measuring the cytolysis and cytotoxicity effect. These two inhibitory tests were performed by a colorimetric method using Viral-ToxGlo method [12, 13]. MT-4 cells ($2 \times 10^5$ cells/well), after infected with HIV, were placed in a 96-well microplate. The various concentrations of *B. medicinalis n*-hexane, ethyl acetate, and water fractions (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL) were then added to the well. The wells were incubated for 6 days at 37 °C temperatures in a 5% $CO_2$ incubator. About 10 µL Viral-ToxGlo was added to each well and was incubated for 60 min at a 37 °C temperature in a 5% $CO_2$ incubator. Duviral as positive control was also prepared with the series concentration of 15.625, 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL. The luminescence was measured using a multimode plate reader (GloMax Explorer

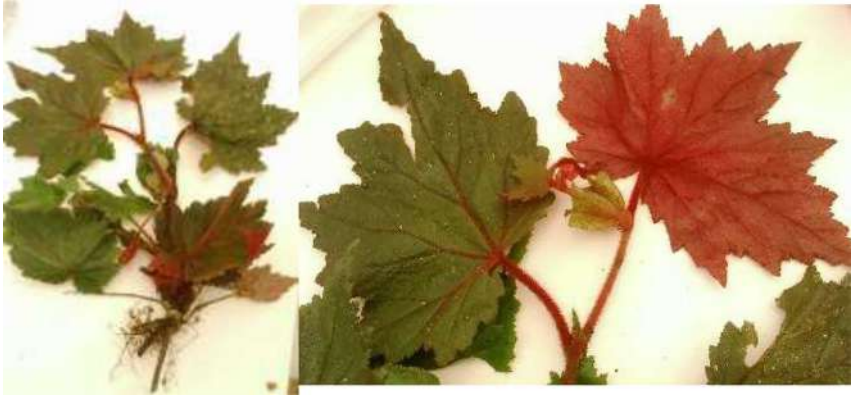

**Figure 1:** *Begonia medicinalis* herb plant.

Promega). The cytotoxicity test was performed in the same procedures without infection to the MT-4 cells. Duviral (Zidovudin and lamivudin) was used as the positive control. The experiment was carried out in duplicates.

### GC-MS analysis

The GC-MS analysis of sample fractions was carried out on a Shimadzu QP-2010 Gas Chromatograph Mass Spectrometer (GC-MS) Ultra, which is equipped with Autosampler AOC-20i and SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm × 0.25 µm) using Helium as carrier gas (1.0 mL/min). The column temperature program was set as follows: an injection temperature of 250 °C; splitless mode; a column oven temperature of 70 °C at the beginning and held for 2 min, then ramped to 200 °C at the rate of 10 °C/min and end temperature 280 °C and held for 9 min at the rate 5 °C/min; an MS ion source temperature of 200 °C, and an interface temperature of 280 °C. The compounds of each chromatogram peaks were identified by comparing the base peak and experimental molecular mass spectra with the database library in the NIST and Wiley.

### Statistical analysis

The $IC_{50}$ for antioxidant activity was calculated by probit analysis plotting the percentage of inhibition and concentrations. Meanwhile, data for 50% virus inhibitory concentration ($IC_{50}$) and 50% cytotoxicity concentration ($CC_{50}$) were analyzed by nonlinear regression analysis using GraphPad Prism® version 8.0.1 software. $IC_{50}$ is the concentration that inhibits 50% of the viral cytopathic effect. $CC_{50}$ is the concentration that generates 50% cytotoxicity on the MT-4 cells.

# Results

## Antioxidant activity

The *n*-hexane, ethyl acetate, and water fractions of *B. medicinalis* were tested for antioxidant activity by using the DPPH method. At a concentration of 7.8125 µg/mL, the *n*-hexane fraction showed a high percentage inhibition of DPPH radical scavenging of 65.98%, followed by the aqueous fraction and the ethyl acetate fraction (Figure 2). Therefore, all fractions have potent DPPH radical scavenging activity attributed to the lower $IC_{50}$ value (<10 µg/mL) in which the *n*-hexane fraction was found to have the lowest value (2.61 ± 0.64 µg/mL). This $IC_{50}$ value is comparable with the positive control of ascorbic acid (1.72 ± 0.26 µg/mL) (Table 1).

## Antiviral (HIV-1) activity

The *n*-hexane, ethyl acetate, and water fractions of *B. medicinalis* were tested on HIV-1 infected MT-4 cells. *N*-hexane fraction of *B. medicinalis* exhibited the strongest potency on inhibiting the cells with the lower $IC_{50}$ of 0.04 ± 0.05 µg/mL than ethyl acetate and water fractions ($IC_{50}$ of 19.05 ± 5.54 and 10.75 ± 15.19 µg/mL, respectively). In addition, all *B. medicinalis* fractions also showed less cytotoxicity and gave high selectivity index (SI) of all fractions in which SI *n*-hexane>SI ethyl acetate>SI water (Table 2).

## GC-MS analysis

Identification by GC-MS was performed to identify secondary metabolites on each fraction based on the NIST and Wiley database libraries for reference. The list of metabolites was noted and tabulated in Table 3, and 10, 15, and 25 compounds in *n*-hexane, ethyl acetate, and water fractions were detected respectively. Hydrocarbons were also found as major compounds in ethyl acetate and water fractions. Meanwhile, the n-hexane fraction contained more carboxylic acid derivate compound i.e. 2-Pentyl 6-(4-pentylphenyl) 2,6-naphthalenedicarboxylate with a total peak area percentage of 76.4%.

The diverse class of natural products of *B. medicinalis* fractions can be seen in Table 4, and in the n-hexane fraction, phenolic compounds were observed in a percentage of 8.38%. Alkaloids were found only in ethyl acetate fraction with a percentage of 4.52% and water fraction contained the highest percentage of terpenoids (10.05%) compared to others.

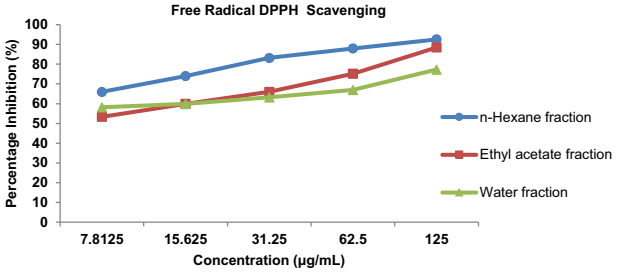


**Figure 2:** Antioxidant activity of *Begonia medicinalis* fractions.

**Table 1:** DPPH radical scavenging activity ($IC_{50}$) of *B. medicinalis* fractions.

| Sample | $IC_{50}$, µg/mL |
|---|---|
| Water fraction | 3.75 ± 0.07 |
| Ethyl acetate fraction | 8.26 ± 4.31 |
| *n*-hexane fraction | 2.61 ± 0.64 |
| Ascorbic acid | 1.72 ± 0.26 |

**Table 2:** $IC_{50}$, $CC_{50}$, and selectivity index (SI) of *B. medicinalis* fractions.

| *B. medicinalis* | $IC_{50}$, µg/mL | $CC_{50}$, µg/mL | SI |
|---|---|---|---|
| Ethyl acetate fraction | 19.05 ± 5.54 | 193.65 ± 232.43 | 10.16 |
| Water fraction | 10.75 ± 15.19 | 7.36 ± 0.83 | 0.68 |
| *n*-hexane fraction | 0.04 ± 0.05 | 11.08 ± 4.60 | 238.80 |
| Duviral[a] | 157.11 ± 174.63 | $1.28 \times 10^{20} \pm 1.81 \times 10^{20}$ | $8.14 \times 10^{17}$ |

[a]Duviral (Zidovudin and Lamivudin) as positive control.

**Table 3:** Gas chromatography-Mass spectroscopy (GC-MS) data of phyto compounds putatively identified in *n*-hexane, ethyl acetate, and water fractions of *Begonia medicinalis*.

| No | Compounds | Peak area, % | | | Retention time, min |
|---|---|---|---|---|---|
| | | n-hexane fraction | Ethyl acetate fraction | Water fraction | |
| 1 | Dodecane, 4,6-dimethyl- | – | 1.47 | 1.86 | 5.271 |
| 2 | Benzene, 1,3,5-trimethyl- | – | 2.29 | 2.58 | 5.359 |
| 3 | Cyclohexane, butyl- | – | 0.82 | 1.06 | 5.521 |
| 4 | Decane, 5-methyl- | – | 2.41 | – | 5.846 |
| 5 | Benzene, (2,3-dimethyldecyl)- | – | 5.15 | 5.53 | 5.919 |
| 6 | 3,6-Dimethyldecane | – | 6.13 | – | 5.985 |
| 7 | Naphthalene, decahydro- | – | 4.92 | – | 6.017 |
| 8 | Nonane, 5-(1-methylpropyl)- | – | 9.49 | – | 6.087 |
| 9 | Cyclohexane, 1-bromo-2-methyl- | – | 1.24 | – | 6.394 |
| 10 | 2,4,4,6-Tetramethyl-6-phenyl-2-heptene | – | 2.22 | – | 6.475 |
| 11 | Dodecane, 2,5-dimethyl- | – | 11.32 | – | 6.587 |
| 12 | Tridecane, 6-methyl- | 1.09 | 25.89 | – | 6.639 |
| 13 | Benzene, methyl(1-methylethyl)- | – | 3.89 | – | 7.009 |
| 14 | N-amylcyclohexane | – | 0.78 | – | 7.24 |
| 15 | Cyclohexane, (1-methylpropyl)- | – | 1.34 | – | 7.292 |
| 16 | Benzenebutanoic acid, 2,5-dimethyl-, methyl ester | – | 1.7 | – | 7.425 |
| 17 | 1,3,7-Trimethyl-8-({1-[2-(4-morpholinyl)-2-oxoethyl]-1h-benzimidazol-2-yl}sulf | – | 0.92 | – | 28.942 |
| 18 | 1h-indole-2,3-dione, 1-(tert-butyldimethylsilyl)-5-chloro-, 3-(o-ethyloxime) | – | 1.11 | – | 34.55 |
| 19 | Silane, 1,3-decadiynyltrimethyl- | – | 1.39 | – | 34.75 |
| 20 | 3,3,7,11-tetramethyltricyclo[5.4.0.0(4,11)]undecan-1-ol | – | 0.95 | – | 34.893 |
| 21 | Methyl 9,10-dideutero-9-octadecenoate | – | 5.53 | – | 35.008 |
| 22 | Z,e-2-methyl-3,13-octadecadien-1-ol | – | 4.91 | – | 35.24 |
| 23 | 2-[4-cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone | – | 2.49 | – | 35.41 |
| 24 | Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl- | – | 0.78 | – | 35.517 |
| 25 | 2,4-Cyclohexadien-1-one, 3,5-bis(1,1-dimethylethyl)-4-hydroxy- | – | 0.86 | – | 39.365 |
| 26 | Spiro[3.5]nona-5,7-dien-1-one, 5,9,9-trimethyl- | 0.19 | – | – | 5.916 |
| 27 | Heptane, 5-ethyl-2-methyl- | 0.78 | – | – | 5.989 |
| 28 | Decane, 2,6,8-trimethyl- | 0.48 | – | – | 6.584 |
| 29 | 2-Pentyl 6-(4-pentylphenyl) 2,6-naphthalenedicarboxylate | 1.08 | – | 1.66 | 33.142 |
| 30 | Physcion-10,10′-bianthrone | 8.38 | – | – | 33.567 |
| 31 | 2-Pentyl 6-(4-pentylphenyl) 2,6-naphthalenedicarboxylate | 13.11 | – | – | 33.817 |
| 32 | 2-dimethyl(pentafluorophenyl)silyloxypentadecane | 12.69 | – | – | 33.975 |
| 33 | 2-Pentyl 6-(4-pentylphenyl) 2,6-naphthalenedicarboxylate | 18.34 | – | – | 34.158 |
| 34 | 2-Pentyl 6-(4-pentylphenyl) 2,6-naphthalenedicarboxylate | 43.87 | – | – | 34.392 |
| 35 | Nonane, 2,5-dimethyl- | 2.64 | – | – | 5.841 |
| 36 | Tetradecane, 1-chloro- | 12.26 | – | – | 5.996 |
| 37 | 6-Octen-1-ol, 3,7-dimethyl- | 10.05 | – | – | 6.086 |

**Table 3:** (continued)

| No | Compounds | Peak area, % | | | Retention time, min |
|---|---|---|---|---|---|
| | | n-hexane fraction | Ethyl acetate fraction | Water fraction | |
| 38 | Cyclopropane, 3-chloro-1,1,2,2-tetramethyl- | 1.51 | – | – | 6.391 |
| 39 | 6-Bromo-2,2-dimethylcyclohexanone | 2.73 | – | – | 6.483 |
| 40 | Undecane | 14.12 | – | – | 6.585 |
| 41 | Undecane, 5-ethyl- | 34.77 | – | – | 6.636 |
| 42 | Benzene, 1,2,3,4-tetramethyl- | 3.55 | – | – | 7.005 |
| 43 | Cyclohexane, pentyl- | 4.19 | – | – | 7.285 |
| 44 | Silicic acid, diethyl bis(trimethylsilyl) ester | 1.5 | – | – | 31.933 |

**Table 4:** The diversity of natural product groups in *B.medicinalis* fractions.

| Compounds class | *n*-hexane fraction, % | Ethyl acetate fraction, % | Water fraction, % |
|---|---|---|---|
| Hydrocarbon | 15.04 | 69.42 | 72.41 |
| Oxygen containing compound | – | 1.64 | 4.23 |
| Alkaloids | – | 4.52 | – |
| Fatty acids | – | 12.14 | – |
| Aromatics | – | 11.33 | 11.66 |
| Terpenoids | 0.19 | 0.95 | 10.05 |
| Carboxylic acids | 76.4 | – | 1.66 |
| Phenolics | 8.38 | – | – |

# Discussion

In this study, antioxidant activity was measured by DPPH radical scavenging activity test. This test was widely applied in evaluating the antioxidants level of plants [14]. Meanwhile, the inhibition of the virus-induced cytopathic effect in MT-4 cells was estimated by using Viral-ToxGlo method. This method was reported as high throughput antiviral screening tool that determines the cellular ATP as a surrogate measure of host cell viability [15]. N-hexane fraction of *B. medicinalis* was reported to have lower $IC_{50}$ on DPPH radical scavenging activity test and cytopathic reduction assay on MT-4 cells infected by HIV. The activity can be categorized as very potent as the $IC_{50}$ was $2.61 \pm 0.64$ μg/mL for antioxidants and $0.04 \pm 0.05$ μg/mL for anti-HIV-1 activity. Moreover, the cytotoxicity was also less toxic ($CC_{50}$ $11.08 \pm 4.60$ μg/mL) affording the highest selectivity index (238.80) comparing to other fractions.

Further identification by GC-MS was conducted to identify the secondary metabolites on each fraction. Interestingly, since phenolic compounds have been reported to possess the antioxidant activity, they are only found in the n-hexane fraction with a percentage of 8.38%. A phenolic compound, Physcion-10,10′-bianthrone, was detected in the retention time of 33.567. This compound was only reported from *Rumex japonicus* H and there was no report for biological activity [16].

Carboxylic acid compound namely 2-pentyl 6-(4-pentylphenyl) 2,6-naphthalene dicarboxylate was found as a major compound in n-hexane fraction with the percentage of 76.4%. The abundance of this compound on n-hexane fraction is suggested to be the main bioactive compound responsible for the antioxidant and antiviral activity. However, further isolation and structure elucidation are needed to confirm the chemical structure.

Compounds of the hydrocarbon class were detected as major compound in the ethyl acetate and water fractions with a percentage of 69.42% and 72.41%, respectively. Terpenoids were found in the highest concentration on water fraction, compared to others. Meanwhile, alkaloids

were only found in ethyl acetate fractions with a percentage of 4.52%. 3,7-dimethyl-6-octen-1-ol, 5,9,9-trimethyl-spiro [3.5]nona-5,7-dien-1-one, and 3,3,7,11-tetra methyl tricyclo[5.4.0.0(4,11)] undecan-1-ol were three terpenoid compounds detected in water fraction, ethyl acetate fraction and n-hexane fraction with the percentage of 10.05, 0.19 and 0.95%, respectively. 1,3,7-trimethyl-8-({1-[2-(4-morpholinyl)-2-oxoethyl]-1h-benzimidazol-2-yl} sulfanyl)-3,7-dihydro-1H-purine-2,6-], 3-(o-ethyloxime), 1-(tert-butyl dimethylsilyl)-5-chloro- 1h-indole-2,3-dione, and 2-[4-cyclohexylbutanoylamino]-3-chloro-1,4-naphthoquinone were alkaloid detected in low concentration on ethyl acetate fraction. The presence of these compounds analyzed by GC-MS might be responsible for antioxidant and anti-HIV-1 activity. Furthermore, Khumaidi et al. (2020) reported that *n*-hexane fractions contained a higher level of total flavonoid compounds (30.226 mg QE/g) than ethyl acetate and water [17]. The flavonoids compounds were attributed to the increasing activity of cell lymphocyte proliferation, which produced an immunomodulatory body's system. Further research on the isolation and purification of the compounds on fractions is in progress.

## Conclusions

It is reasonable to conclude that *B. medicinalis* fractions have potency as antioxidant and antiviral agents. Also, *n*-hexane fraction was found to have the lowest $IC_{50}$ on both DPPH radical scavenging activity and cytopathic reduction assay on MT-4 cells infected by HIV-1 with the $IC_{50}$ of 2.61 ± 0.64 and 0.04 ± 0.05 μg/mL, respectively. GC-MS analysis identified carboxylic acid derivates, phenolics, terpenoids, and alkaloids, which may be responsible for the antioxidant and antiviral activities.

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**Competing interests:** Authors state no conflict of interest.

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**Ethical approval:** Not applicable.

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Lidya Tumewu, Lutfah Qurrota A'yun, Hilkatul Ilmi, Achmad Fuad Hafid and Aty Widyawaruyanti*

# *Artocarpus sericicarpus* stem bark contains antimalarial substances against *Plasmodium falciparum*

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**Abstract**

**Objectives:** The finding of alternative medicine for malarial treatment still has become a substantial demand. The plant is one of the potential sources of drugs, among other natural sources. Artocarpus species showed great potential as the antimalarial source. This study aims to obtain active antimalarial fractions from *Artocarpus sericicarpus* stem bark.

**Methods:** Stem bark of *A. sericicarpus* was extracted by ultrasonic-assisted extraction method using n-hexane, dichloromethane, and methanol as solvents. Fractionation of dichloromethane extract was conducted by open column chromatography using octadecyl silica as a stationary phase and gradient acetonitrile-water as a mobile phase. The antimalarial activity was determined by lactate dehydrogenase (LDH) assay against *Plasmodium falciparum* 3D7 strain.

**Results:** *A. sericicarpus* n-hexane, dichloromethane, and methanol extracts were showed antimalarial activity with an $IC_{50}$ value of >4, 2.11, and >4 μg/mL, respectively. Fractionation of dichloromethane extract was obtained 13 fractions. Seven of the 13 fractions tested showed antimalarial activity. Fraction-6 performed the highest inhibition with an $IC_{50}$ value of 1.53 ± 0.04 μg/mL. Phytochemistry screening revealed that Fraction-6 contains flavonoid, polyphenol, and terpenoid compounds that can take a role in its antimalarial activity.

**Conclusions:** *A. sericicarpus* contains antimalarial substances mainly in Fraction-6, which strongly inhibited the growth of *P. falciparum*. The flavonoid, polyphenol, and terpenoid compounds were identified in Fraction-6, which need to be further isolated to obtain and elucidate the active antimalarial compounds.

**Keywords:** active fraction; antimalarial; *Artocarpus sericicarpus*; lactate dehydrogenase assay.

## Introduction

According to the World Malaria Report 2019, there were 228 million malaria cases globally and 405,000 malaria deaths in 2018 [1]. Although the incidence rate of malaria deaths has declined over the past few years, the progress of malarial eradication is beginning to slow. Antimalarial drug resistance emphasizes the need for continued research in antimalarial drug development [2].

Natural products were contributed to the development of many drugs for varied indications. Mainly plants have played a leading medical role in most cultures [3]. In malaria, numerous plants have been reported as a source of antimalarial based on their phytochemical content, such as alkaloids, flavonoids, terpenoids, and other metabolites. A large number of antimalarial compounds had isolated and identified from plants [4, 5]. Herbal medicine has become the primary source of antimalarial drugs since quinine discovery from *Cinchona* spp bark and artemisinin from *Artemisia annua* L. leaves and stems [6, 7]. Those plants were traditionally used medication for thousands of years. The traditional medicines were significantly provided potential candidates who possibly become sources of bioactive compounds [8].

***Corresponding author: Aty Widyawaruyanti**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 31 5933150, E-mail: aty-w@ff.unair.ac.id
**Lidya Tumewu and Achmad Fuad Hafid,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
**Lutfah Qurrota A'yun,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
**Hilkatul Ilmi,** Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia

*Artocarpus champeden* is one of the plants used as a traditional medicine in the indigenous culture at Papua. The stem bark was used to treats fever and malaria. A previous study reported several antimalarial compounds isolated from *A. champeden* stem bark extract [9–11]. Two new compounds, along with seven known compounds, were isolated and identified as an active antimalarial with a new permeation pathway mechanism of action [9]. The *A. champeden* stem bark extract was formulated as a capsule dosage form and tested on *Plasmodium berghei* infected mice. The results suggested that formulated *A. champeden* stem bark extract was decreased parasitemia level, extended survival time of infected mice, enhanced the production of IFN-ɣ and TNF-α, and no effect on liver function. It may provide a new therapeutical strategy for malaria treatment [12]. Another species of Artocarpus which potentially antimalarial was *Artocarpus altilis*. The ethanol extract of *A. altilis* leaves was exhibited antimalarial activity against *Plasmodium falciparum* and *P. berghei* [13]. The antimalarial compound from the dihydrochalcone class was also isolated from *A. altilis*, and the compound was possessed a cysteine protease inhibitor of falcipain-2 through in silico study [14].

Drug discovery which was derived from plants was often followed the chemotaxonomic path as a potentially useful strategy. It relies on the fact that taxonomically related plants often biosynthesize chemically similar secondary metabolites [8]. Due to the chemotaxonomy approach, another Artocarpus species likely exhibit similar bioactivity.

Although many reports on antimalarial activity of Artocarpus species, little attention has been directed to *Artocarpus sericicarpus*. A preliminary study revealed that *A. sericicarpus* stem bark extract showed potency as an antimalarial source. The n-hexane, dichloromethane, and methanol extracts of *A. sericicarpus* stem bark were inhibited 99–100% of *P. falciparum* growth at a concentration of 100 µg/mL [15]. Therefore, this study aims to explore the antimalarial activity of *A. sericicarpus* stem bark extracts.

# Materials and methods

### Plant material

*A. sericicarpus* stem bark was obtained from Balikpapan Botanical Garden in East Kalimantan, Indonesia. The plant was identified at Purwodadi Botanical Garden, East Java, with determination No.0074 IPH.06/HM/XII/2015.

### Extraction and fractionation methods

*A. sericicarpus* stem bark was dried and ground into powder form. The powder was extracted using n-hexane as a solvent by ultrasonic-assisted extraction. The extract was filtered, and filtrate then evaporated using a rotary evaporator to obtain n-hexane extract. The residue was further extracted using dichloromethane as a solvent to obtain dichloromethane extract. Then the residue again extracted using methanol as a solvent to obtain methanol extract. All extracts were tested against *P. falciparum* 3D7 strain. The most active extract was fractionated by open column chromatography using octadecyl silica as a stationary phase and gradient acetonitrile-water as a mobile phase. The fractions were further tested for their antimalarial activity to determine the active fraction.

### *Plasmodium falciparum* culture

Frozen stock of *P. falciparum* strain 3D7 was obtained from the Eijkman Institute for Molecular Biology, Jakarta. The parasite was cultured in 2% hematocrit of human red blood cells (RBCs) type O in RPMI-1640 medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA). The medium was supplemented with 25 mM HEPES buffer, 2 g/L sodium hydrogen bicarbonate, 50 µg/mL hypoxanthine, 50 µg/mL gentamicin sulfate, and 0.5% (w/v) Albumax II (Gibco, ThermoFisher Scientific, Waltham, MA., USA). Plasmodium cultures were placed in 5% $O_2$, 90% $N_2$, and 5% $CO_2$ mixed gas incubator at 37 °C. Human RBCs were obtained from the Indonesian Red Cross. Culture medium was changed daily, and parasitemia was maintained below 5% for routine subcultures [16]. Parasitemia was determined by examining a thin blood smear of infected erythrocytes using 5% of Giemsa's staining. Infected erythrocytes per 1,000 total erythrocytes were counted under a light microscope to determine the percentage of parasitemia.

### Antimalarial LDH assay

Determination of the antimalarial activity of extracts and fractions was carried out by the lactate dehydrogenase (LDH) assay against *P. falciparum* strain 3D7. Parasite culture synchronization using 5% (w/v) D-sorbitol was done as previously described [17]. Ring-stage parasites at 0.3% parasitemia (100 µL/well) were placed in a 96-well plate. The 50% growth inhibition concentration of extracts and fractions was determined by adding 0.4 µL serial dilution of each sample into the well. A well-containing culture media, infected RBCs, RBCs alone, and chloroquine (10 µM) was used as a control. Determination of parasite growth was carried out after 72 h of incubation by diaphorase-coupled lactate dehydrogenase (LDH) assay [18]. The absorbance of each well was measured at 650 nm using a Multiskan Skyhigh microplate spectrophotometer (Thermo Fisher Scientific, USA).

### Statistical analysis

The inhibition rate was calculated with the absorbance of uninfected wells defined as 100% inhibition. The $IC_{50}$ values were analyzed and calculated with GraphPad PRISM 7.0 by applying the "log (inhibitor) vs. response – Variable slope (four parameters)" in the "Dose-response-inhibition" equation family.

## Results

The preliminary study of antimalarial activity screening of *A. sericicarpus* stem bark extracts was solely provided data on inhibition, and determination of effectivity was not yet conducted [15]. Therefore, this study was further determined the $IC_{50}$ of those extracts. The antimalarial activity assay of n-hexane, dichloromethane, and methanol extracts revealed dichloromethane extract as the most active extract among others with an $IC_{50}$ value of 2.11 µg/mL. Meanwhile, n-hexane and methanol extract had an $IC_{50}$ value of more than 4 µg/mL.

The bioassay-guided separation was suggested as the most active extract as the most potential candidate for further separation. The separation by open column chromatography method was taken further for dichloromethane extract, and 13 fractions (F1–F13) were obtained. The fractions were screened for their antimalarial activity by LDH assay at a concentration of 4 µg/mL. The screening result showed seven fractions had inhibition at a range of 62–87%, namely F3–F8 and F12 (Figure 1). The $IC_{50}$ determination of active fractions was conducted and showed the $IC_{50}$ value of active fractions at a range of 1.53–3.65 µg/mL. Meanwhile, F6 showed the highest activity with an $IC_{50}$ value of 1.53 ± 0.04 µg/mL and followed by F7 with an $IC_{50}$ value of 1.58 ± 0.04 µg/mL (Figure 2).

The antimalarial assay was conducted by LDH assay, which determined the level of Plasmodial lactate dehydrogenase (pLDH) [19]. pLDH was become a potential antimalarial drug target assay due to highly expressed on Plasmodium erythrocytic stage and can be distinguished from human LDH [20]. pLDH is a terminal enzyme in the glycolysis of malaria parasite and distinguishable from human LDH activity by using 3-acetylpiridine adenine dinucleotide (APAD), instead of NAD by human LDH [21].

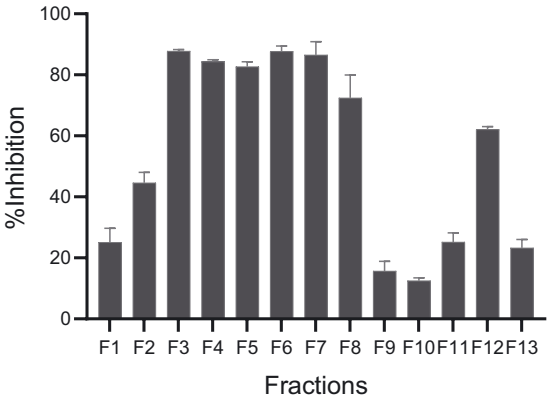


**Figure 1:** Percentages of parasites growth inhibition at a concentration of 4 µg/mL.

The phytochemical observation of fractions by Thin Layer Chromatography (TLC) was conducted to estimate the profile of the active substance. The result indicated that fractions mostly contain polyphenols and flavonoids (Figure 3). Since the F6 was revealed as the most active fraction, the phytochemical screening was conducted by TLC using spray reagent anisaldehyde-sulfate, citric boric, $FeCl_3$, and KOH for detection of terpenoids, flavonoids, polyphenols, and anthraquinones, respectively. The results showed violet spot, yellow spot, and dark gray spot, indicating terpenoids, flavonoids, and polyphenols [22]. Meanwhile, no anthraquinone was detected in F6.

## Discussion

*A. sericicarpus* is indigenous plant to Indonesia (Kalimantan, Sulawesi, and Maluku), Malaysia (Sarawak), and Philippines. It has the local name peluntan or pedalai [23]. The fruit is eaten fresh, and seeds are edible as well. In Kalimantan, *A. sericicarpus* grows wild in the forest, and there are also in the yards of inland residents [24]. This study revealed the potency of *A. sericicarpus* stem bark as a source of antimalarial bioactivity. The choice of *A. sericicarpus* was based on the chemotaxonomic approach since several Artocarpus species were reported for their antimalarial activity. Dichloromethane extract of *A. sericicarpus* was exhibited antimalarial activity with an $IC_{50}$ value of 2.11 µg/mL. Meanwhile, dichloromethane extract of *A. champeden* and ethanol extract of *A. altilis* were performed $IC_{50}$ value of 0.99 and 1.32 µg/mL, respectively [9, 13]. Although *A. sericicarpus* had lower activity than *A. champeden* and *A. altilis*, it's still classified as a strong active antimalarial substance based on previously reported criteria. Plants extract with $IC_{50}$ value of <5, 5–50, 50–100, and >100 µg/mL was classified as strong active, active, weakly active, and inactive, respectively [25].

The separation of dichloromethane extract of *A. sericicarpus* resulted in 13 fractions, and 7 fractions were active among them. The $IC_{50}$ value of active fractions at a range of 1.53–3.65 µg/mL included in strong active antimalarial substance. F6, as the most active fraction, was contained terpenoids, flavonoids, and polyphenol based on TLC screening using specific spray reagents. A previous study reported secondary metabolites isolated from Artocarpus to involve terpenoids, flavonoids, stilbenoid, neolignan, arylbenzofuran, and Diels-Alder adducts compounds. Flavonoid compounds are the most abundant compound contains in Artocarpus [26]. Flavonoids have been reported as a potential source of future antimalarial lead [27]. Numerous terpenoids, flavonoids, and

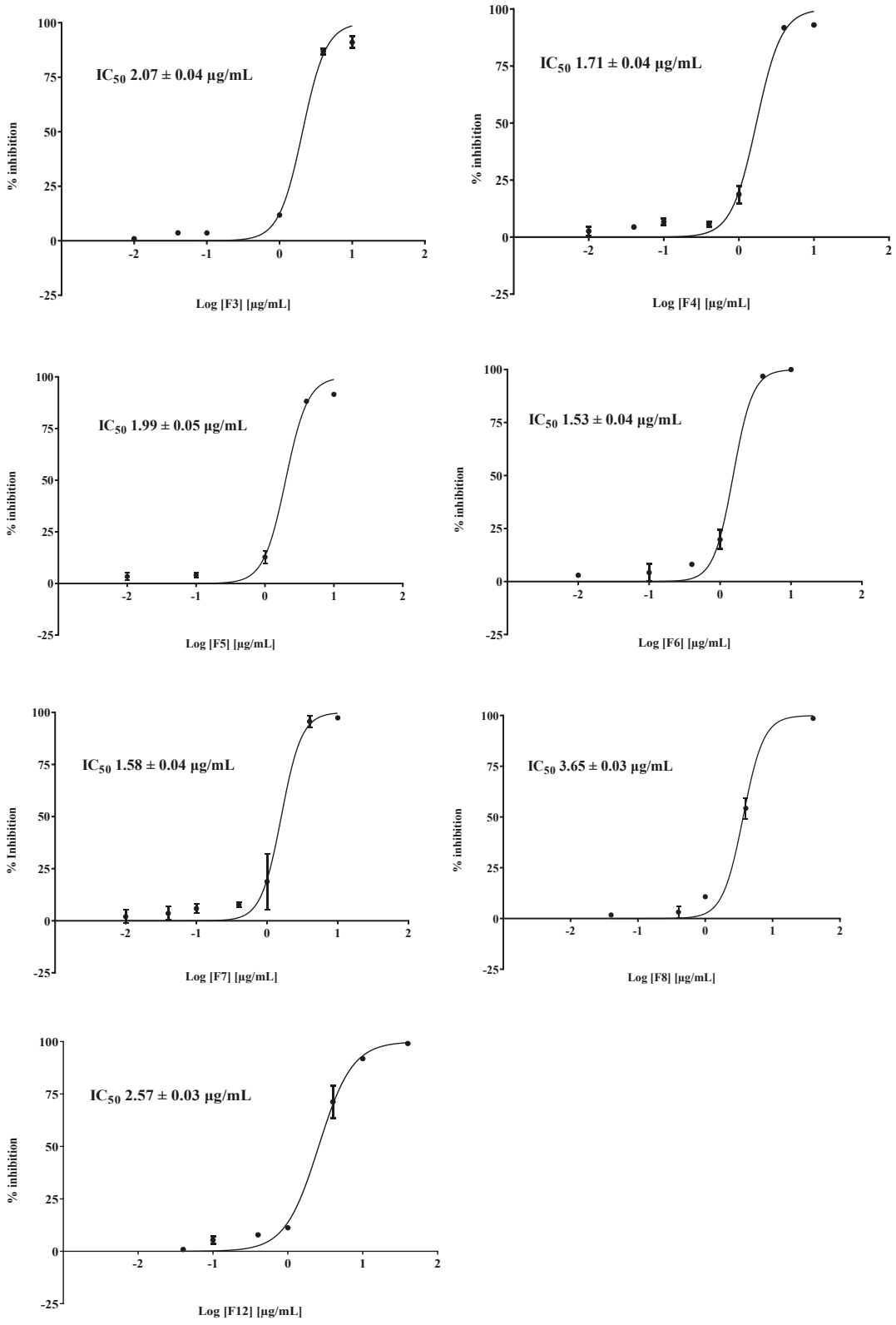


**Figure 2:** Dose response curve of active fractions against *Plasmodium falciparum* and the $IC_{50}$ value of active fractions.

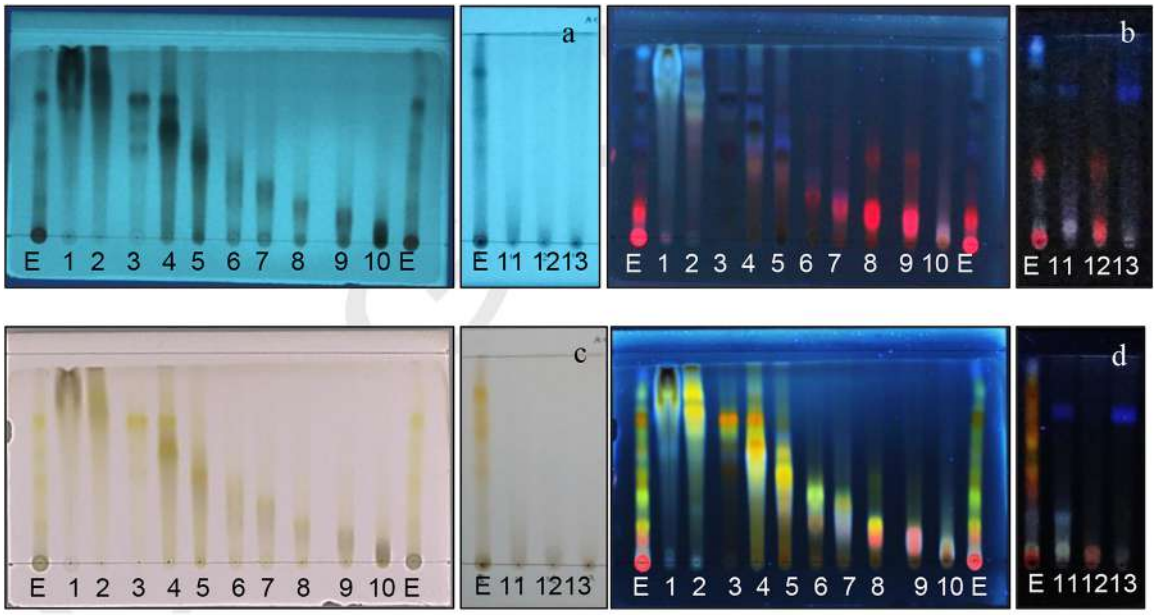


**Figure 3:** TLC profile of dichloromethane extract (E) and fractions (F1–F13) using silica gel RP-18 as a stationary phase and acetonitrile-water (8/2 v/v) (for F1–F10) and acetonitrile 100% (for F11–F13) as a mobile phase. The TLC spots were observed under UV 254 nm (a), UV 365 nm (b), white light after sprayed with $H_2SO_4$ 10% and heated 105 °C for 5 min (c), UV 365 after sprayed with $H_2SO_4$ 10%, and heated 105 °C for 5 min (d).

polyphenols compounds showed their activity as potent antimalarial [4, 5]. The flavonoids, terpenoids, and polyphenols compounds contain in F6 were take a role in its antimalarial activity performance.

This research reported the active fractions from *A. sericicarpus* stem bark extract. To our knowledge, no antimalarial compounds had reported from *A. sericicarpus* extract to date. Therefore, F6 needs to be further separated to obtain and elucidate the molecular structures of active compounds. Furthermore, F7 could be the next potential fraction for further separation based on its $IC_{50}$ value. The mechanism action of active compounds was interesting to be identified as well. Some flavonoids and chalcones were proposed to act by interfering with hemin degradation and inhibiting cyclin-dependent protein kinases and plasmepsin II [28]. The mechanism action of active compounds could be further determined based on these proposed mechanisms.

## Conclusions

Antimalarial substances of *A. sericicarpus* dichloromethane extract were mainly contained in Fraction-6, which strongly inhibited *P. falciparum*'s growth. The antimalarial substances need to be further isolated and identified to obtain active antimalarial compounds.

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Rahmi Annisa, Mochammad Yuwono and Esti Hendradi*

# Formulation and characterization of *Eleutherine palmifolia* extract-loaded self-nanoemulsifying drug delivery system (SNEDDS)

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**Abstract**

**Objectives:** This study aimed to determine the effect of different components and ratios of oil, surfactant, and cosurfactant on *E. palmifolia* extract-loaded SNEDDS.
**Methods:** *E. palmifolia* extract loaded SNEDDS was formulated from virgin coconut oil, Miglyol 812 as oil, using Tween 80 and Transcutol as surfactants, as well as propylene glycol and PEG 400 as cosurfactants. The optimization design formula consisted of eight design formulas in five ratio formulas, thus a total of 40 formulas were optimized using different components and ratios of oil, surfactant, and cosurfactant. These ratios used were 1:1:1, 1:2:1, 1:3:1, 1:4:1, as well as 1:5:1, and the formula's components were determined based on the optimization results.
**Results:** The optimal formula of *E. palmifolia* extract loaded SNEDDS had the ratio 1:1:1 (formula A) of Miglyol 812:Tween 80:PEG 400 and 1:3:1 (formula E) of Miglyol 812:Tween 80:propylene glycol. Meanwhile, the optimal formulation characteristics showed a transmittance value above 90%, pH range of 5.10–5.20, 2.21–14.51 cP viscosity, emulsification time below 120 s, and particle size of 24.71–136.77 nm.
**Conclusions:** The optimal formula of *E. palmifolia* extract-loaded SNEDDS, were obtained using different components and ratios. These are Miglyol:Tween 80:PEG 400 at a component ratio of 1:1:1 (formula A) and Miglyol 812:Tween 80:propylene glycol at a component ratio of 1:3:1 (formula E).

***Corresponding author: Esti Hendradi**, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, E-mail: esti-h@ff.unair.ac.id
**Rahmi Annisa,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Department of Pharmacy, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim, Malang, Indonesia
**Mochammad Yuwono,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

**Keywords:** characterization; drug delivery system; *Eleutherine palmifolia* (*E. palmifolia*); formulation; self-nanoemulsifying; SNEDDS.

## Introduction

The self-nanoemulsifying drug delivery system (SNEDDS) is an oil-based isotropic nanoparticle formulation containing surfactants and cosurfactants. This system formed fine oil-in-water nanoemulsion upon mild agitation, followed by administration in aqueous media, for instance, GI fluids [1–3]. Furthermore, the dosage formulation increases active substance dissolution by facilitating solubilized phase formation and increasing transport through the intestinal lymphatic system. SNEEDS improves drugs bioavailability (for instance, lipophilicity) [4] and increases the solubility of water-insoluble medicines, to aid gastrointestinal tract absorption [5].

The natural ingredients solubility is estimated at 40% as a new drug source, and therefore only slightly soluble in water [6]. Low water solubility and permeability in penetrating through the absorption barrier tends to alter the bioavailability of a naturally occurring compound in the body [7]. Meanwhile, *Eleutherine palmifolia,* also known as Dayak onion, is an indigenous plant of Kalimantan, used to cure various diseases. The phytochemical constituent of Dayak onion with the ability to function as an anticancer agent is naphthoquinone, and this has bioactivity as an antioxidant [8]. In addition, naphthoquinone is a lipophilic compound with a log p-value of 3.933, and therefore of low solubility in water media. Thus, there is a need to develop a SNEEDS, to improve naphthoquinone bioavailability in *E. palmifolia* extract.

SNEEDS has an advantage of delivering drugs in dissolved form into the gastrointestinal tract lumen (GI). Therefore, providing a more extensive interface area for drug absorption, and the characteristics are influenced by constituent components (oil, surfactant, and cosurfactant) [9]. The oil component is the primary carrier of active substances, and therefore plays a role in determining the emulsion size [10]. For this reason, this study aims to assess

the effect of comparing oils, surfactants, and cosurfactants in the SNEEDS formula of *E. palmifolia* extract, using Miglyol 812 and VCO as a carrier oil. The surfactants used were Tween 80 and Transcutol, while the cosurfactants used were PEG 400 and propylene glycol. Furthermore, the optimal formula was determined based on the oil, surfactant, and cosurfactant mixture's homogeneity, with a transmittance value >90%, while the characteristic formulas consist of emulsification time, pH, particle size, and viscosity. Therefore, this study is an optimization stage to obtain the optimal SNEEDS.

## Materials and methods

The *E. palmifolia* samples were purchased from vendors in East Kalimantan and identified at the Materia Medica in Batu, East Java, with access number 074/342A/102.7/2018. Subsequently, the specimens were stored in the Pharmacognosy Laboratory, Pharmacy Department, Faculty of Medicine and Health Science, Universitas Islam Negeri Maulana Malik Ibrahim, Malang, Indonesia.

The *E. palmifolia* samples were extracted thrice with 500 mL ethanol at ambient temperature, through sonication Q2400 (Sonica, USA), at 10 min intervals. About 25 g of the sample was dissolved in 500 mL of 96% ethanol at a ratio of 1:20. Subsequently, the filtrate was separated from the solvent using a rotary evaporator (Heidolph, Germany), to obtain a thick, dark brown extract with a distinctive *E. palmifolia* extract odor.

Meanwhile, the materials for this study include, Miglyol 812 procured from Sigma Aldrich, Tween 80, PEG 400 and propylene glycol purchased from Merck, Transcutol gift from Gattefose, and virgin coconut oil (VCO) acquired from Herbal Bagoes.

The SNEEDS design was formulated from oil, surfactants, and cosurfactants with suitable compositions, to form stable isotropic mixtures. Furthermore, optimization of oil: surfactant: cosurfactant was performed at ratios of 1:1:1, 1:2:1, 1:3:1, 1:4:1, 1:5:1 using Miglyol 812 and VCO as oil phase, as well as, Tween 80 and Transcutol as surfactants, while PEG 400 and propylene glycol were used as cosurfactants. The component mixture produced a clear, transparent solution and no phase separation (homogeneous). Table 1 shows the optimization formula's design.

For this preparation, 50 mg of *E. palmifolia* extract was mixed with 10 g of SNEEDS and stirred continuously for 10 min, using a magnetic stirrer (Heidolph, Germany) at 25 ± 2 °C, and a speed of 300 rpm. Subsequently, the mixture was characterized, and the SNEEDS formula for characterization was selected based on clarity and the absence of phase separation.

Meanwhile, the pH of each formula was measured using a pH meter S220 (Mettler Toledo, USA) and the electrodes were inserted into 10.00 mL of *E. palmifolia* SNEEDS. Each measurement was conducted in triplicates and recorded.

The percentage transmittance was measured using a UV–vis-1,800 spectrophotometer (Shimadzu, Japan). For this analysis, 100 µL of *E. palmifolia* extract-loaded self-nanoemulsifying drug delivery system was dissolved in 100 mL of homogenous aquadest for 1 min, with an vortex mixer absorption, at a wavelength of 650 nm. Absorbance values close to 100% indicated the dispersion droplets produced by the SNEEDS reached nanometer size.

The SNEEDS' emulsification time test was highly necessary and aimed at determining the process and time required to form nanoemulsion in vitro by visual observation. For this analysis, artificial gastric fluid (AGF) without pepsin, and with a pH of 1.2 ± 0.5 and artificial intestinal fluid (AIF) without pancreatin, and with a pH of 6.8 ± 0.5 (Table 2), were used as test media. Generally, SNEEDS is a system in the form of a pre-concentrate consisting of oil, surfactants, and cosurfactants spontaneously forming nanoemulsions on mixing with the media through light agitation in the digestive tract. The test was performed by dropping 100 µL of *E. palmifolia* loaded SNEEDS into 200 mL of media using a stirrer (Heidolph, Germany) at 37.0 ± 0.5 °C and a speed of 100 rpm. The emulsification time or time required to form nanoemulsion, was recorded and repeated thrice.

The viscosity test was conducted with a cone and plate viscometer (Brookfield, USA), using stationary plates (CP-40) filled with 0.50–2.00 mL *E. palmifolia* extract-loaded SNEEDS in the sample cup. This ensures the sample is bubble-free and evenly spread, and the cup was also reassembled on the viscometer and turned on. The process was repeated thrice until the sample became stable.

Furthermore, the particle size of the emulsion formed after reconstructing *E. palmifolia* extract-loaded SNEEDS was determined using dynamic light scattering Nanowave II (Microtrac, USA), and the formulations were diluted at a ratio of 1:10 w/w with aquadestas, and mixed well for 1 min. The diluted samples were transferred into cuvettes (model nano PTFE) with a refractive index of 1.20 (ratio of the indices between the oil and water phases), and the process was repeated thrice.

The test was conducted with Fourier Transform Infrared (FTIR) carry 630 (Agilent, USA). About 2 mL component of *E. palmifolia* extract-loaded SNEEDS was placed on the lens samples, and analyzed at a wavelength of 4,000–400 $cm^{-1}$.

## Results

The use of Miglyol 812 and VCO at a ratio of 1:1:1, 1:2:1, 1:3:1, 1:4:1, 1:5:1 yielded 40 formula designs tested for clarity, absence of phase separation (homogeneous), and transmittance. Table 3 shows the formula design optimization results, used as the basis for *E. palmifolia* extract-loaded SNEEDS formulation. According to these results, eight out of 40 formulas progressed to the next stage. The formulas were a formulation of VCO oil with a mixture of surfactants (Tween 80, Transcutol) and cosurfactants (PEG 400, propylene glycol). Table 3 shows the *E. palmifolia* extract-loaded SNEEDS produced in nanoemulsion with a consistency of about 100% clarity, determined using percent transmittance.

According to Table 3, the percent value of formulas A and E has a transmittance of about 100%, in the absence of phase separation. Therefore, the prepared SNEEDS are

**Table 1:** Formulation ratio of *E. palmifolia* extract-loaded SNEEDS.

| Formula | Ratio | Miglyol 812 | VCO* | Tween 80 | Transcutol | PEG 400 | Propylene glycol |
|---|---|---|---|---|---|---|---|
| A | 1:1:1 | 1 | – | 1 | – | 1 | – |
| | 1:2:1 | 1 | – | 2 | – | 1 | – |
| | 1:3:1 | 1 | – | 3 | – | 1 | – |
| | 1:4:1 | 1 | – | 4 | – | 1 | – |
| | 1:5:1 | 1 | – | 5 | – | 1 | – |
| B | 1:1:1 | – | 1 | 1 | – | 1 | – |
| | 1:2:1 | – | 1 | 2 | – | 1 | – |
| | 1:3:1 | – | 1 | 3 | – | 1 | – |
| | 1:4:1 | – | 1 | 4 | – | 1 | – |
| | 1:5:1 | – | 1 | 5 | – | 1 | – |
| C | 1:1:1 | 1 | – | – | 1 | 1 | – |
| | 1:2:1 | 1 | – | – | 2 | 1 | – |
| | 1:3:1 | 1 | – | – | 3 | 1 | – |
| | 1:4:1 | 1 | – | – | 4 | 1 | – |
| | 1:5:1 | 1 | – | – | 5 | 1 | – |
| D | 1:1:1 | – | 1 | – | 1 | 1 | – |
| | 1:2:1 | – | 1 | – | 2 | 1 | – |
| | 1:3:1 | – | 1 | – | 3 | 1 | – |
| | 1:4:1 | – | 1 | – | 4 | 1 | – |
| | 1:5:1 | – | 1 | – | 5 | 1 | – |
| E | 1:1:1 | 1 | – | 1 | – | – | 1 |
| | 1:2:1 | 1 | – | 2 | – | – | 1 |
| | 1:3:1 | 1 | – | 3 | – | – | 1 |
| | 1:4:1 | 1 | – | 4 | – | – | 1 |
| | 1:5:1 | 1 | – | 5 | – | – | 1 |
| F | 1:1:1 | – | 1 | 1 | – | – | 1 |
| | 1:2:1 | – | 1 | 2 | – | – | 1 |
| | 1:3:1 | – | 1 | 3 | – | – | 1 |
| | 1:4:1 | – | 1 | 4 | – | – | 1 |
| | 1:5:1 | – | 1 | 5 | – | – | 1 |
| G | 1:1:1 | 1 | – | – | 1 | – | 1 |
| | 1:2:1 | 1 | – | – | 2 | – | 1 |
| | 1:3:1 | 1 | – | – | 3 | – | 1 |
| | 1:4:1 | 1 | – | – | 4 | – | 1 |
| | 1:5:1 | 1 | – | – | 5 | – | 1 |
| H | 1:1:1 | – | 1 | – | 1 | – | 1 |
| | 1:2:1 | – | 1 | – | 2 | – | 1 |
| | 1:3:1 | – | 1 | – | 3 | – | 1 |
| | 1:4:1 | – | 1 | – | 4 | – | 1 |
| | 1:5:1 | – | 1 | – | 5 | – | 1 |

*VCO, virgin coconut oil.

**Table 2:** Formulation of artificial gastric fluid (AGF) and artificial intestinal fluid (AIF).

| **Formula AGF pH 1.2 ± 0.5** | | **Formula AIF pH 6.8 ± 0.5** | |
|---|---|---|---|
| NaCl | 20.00 mg | $MgCl_2$ | 0.15 g |
| HCl 37% | 0.70 mL | $CaCl_2$ | 0.15 g |
| Aquadest | ad 100.00 mL | KCl | 0.09 g |
| | | NaCl | 1.76 g |
| | | $NaHCO_3$ | 0.42 g |
| | | Aquadest | ad 500 mL |

**Table 3:** Results of *E. palmifolia* extract-loaded SNEEDS formula design optimization characterization.

| Formula | | Physical characteristics | Transmittance (%) | Formula | | Physical characteristic | Transmittance (%) |
|---|---|---|---|---|---|---|---|
| 1 | A (1:1:1) | + | 99.57 | 21 | E (1:1:1) | - | 10.55 |
| 2 | A (1:2:1) | + | 99.88 | 22 | E (1:2:1) | + | 69.71 |
| 3 | A (1:3:1) | + | 94.98 | 23 | E (1:3:1) | + | 92.45 |
| 4 | A (1:4:1) | + | 96.17 | 24 | E (1:4:1) | + | 95.80 |
| 5 | A (1:5:1) | + | 96.95 | 25 | E (1:5:1) | + | 99.10 |
| 6 | B (1:1:1) | - | 97.06 | 26 | F (1:1:1) | - | 4.97 |
| 7 | B (1:2:1) | - | 66.06 | 27 | F (1:2:1) | - | 8.51 |
| 8 | B (1:3:1) | - | 87.75 | 28 | F (1:3:1) | - | 73.98 |
| 9 | B (1:4:1) | - | 71.05 | 29 | F (1:4:1) | - | 83.44 |
| 10 | B (1:5:1) | - | 86.40 | 30 | F (1:5:1) | - | 98.96 |
| 11 | C (1:1:1) | - | 97.92 | 31 | G (1:1:1) | - | 97.21 |
| 12 | C (1:2:1) | - | 95.71 | 32 | G (1:2:1) | - | 95.31 |
| 13 | C (1:3:1) | - | 85.32 | 33 | G (1:3:1) | - | 84.61 |
| 14 | C (1:4:1) | - | 89.68 | 34 | G (1:4:1) | - | 88.61 |
| 15 | C (1:5:1) | - | 86.69 | 35 | G (1:5:1) | - | 85.91 |
| 16 | D (1:1:1) | - | 97.54 | 36 | H (1:1:1) | - | 98.09 |
| 17 | D (1:2:1) | - | 66.67 | 37 | H (1:2:1) | - | 95.77 |
| 18 | D (1:3:1) | - | 88.46 | 38 | H (1:3:1) | - | 97.25 |
| 19 | D (1:4:1) | - | 71.54 | 39 | H (1:4:1) | - | 91.31 |
| 20 | D (1:5:1) | - | 87.02 | 40 | H (1:5:1) | - | 91.86 |

\+ : clear, transparent, no phase separation (homogeneous)
\- : clear, transparent, phase separation (not homogeneous)

expected to be clear and transparent because the particle size is classified as nanoemulsion. In small proportions, a good formula ought to be a clear, transparent mixture, with transmittance values above 90%. Based on these considerations, *E. palmifolia* extract-loaded SNEEDS were formulated with the following characteristics of Miglyol 812:Tween 80:PEG 400 (formula A) as well as Miglyol 812:Tween 80:propylene glycol (formula E) in a ratio of 1:1:1 and 1:3:1, respectively.

The formula's pH were in the range of 5.10–5.20, while viscosity was measured with a viscosimeter. Viscosity shows the characteristics of a liquid, and is described as the resistance to flow. Increased viscosity of preparation leads to higher strength. Table 4 showed the results of pH and viscosity.

Table 5 indicates *E. palmifolia* extract-loaded SNEEDS formed an emulsion in aquadest, AGF, and AIF without enzymes in less than 2 min. Therefore, the SNEEDS are well emulsified in all three media, where the emulsification time of formula A and formula E was compared. Formula A has short emulsification time mediated by the action of surfactants and cosurfactants, and this tends to form the interface layer of oil and water immediately. The cosurfactants play a more critical role in determining the time of emulsification, rather than reducing droplet size. Furthermore, a space between surfactants was formed because the structure was more swollen due to high fluidity, to form nanoemulsions faster. The ability to increase emulsification in cosurfactants was also determined at the hydrophobic alkyl chain length, with better emulsification ability [11].

Table 6 shows formulas A (1:1:1) and E (1:3:1) of *E. palmifolia* extract-loaded SNEEDS had a droplet size below 200 nm. The small size expands the droplet's contact surface with the gastric fluid, allowing the lipase enzyme to break the system down more easily, as drug release is faster with small size. Therefore, smaller the particle size ensures the drug reaches the specific target cells more easily. Figure 1 shows the result of particle size distribution *E. palmifolia* extract-loaded SNEEDS, on AIF and AGF media.

Theoretically, the oil was used at a fixed ratio, because high proportion tends to increase *E. palmifolia* extract-loaded SNEEDS's droplet size [13]. Also, the size distribution or polydispersity index (PDI) is the standard deviation of the average particle size, and indicates the nanoemulsion's uniformulation. A PDI value below one indicates uniformity of the size formed.

Figure 2 shows the results of characterization using FTIR, an instrument with the ability to detect chemical interactions in a mixture of components based on changes in functional groups. In this study, FTIR spectroscopy was performed to investigate the interaction between any ingredients used in the formulation. Figure 2 also shows the chemical profile forming different patterns of differences and characteristics. According to the results, there was no difference in the functional groups of *E. palmifolia* extract-loaded SNEEDS in formula A (1:1:1) and formula E

**Table 4:** Test results of pH and viscosity *E. palmifolia* extract-loaded self-nanoemulsifying drug delivery system formula.

| Selected formula | | pH ± SD | Viscosity (Cps) ± SD* |
|---|---|---|---|
| 1 | A (1:1:1) | 5.20 ± 0.05 | 2.21 ± 0.77 |
| 23 | E (1:3:1) | 5.10 ± 0.10 | 14.51 ± 0.50 |

*The value of SD for three times replication. SD, standard deviation.

**Table 5:** Test results of emulsification time for *E. palmifolia* extract-loaded self-nanoemulsifying drug delivery system.

| Selected formula | | Emulsification time (second) ± SD* | | |
|---|---|---|---|---|
| | | Aquadest | AIF | AGF |
| 1 | A (1:1:1) | 105.00 ± 0.01 | 69.00 ± 0.01 | 69.00 ± 0.05 |
| 23 | E (1:3:1) | 72.00 ± 0.02 | 52.00 ± 0.03 | 49.00 ± 0.03 |

*The value of SD for three times replication. AGF, artificial gastric fluid; AIF, artificial intestinal fluid; SD, standard deviation.

**Table 6:** Test results of particles size analysis for *E. palmifolia* extract-loaded self-nanoemulsifying drug delivery system formula.

| Selected formula | | Particles size (nm) ± SD | | Polydispersity index ± SD* | |
|---|---|---|---|---|---|
| | | AIF | AGF | AIF | AGF |
| 1 | A (1:1:1) | 74.02 ± 3.97 | 24.71 ± 0.93 | 0.13 ± 0.01 | 0.56 ± 0.01 |
| 23 | E (1:3:1) | 134.77 ± 2.83 | 75.86 ± 2.63 | 0.13 ± 0.01 | 0.38 ± 0.01 |

*The value of SD for three times replication. AGF, artificial gastric fluid; AIF, artificial intestinal fluid; SD, standard deviation.

(1:3:1). SNEEDS with *E. palmifolia* extract was therefore confirmed to have been successfully established as an active ingredient. FTIR is a suitable method for examining extracts, and the infrared spectrum may be approved as a functional group for complications in the sample [14]. In this study, the FTIR analysis used a wavelength range of 4,000–400 $cm^{-1}$.

## Discussion

The components of SNEEDS are oil as a drug carrier, surfactants as emulsifiers through the interface film layer's formulation and stability, and cosurfactants, to assist the task of others. Furthermore, the formula's characteristics were influenced by the ratio of oil, surfactant, polarity, and emulsion droplet load. The Nanoemulsion was formed as oil, covered by the surfactant's hydrophobic part. Conversely, the surfactant's hydrophilic portion interacted with water molecules to form emulsions. Self-emulsification also occurred in cases where the dispersed phase's entropy energy is greater, compared to the energy required to increase the dispersed phase's surface area.

The SNEEDS preparation's formulation will increase active substance dissolution by facilitating the formation of a solubilized phase. This is also possible by increasing the intestinal lymphatic system, and avoiding P-GP efflux, due to the absorption as well as bioavailability of active substances from the gastrointestinal tract.

The virgin coconut oil with surfactants (Transcutol) and cosurfactants (PEG 400, propylene glycol) is unable to form clear, transparent, and phase separation (formula B, C, D, F, G, and H). This is because VCO is rich in chain fatty acids such as capric (C10:0) (5.21%), lauric (C12:0)

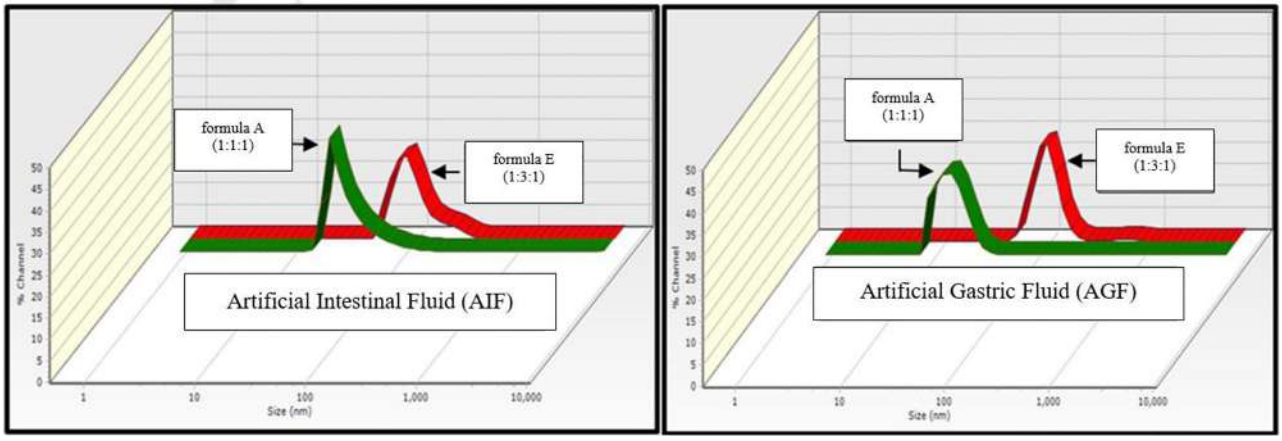


**Figure 1:** Diagram of *E. palmifolia* extract-loaded self-nanoemulsifying drug delivery system formula's particle size distribution in AIF and AGF.

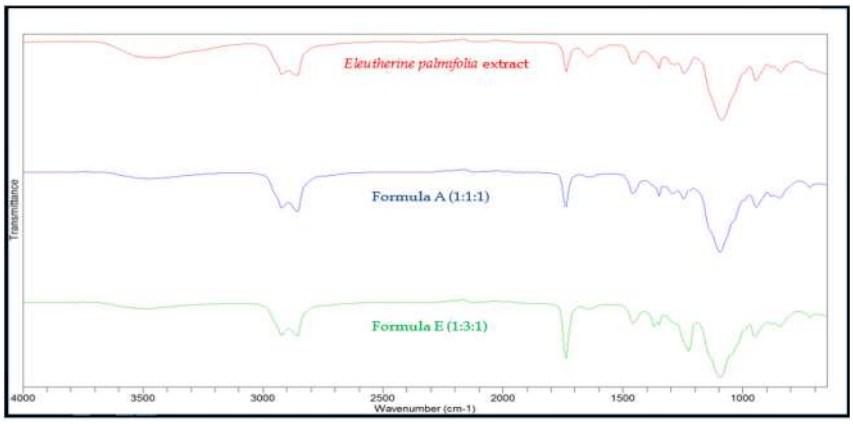


**Figure 2:** Infrared spectrum of *Eleutherine palmifolia* extract, formula A (1:1:1), formula E (1:3:1).

(48.66%), and myristate (C14:0) (17.82%) acids. In cases where the amount of surfactant is greater, compared to the oil, communication becomes difficult, due to the branched structure, leading to a nonhomogeneous mixture. In addition, homogeneity is also caused by the cosurfactants (PEG 400 and propylene glycol), and is often insufficient for reducing surface tension. However, this tends to penetrate the single surfactant layer and provide new fluidity to interfere with the liquid crystal phase formed in cases where the film is too rigid.

SNEDDS are well known for the potential to enhance lipophilic drugs' solubility and absorption, by increasing the surface area and decreasing the size of oil droplets, readily in terms of change in particle size. The primary mechanism lipids and lipophilic excipients undergo to formulate SNEDDS tend to affect drug absorption, bioavailability, and disposition, following oral administration. Currently, SNEDDS have been reported to influence pre-enterocyte level (alteration of the intestinal milieu's composition and character, as well as increased solubilization), intraenterocyte level (interaction with enterocyte-based transport processes, including permeability through the cell membrane and effects on intraenterocyte metabolism as well as drug transporters); and post-enterocyte level (recruitment of intestinal lymphatic drug transport).

Meanwhile, emulsification time describes the minutes required for SNEEDS to form nanoemulsion, upon contact with gastrointestinal fluid. Table 5 shows the profile results of the emulsification time test, conducted to measure the rate self-nanoemulsifying drug delivery system use to form nanoemulsion after contact with stomach intestinal fluids. However, SNEEDS is said to meet the requirements in cases where a nanoemulsion is directly formed with light stirring. Therefore, the selection of oil, surfactants, and cosurfactants is very important to the emulsification of the digestive tract, and the faster time implies higher drug absorption. Based on the results of test interpretation, a good emulsification time ought to be within a range of 1–2 min, for a clear and transparent liquid to be produced.

The particle size analyzer determines the drug's speed, stability, and ease of optimal absorption, and is therefore a critical factor in self-emulsification. Table 6 showed the test results. Generally, SNEEDS has particle sizes below 200 nm and is a suitable drug delivery system, with high clarity and stability. Also, the small particle size provides a clear dosage while testing the percent transmittance, in addition to preventing sedimentation and improving stability [12].

The characteristics results obtained with carrier oil show Miglyol 812 is more effective in SNEEDS formation, because a smaller surfactant amount is required, compared to VCO. Conversely, VCO shows difficulty in forming nanoemulsion and a larger droplet size, compared to Miglyol 812. Oil medium-chain triglycerides, including Miglyol 812, easily form nanoemulsion compared to long-chain triglycerides, for instance, soybean, coconut, and palm oil. Furthermore, chain triglycerides increase drug transport to intestinal lymphatics, in order to prevent first-pass metabolism, compared to intermediate-chain tri-, di- and monoglycerides.

## Conclusions

In this study, the optimal formula of *E. palmifolia* extract-loaded SNEDDS, were obtained using different components and ratios. These are Miglyol:Tween 80:PEG 400 at a component ratio of 1:1:1 (formula A) and Miglyol 812:Tween 80:propylene glycol at a component ratio of 1:3:1 (formula E).

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Helmy Yusuf*, Nina Wijiani, Rizka Arifa Rahmawati, Riesta Primaharinastiti, M. Agus Syamsur Rijal and Dewi Isadiartuti

# Analytical method for the determination of curcumin entrapped in polymeric micellar powder using HPLC

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**Abstract**

**Objectives:** Curcumin belongs to the family of curcuminoids, natural polyphenolic compounds that possesses neuroprotective properties, anti inflammatory and anticancer. Its entrapment in the developed casein-based micellar powder (CMP) and poloxamer-based micellar powder (PMP) was to enhance the solubility and improve the bioavailability. Henceforth, the present study aimed to acquire an efficient analytical method for the curcumin analysis in polymeric micellar formulations.

**Methods:** A fast and specific HPLC method was developed for analyzing curcumin in two different micellar matrices using casein and poloxamer. The HPLC was equipped with a C18 column (250 × 4 mm, 5 µm) and diode array detector. A designated isocratic elution of curcumin was employed using mobile phase with a composition of water (1%, v/v acetic acid) and acetonitrile in a ratio of 50:50 v/v. The employed flow rate was 1.0 mL/min and the analyte was examined at 421 nm.

**Results:** An effective analysis in HPLC was successfully achieved by the predetermined HPLC condition. A good resolution of peaks at the employed flow rate was achieved. The linearity was excellent in two different range of concentrations, 2–20 and 10–50 µg/mL. The selectivity, accuracy and precision fulfilled the acceptable requirements.

**Conclusions:** The developed method was practically effective to qualitatively identified curcumin. In addition, the assay also effectively quantified the amount of curcumin in the polymeric entrapping matrices which demonstrates that it has great potential to be used in natural compound analysis.

**Keywords:** curcumin; HPLC assay; micellar powder.

## Introduction

Phenolic compounds, namely, curcuminoids of *Curcuma longa* L. have been reported for its various pharmacological activities. The main constituent curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is known for neurodegenerative diseases [1], anti inflammatory [2] and anticancer properties [3]. Curcumin with the analogs have been recently reported to synergistically exhibit therapeutic activities including inhibition of tumor cells proliferation [4]. Commercial products of curcumin consists of three constituents of curcuminoids with curcumin as the main constituent (77%), followed by its two analogs i.e. demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) [5].

In spite of the therapeutic potential, there are some drawbacks in the clinical development for its natural short biological half-life that led to a low bioavailability following oral administration [6]. Many clinical studies showed the extremely low serum concentration after oral administration of curcumin [7, 8]. These findings have triggered fast development in the area of formulation addressing such problems including the use of solid dispersion and polymeric micelles [9, 10]. Henceforth, the fast development in the area of curcumin formulations should come along with the rapid development of validated quantification analysis of curcumin.

Available reported methods for the quantification of the curcuminoids use spectrophotometric methods at the most [11]. On the other hand, the analysis of separable curcuminoids is promising by high-performance liquid chromatographic (HPLC) either on reversed phase or non-reversed phase columns [12, 13]. C18 columns are preferred for HPLC analysis for labile characteristics of compounds such as curcuminoids [13]. Until now, official chromatographic methods for the estimation of curcumin are not

***Corresponding author: Helmy Yusuf**, Department of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 31 5933150, E-mail: helmy-yusuf@ff.unair.ac.id
**Nina Wijiani, Rizka Arifa Rahmawati, Riesta Primaharinastiti, M. Agus Syamsur Rijal and Dewi Isadiartuti,** Department of Pharmaceutical Sciences, Universitas Airlangga, Surabaya, Indonesia

available. Thus, there is a fundamental need to develop a validated HPLC method that is rapid, accurate and specific for the quantitative estimation of curcumin in various dosage forms. In terms of quality control, this is in order to assure the quality of pharmaceutical products and minimize batch-to-batch variation.

The present study developed a method for the extraction and analytical of curcuminoids from two different micellar powder formulations. The novelty of the developed analysis method was due to the complexity of these two formulations. CMP formulation contained casein, D-α tocopherol succinate (TPGS) and sucrose. Whilst, PMP formulation contained poloxamer 407 and eudragit S-100. As curcumin was incorporated in these matrices, the analytical aspect which requires a rapid and valid process has been the challenging aspect of the study. Thus, the present study discussed the analysis of curcuminoids by describing the HPLC analytical conditions for curcumin, demethoxycurcumin, and bisdemethoxycurcumin. An isocratic reversed-phase was used for a selective and sensitive assay of curcumin in casein-based and poloxamer-based micelles as vehicles for oral delivery of curcumin.

# Materials and methods

## Materials

Curcumin, (raw and reference standard), acetonitrile and methanol (HPLC grade), and acetic acid were purchased from Sigma-Aldrich (Singapore). The CMP and PMP powder formulations were produced in the Laboratory of Department of Pharmaceutics, Universitas Airlangga (Indonesia).

## Instrument

The HPLC system comprised of an Agilent 1,100 series (Agilent Technologies, Palo Alto, USA) with an automatic sampler and a PDA detector (Waters Corporation, USA). Analysis was carried out using a stationary phase of C18 column with dimensions of 250 mm × 4 mm, 5 µm. The column temperature was preserved at 35 °C. The mobile phase was water (with 1%, w/v of 1-acetic acid) in combination with acetonitrile (50:50, v/v) with a flow rate of 1.0 mL/min. Prior pumping into the HPLC system, the mobile phase was passed through a 0.2 µm membrane filter. Wavelength for detection was set at 421 nm with volume of injection of 30 µL.

## Preparation of standard stock solution

The standard stock solution of curcumin was prepared by dissolving a weighed amount of 10.0 mg of the drug in 10 mL of methanol to make up a concentration of 1,000 ppm. From this stock solution, two serial concentrations within the range of 10–50 µg/mL was prepared for CMP samples analysis, and 2–20 µg/mL for PMP samples analysis which were carefully diluted with proper amounts of methanol.

## Sample preparation

Two different micellar powder formulations were used in the study, i.e. CMP Powder and PMP Powder. The CMP formulation contained casein, TPGS and sucrose, while the PMP formulation contained poloxamer 407 and eudragit S-100. These two formulations have been one of particular interests especially in the search for the most potential formulations to bring curcumin into clinical use. Curcumin was loaded at 2 and 0.5% w/w to prepare CMP and PMP formulations respectively due to the nature of the matrices used in each formulation. The matrices used in the formulation have brought challenges for the curcumin estimation in these micellar powders. In order to obtain reliable results, 12 powders from each formulation were selected. An amount of 50 mg of curcumin micellar powder from each formulation was accurately weighed and brought into a 25.0 mL volumetric flask. The sample was dissolved with methanol to get a concentration of 2,000 ppm. This mixture was sonicated until a clear solution was obtained. A filtration using 0.22 µm membrane filter was applied to the clear solution and the HPLC analysis required 30 µL out of this solution.

## Selectivity

The ability to separate the curcumin peak from other components in the sample was the objective of selectivity determination. In the present study, the selectivity was evaluated by injecting of standard solution, blank solvent, matrix solvent and samples solution (CMP and PMP) to the HPLC system.

## Linearity

Two different linearity curves were prepared for each sample by using standard solutions to get the linearity within the range of 10, 20, 30, 40, and 50 µg/mL for CMP samples; and within the range of 2, 5, 10, 15, and 20 µg/mL for PMP samples. Filtrations using a 0.22 µm of these standard solutions were employed prior injection to the HPLC system. Further linear regression analysis was used to evaluate the linearity, which was determined by the least square regression method from the serial data of standard solution concentrations vs. their peak area.

## Limit of detection (LOD) and quantification (LOQ)

The signal-to-noise ratio was used to evaluate the LOD and LOQ. The LOD is the lowest concentration level indicated by a peak area of three times the baseline noise. The LOQ is the lowest concentration level that indicated by a peak area with a ratio of signal to noise not less than 10. Both were determined by introducing the low concentration of standard solutions of 2, 5, 10, 15, and 20 µg/mL and 10, 20, 30, 40, and 50 µg/mL to the HPLC system. Linear regression analysis was applied to get the slope and standard deviation of y-intercepts of the obtained linear regression (Sy). The LOD and LOQ were calculated by following formula:

$$\mathrm{LOD} = (3(\mathrm{Sy}))/\mathrm{Slope}; \mathrm{LOQ} = (10(\mathrm{Sy}))/\mathrm{Slope}$$

### Accuracy

Recovery was evaluated by spiking three different known amounts of curcumin standard solutions (80, 100, and 120%) according to concentration in the target sample in the micellar powder. The experiments were carried out in three replicates for each concentration. Calculation of the percent recovery was based on the following formula:

$$\text{Recovery}\,(\%) = (\text{Spiked concentration} - \text{Original concentration})/(\text{Spike value}) \times 100\%$$

### Precision

The precision was carried out by spiking the micellar powder with a 100% known amount of curcuminoid standard prepared in six replicates. The intraday precision (repeatability) was determined by analyzing samples in six replications within one day. The precision is presented as RSD value.

## Results

HPLC was developed using a stationary phase of C18 column with dimension of 250 × 4 mm, i.d. 5 µm. An isocratic mobile phase was employed using water (with 1%, w/v of 1-acetic acid) and acetonitrile (50:50, v/v). Optimizing condition was carried out in terms of volume injection, flow rate, and temperature of column to analyze curcumin in the samples. The mobile phase was selected in which not only a good resolution for curcumin in the sample was obtained, but also it showed a good peak shape (Figure 1). The injection volume was 30 µL to improve the column efficiency.

Maintaining the flow rate at 1.0 mL/min and the temperature of column at 35 °C increased the resolution of curcumin peak relative to the other components in the sample. The chromatograms of standard curcuminoids and those obtained from the CMP and PMP samples are presented in Figure 1. Common commercial curcumin products contain pure curcumin around 77%, demethoxycurcumin around 17%, and bisdemethoxycurcumin around 3% [5], henceforth, the obtained retention times of standard curcumin were 4.331; 4.801; and 5.334 min representing the bisdemethoxycurcumin, demethoxycurcumin and curcumin, respectively (Table 1). The analysis of these compounds in the CMP sample showed retention times of 4.371; 4.835; and 5.363 min; whilst, in the PMP sample were 4.356; 4.821; and 5.355 for the bisdemethoxycurcumin, demethoxycurcumin, and curcumin, respectively. These results showed that the developed HPLC condition successfully identified the curcumin with the two analogs, demethoxycurcumin, and bisdemethoxycurcumin. Moreover, the resulting retention time also demonstrated that the developed method requires a shorter time of analysis.

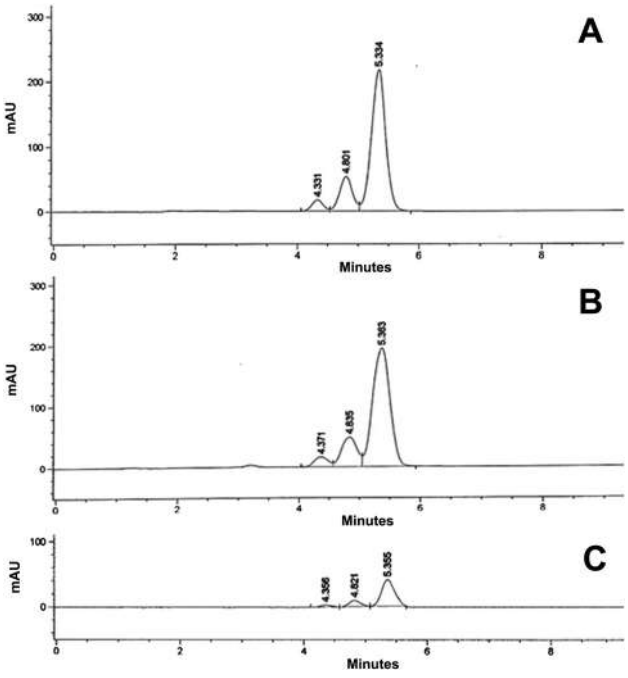


**Figure 1:** The chromatogram of curcuminoids: (A) Standard curcumin and the analogs, (B) curcumin and the analogs in the CMP sample, (C) curcumin and the analogs in PMP sample.

## Selectivity

The developed HPLC method separated the analyte curcumin with the two analogs. Table 1 showed the result of the selectivity test. Separation of curcumin and two analogs was achieved. The retention times of curcumin and the

**Table 1:** The results of the selectivity test.

| Sample solution | Curcuminoids | tR | RS |
|---|---|---|---|
| Curcumin standard | Bisdemethoxycurcumin | 4.331 | 1.31 |
| | Demethoxycurcumin | 4.801 | |
| | Curcumin | 5.334 | |
| CMP | Bisdemethoxycurcumin | 4.371 | 1.0 |
| | Demethoxycurcumin | 4.835 | |
| | Curcumin | 5.363 | |
| PMP | Bisdemethoxycurcumin | 4.356 | 1.43 |
| | Demethoxycurcumin | 4.821 | |
| | Curcumin | 5.355 | |
| Methanol blank | | – | – |
| Matrix solvent | | – | – |

two analogs were approximately 4 and 5 min which were relatively short. The selectivity of the separation of curcumin with the two analogs might be close to RS value of 1.5 that is considered as a total separation. Nevertheless, peak shapes were remarkably good. The analysis successfully resolved the individual peaks of curcumin, demethoxycurcumin, and bisdemethoxycurcumin in both CMP and PMP samples analyzed without any interference from the other compounds. The analytes retention times and the use of standard solution for spiking were used to confirm each peak. The methanol blank and micellar powder matrix did not interfere with these three components (Figure 1). In contrast, some studies reported the failure in obtaining good resolution of these three compounds using similar C18 stationary phases [12, 13]. Another study reported tailing peaks that led to a poor resolution come out as the problem of such analysis [14]. Moreover, the selectivity in the present study was also evaluated by using match factor (MF) and peak purity that require values of >990. The results demonstrated that both values were >990 for both CMP and PMP samples analysis (data not shown).

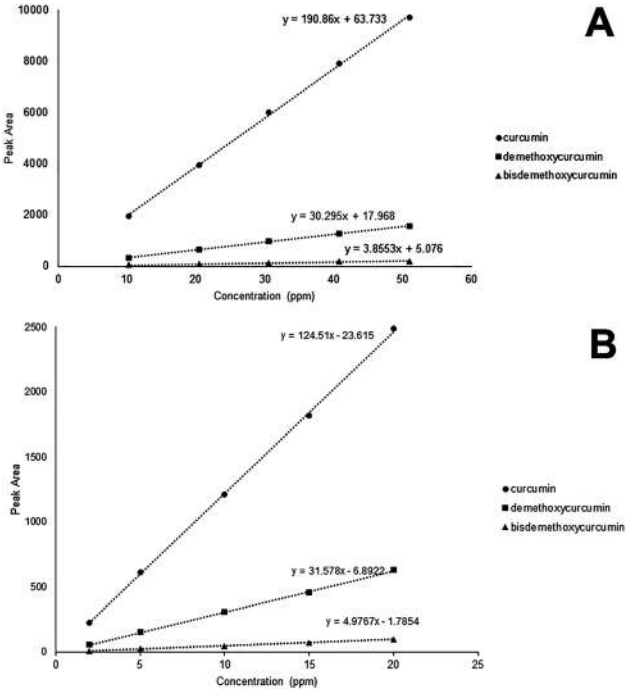


**Figure 2:** Calibration curves were prepared for two different range of concentrations.
(A) At range of 10–50 µg/mL for CMP samples, and (B) at range of 2–20 µg/mL for PMP samples.

## Linearity

Calibration curves were prepared to quantify the curcuminoids content in different CMP and PMP samples. Three independent injections of serial predetermined concentrations of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were used to derive the peak areas. Two different linear correlation curves were prepared for the two CMP and PMP samples based on the predetermined concentration of curcuminoids in the samples. The plotting of serial concentrations (ppm) of curcumin with the two analogs vs. the obtained peak areas were presented in Figure 2A, B. Acceptable correlation coefficients ($R^2$) of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were obtained as results of regression analysis of those concentration points to the corresponding peak areas (Table 2). The corresponding peak areas were found to be linearly correlated with the concentrations of the standard solution in both range of concentrations.

The obtained regression equations together with correlation coefficient and LOD/LOQ of CMP and PMP formulations are presented in Table 2. Both $R^2$ values fulfilled those required by AOAC i.e. >0.99 [15].

## LOD and LOQ

Curcuminoids concentrations in both CMP and PMP samples were reflected in the two calibration ranges. Based on the data presented in Table 2, it can be further calculated the LOD of curcumin and the two analogs i.e. demethoxycurcumin and bisdemethoxycurcumin. The results showed

**Table 2:** The linearity and LOD/LOQ results derived from the calibration curves.

| Prepared calibration curve for | Curcuminoids | Regression equation | $R^2$ | LOD/LOQ, µg/mL |
|---|---|---|---|---|
| CMP samples, 10–50 µg/mL | Bisdemethoxycurcumin | $Y=3.8553x + 5.076$ | 0.988 | 3.95/13.17 |
| | Demethoxycurcumin | $Y=30.295x + 17.968$ | 0.999 | 1.78/5.93 |
| | Curcumin | $Y=190.86x + 63.733$ | 0.992 | 1.01/3.34 |
| PMP samples, 2–20 µg/mL | Bisdemethoxycurcumin | $Y=4.9767x - 1.7854$ | 0.993 | 1.08/3.59 |
| | Demethoxycurcumin | $Y=31.578x - 6.8922$ | 0.995 | 0.65/2.22 |
| | Curcumin | $Y=124.51x - 23.615$ | 0.995 | 0.57/1.89 |

that the LOD for curcuminoids in the CMP samples were 1.01, 1.78, and 3.95 μg/mL in respective order. Whilst, the LOD of these compounds in PMP samples were 0.57, 0.65, and 1.08 μg/mL, respected to the same order as the CMP sample. Next, through the data provided in Table 2, it can be also derived for the determination of LOQ, the lowest concentration of an analyte which can be consistently quantified by the developed analytical procedure. The results were 3.34, 5.93, and 13.17 μg/mL in the CMP sample, while for the PMP sample were 1.89, 2.22, and 3.59 μg/mL, respectively.

### Accuracy

Accuracy of the developed analytical method was represented as percent recovery (Table 3). The percent recovery of spiking standard solution to the micellar powder matrices was in range of 103.48–104.56% for CMP samples and 82.86–99.36% for PMP samples. These results showed that the developed method accurately measured curcumin in the presence of these various excipients. No interference from the excipients used in the two formulations.

### Precision

The precision was evaluated by six determination curcumin concentrations in the CMP and PMP micellar powder samples within a day. The CMP and PMP samples were loaded with two different concentrations due to the nature of the used matrices as those described in the sample preparation section. Therefore, the measured concentrations were different as shown in Table 4. The intraday precision showed %RSD of 1.77 and 1.57 for CMP and PMP samples, respectively. Both values were less than 2, which met the acceptance criteria for precision recommended by AOAC [15].

**Table 3:** The accuracy of the developed method in both CMP and PMP samples.

| Samples | % Recovery at each addition level to target concentration | | |
|---|---|---|---|
| | 80% | 100% | 120% |
| CMP | 104.11 ± 4.26 | 104.56 ± 4.51 | 103.48 ± 5.88 |
| PMP | 93.57 ± 9.33 | 82.86 ± 3.15 | 99.36 ± 4.86 |

**Table 4:** The precision of the developed method in both CMP and PMP samples.

| Samples | Measured concentration, % w/w ± RSD, %, n=6 |
|---|---|
| CMP | 0.67 ± 1.77 |
| PMP | 0.11 ± 1.57 |

## Discussion

The developed HPLC method was fast, yet simple and profound, accurate, and specific for the quantitative analysis of curcumin in the CMP and PMP formulations. The method was validated for a series of parameters, therefore, can be applied for the assay of curcumin in such micellar powder products. The developed HPLC method was using an isocratic mobile phase and PAD detector. Various validation parameters including selectivity, linearity, LOD/LOQ, accuracy and precision were employed in the developed method. The run time was less than 7 min using a relatively low flow rate (1.0 mL/min) which can be cost-effective and suitable for analysis of samples in large numbers.

For the curcumin estimation in the two CMP and PMP micellar powder formulations, samples were analyzed in triplicate following extraction of the drug as described in the sample preparation section. Calibration curves were prepared together with the samples of the curcuminoids extracted from the micellar powder samples. Three independent injections to the HPLC were implemented from six different curcuminoids concentrations and plotted vs. the obtained peak areas. The results showed tremendous correlations between the peak area and the concentration of curcuminoid standard within the two concentration ranges. A linear increased of the peak areas were in line with the increased concentrations within the range of 2–20 and 10–50 μg/mL, with high reproducibility and accuracy (Figure 2 and Table 2).

The amount of curcumin in the CMP powder was 1.99 ± 0.93% w/w and in the PMP powder was 0.3 ± 0.02% w/w. This was similar with the loaded curcumin in the prepared formulations i.e. 2 and 0.5% w/w for respective CMP and PMP formulations. The result in CMP samples indeed reflected the actual concentration of the curcumin in the prepared formulation. However, this was different from those in PMP samples that exhibited a noteworthy decrease from the initial amount of curcumin loaded to the formulation. A substantial amount of the curcumin might be lost during the preparation. Yet, it implied that the analysis condition was accurately estimated the curcumin concentration in the samples. None of the excipients used in the two formulations interfered with the analyte peak as depicted in Figure 1. The spectrum of curcumin extracted from the micellar powder matrices identical with that of

standard curcumin (Figure 1) showing the purity of curcumin peak in the micellar powders. In this case, the complexity of the matrices used in the two developed formulations was successfully coped by the developed HPLC analysis condition.

There are no individual commercial products of pure curcumin and the two analogs demethoxycurcumin, and bisdemethoxycurcumin. Hence, HPLC analysis of the standard curcuminoids exhibited single peaks at retention times of 4.331, 4.801 and 5.334 min for bisdemethoxycurcumin, demethoxycurcumin, and curcumin, respectively (Figure 1 and Table 2). The performed analysis has identified curcumin as the major curcuminoid, and another two analogs which were identified as demethoxycurcumin and bisdemethoxycurcumin as compared to the standard curcuminoids. Many reported analysis confirmed the compositions of these curcuminoids which were curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) [5]. The individual peaks of these three curcuminoids were resolved with no interference from other compounds in all of the samples analyzed. Retention time determination and spiking with standard were used to confirm each peak.

The developed HPLC analysis resulted in acceptable parameters for the determination of curcumin with the two analogs, demethoxycurcumin and bisdemethoxycurcumin. However, not all C18 stationary phase successfully separated those three components. Some studies reported that those three curcuminoids were not completely resolved using the same C18 column in the HPLC system [12, 13]. In contrast, the use of an amino-bonded stationary phase and fluorescence detector have been reported successfully separated all those analytes [16, 17]. Nevertheless, reproducibility and poor resolution are often addressed to such methods.

The critical aspect was the selection of the mobile phase to obtain an optimum chromatographic separation of analytes. Studies reported the use of acetonitrile/acetate buffer as the mobile phase [18, 19] while other studies used mobile phase of sodium hydroxide and citric acid [20, 21]. However, they are not flexible in terms of volatility if liquid chromatography–mass spectrometry (LC–MS) is being used as a set of extended methods. In this study, the resolution of curcumin, demethoxycurcumin, and bisdemethoxycurcumin was well achieved by using a ratio of 50:50 between acetic acid (1%) and acetonitrile (Figure 1) which can be extended to LC–MS that requires a volatile solvent. This is a value-added to many other individual curcuminoids determination such as fluorometric detection [17], high-performance thin-layer chromatography (HPTLC) [22] and amino-bonded silica columns [23].

All the results of the present study disclosed the utilization of HPLC to estimate curcumin in both prepared casein-based and poloxamer-based micellar powder formulations by a simple procedure in terms of aspects including sample preparation and the analytical. The method might be appropriate for routine analysis of micellar products containing curcuminoids in a much larger scale.

## Conclusions

The developed HPLC method was validated for parameters including selectivity, linearity, accuracy and precision in order to determine curcumin in polymeric micellar powder formulations. The mobile phase composition was simple and easy to prepare with a fast run time which is appropriate for the analysis of samples in larger number. Therefore, this HPLC method can be used for curcumin analysis in samples those using micellar polymeric matrices such as casein-TPGS-sucrose, poloxamer, and/or eudragit.

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Hanni P. Puspitasari*, Dhita Fatmaningrum, Sa'adatus Zahro, Shofi Salsabila, Zulfia A. Rizqulloh, Ana Yuda, Mufarrihah, Anila I. Sukorini and Neny Purwitasari

# Challenges in the provision of natural medicines by community pharmacists in East Java Province, Indonesia

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**Abstract**

**Objectives:** Community pharmacist has been widely known as a health professional who can be easily accessed to provide medicines and reliable medicine information. However, this was not always in the case of dispensing natural medicines. Several international studies revealed that community pharmacists were less likely to deliver natural medicines accompanied with detailed information. Therefore, this study aimed to investigate factors influencing Indonesian community pharmacists in the supply of, delivery of, and provision of information about natural medicines.

**Methods:** A qualitative study with purposively selected community pharmacists in four areas (district or municipality) in East Java Province was designed. In-depth, semi-structured interviewed were conducted using a Capability-Opportunity-Motivation-Behaviour approach. All interviews were audio-recorded, transcribed *ad verbatim*, and thematically analysed.

**Results:** Data saturation was reached after interviewing 14 community pharmacists. All informants reported dispensing non-prescribed natural medicines. Nine had experienced dispensing prescribed natural medicines, mainly fulfilling paediatricians' requests. The most common information given was about product usage, while information about safety (i.e. side effects, interaction) was rarely provided. Although numerous registered natural medicines have been available, informants had low motivation to supply a variety of types, primarily because little opportunity to receive requests from doctors and the community. Limited capability due to a lack reliable source of information about natural medicines was another reason.

**Conclusions:** Poor motivation to supply natural medicines was because community pharmacists had little opportunity for such requests and limited capability due to scarcity of information. This indicated support from natural medicine manufacturers, researchers, and the government is highly required.

**Keywords:** community pharmacy; information; natural medicine.

***Corresponding author: Hanni P. Puspitasari**, Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62315933159, E-mail: hanni-p-p@ff.unair.ac.id

**Dhita Fatmaningrum, Sa'adatus Zahro, Shofi Salsabila, Zulfia A. Rizqulloh, Ana Yuda, Mufarrihah and Anila I. Sukorini,** Department of Pharmacy Practice, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

**Neny Purwitasari,** Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

## Introduction

The international literature has consistently reported the common use of natural medicines among health care professionals (79%) and lay persons (46%) [1–9]. The 2018 Indonesian national survey also showed that 32% people usually consumed traditionally-made natural medicines while 48% preferred to take industrial-made natural medicines [10]. The survey also revealed that about 58% people in East Java Province used natural medicines as an alternative treatment or for maintaining health. The high proportion of natural medicine users was reported because they agreed about its beneficial effects, they were under certain conditions, such as pregnancy or diagnosed with chronic diseases who felt unsafe to use conventional medicines, they never experienced side effects after taking natural medicines, the ingredients were available surround them, or due to their family traditions [1–3, 6–9] (Table 1).

In Indonesia, natural medicines have been classified into three groups, namely Jamu, Obat Herbal Terstandar (OHT) and Fitofarmaka (FF) [11]. Jamu is traditional medicine made from natural materials (plant, animal or mineral substances) in which the efficacy claim is proven based on empirical data. This group of natural medicines includes the one which are traditionally made by home industries, traditional medicine industries or formulated

**Table 1:** Characteristics of informants.

| Characteristics | Category | Number of informants | | | | |
|---|---|---|---|---|---|---|
| | | Area A, n=7 | Area B, n=3 | Area C, n=2 | Area D, n=2 | All areas, n=14 |
| Age | <30 years old | 4 | 1 | 2 | 1 | 8 |
| | ≥30 years old | 3 | 2 | 0 | 1 | 6 |
| Gender | Female | 4 | 3 | 1 | 2 | 10 |
| | Male | 3 | 0 | 1 | 0 | 4 |
| Type of community pharmacy | Independent | 3 | 2 | 1 | 1 | 7 |
| | Chain | 4 | 1 | 1 | 1 | 7 |

by pharmaceutical industries. OHT or standardised herbal medicines are natural medicines which efficacy claims are scientifically proven through pre-clinical studies. Meanwhile, the efficacy claims of FF or phytopharmaca are both scientifically and clinically proven through pre-clinical and clinical studies. Up to September 2020, the number of registered natural medicines in Indonesia was more than 11.000 jamu products, 71 OHT products and 24 FF products [12, 13].

Although there was a large number of registered natural medicines in Indonesia, a preliminary survey investigating East Java community pharmacists' practice in the area of natural medicines showed that the proportion of natural medicines supplied in the studied pharmacies to the number of registered natural medicines available in Indonesia was less than 1% Jamu, 11–16% OHT and 13–19% FF [unpublished work]. Other studies also found that despite the high proportion of users among pharmacists (65%), less than 15% reported dispensed natural medicines for their clients [1, 4]. Community pharmacists have been widely known as a health professional who can be easily accessed to provide medicines and reliable medicine information. However, some barriers in relation to natural medicines have been identified, including limited knowledge on and access to information. Little was known about barriers faced by East Java community pharmacists in this particular area. Therefore, this study aimed to investigate factors influencing East Java community pharmacists in the supply of, delivery of, and provision of information about natural medicines.

## Materials and methods

Historically, East Java Province was established by two main cultures, Javanese ethnic, which consisted of two subculture: Jenggala-Singhasari and Panjalu-Kadiri, and Madura tribe [14]. To minimise heterogeneity, a preliminary survey was conducted in four purposively selected areas representing Javanese ethnic, i.e. Surabaya and Lamongan (Jenggala-Singhasari) as well as Kediri and Tulungagung (Panjalu-Kadiri) [unpublished work]. Lists of community pharmacies from each area and permission to conduct the study were obtained from local pharmacy professional organisations. Approval from the National and Political Unity Agency, East Java Province was also received prior to the study (Number 070/6523/209.4/2019). Following up the survey findings, a qualitative study was designed to explore factors influencing community pharmacists' practice in the area of natural medicine services.

Data from all community pharmacists participating in the preliminary survey were tabulated to enable identification of data variability. Survey respondents with different characteristics (i.e. age, gender), type of pharmacy, and reported interesting responses were used as considerations to purposively select participants in the qualitative study. Selected community pharmacists were visited, explained about the study, and invited to participate. Those who expressed interest to take part were asked about time and venue for conducting a semi-structured interview at their convenience. Although face-to-face interviews were preferred, during pandemic Covid-19 some participants were willing to be interviewed using WhatsApp platform. Written consent was obtained from all participants prior to interviews.

A Capability-Opportunity-Motivation and Behaviour (COM-B) approach was used to guide all interviews [15]. Based on this approach, three essential conditions: i.e. capability, opportunity, and motivation affect behaviour (in the context of this study: community pharmacists' practice in supplying, delivering and providing information about natural medicines). Open-ended questions indicating each variable were listed in the interview guide, developed from the survey findings. Interviews were conducted until reaching data saturation. All face-to-face interviews were audio-recorded and transcribed *ad verbatim*, while records based on WhatsApp interviews were documented. Data were coded in parallel with interviews. Coding from each transcript was then verified at least by two investigators.

## Results

Data were considered reaching saturation after conducting 14 interviews. Equal proportion of participants was obtained from the type of pharmacy (independent vs. chain pharmacies). The majority of selected participants were female (n=10, 71.4%) and aged less than 30 years old (n=8, 57.2%), indicating a high proportion of female and young

practicing community pharmacists in East Java Province. Participants responses were classified into four COM-B key themes as outlined below.

## Behaviour

The survey results showed the average number of services using natural medicines either with or without prescription requests. However, detailed of such services were explored in the interviews.

Nine of 14 participants reported dispensing natural medicines based on prescription orders. However, the majority of such prescribers was paediatricians who asked for natural medicines for relieving cough or boost children immune system. During pandemic Covid-19, when paediatricians did not prescribe natural medicines, some participants recommended the one indicated as immunostimulants.

All participants had experienced dispensing natural medicines in response to individuals who requested specific products for self-medication purposes. The requests were mainly based on advertisements in mass media or experiences passing from mouth to mouth. Participants were unlikely to recommend natural medicines when individuals asked for products to relieve symptoms or cure disease. Participants believed that such conditions could be solved with conventional medicines as natural medicines were only for immune booster or complimentary purposes. In other cases, recommendations for natural medicines were not given because products for certain conditions were not available in the market or were not supplied in the pharmacy due to uncommon requests.

"*On average, people ask for a (natural medicine) product by showing the packaging … (we are) unlikely to recommend natural medicines unless the condition can only be relieved by using natural medicines … but if conventional medicines are available, I tend to give the conventional one because it gives quicker response*" [#2, 28 yo, female]

"*It's a bit hard when someone with cough and cold ask for a natural medicine … we only supply natural medicines for relieving cough (not cold symptoms) … in this case, I give a conventional medicine, but also suggest a natural medicine as complementary to boost the immune system*" [#7, 31 yo, female]

When delivering natural medicines either with or without prescriptions, almost all participants reported providing information about how to use the medicine. The source of such information was obtained from product packaging. The other information provided was about the purpose of medicine, particularly when the participants highlighted that natural medicines were only complementary. Meanwhile, information about safety, such as side effects and interaction, were rarely given. Some participants disclosed that they had difficulty in finding reliable source of information regarding natural medicine safety, unlike the one for chemical medicines that could be easily accessed from monographs or standard references. Furthermore, safety was not of great concern to people who consumed natural medicines. Therefore, most participants exhibited little efforts to spend much of their time to find relevant source of information.

"*Generally people choose to take natural medicines because almost 100% believe that the medicines do not have side effects, so they never ask (about its safety)*" [#8, 33 yo, female]

## Capability

Some participants stated that knowledge about natural medicines they obtained during formal education at university was enough despite superficial. In fact, when they were asked about definition and types of natural medicines available in Indonesia, the majority was unable to answer correctly.

Although they expected to upgrade knowledge, when there were little requests about the detailed, they did not feel enthusiastic to obtain further information. Moreover, source of information was limited from brochure or product packaging with no support from scientific publications or standard references. Searching literature from the Internet was considered time consuming, while training or seminars about natural medicines were almost never offered by manufacturers, pharmacy professional organisation or the health department.

"*Information was ideally received from (natural medicine) manufacturers, otherwise we find it difficult to provide explanation. Sometimes information brought by salesman was not accompanied with scientific evidence. At the end, we can only refer to limited information available at product packaging*" [#7, 31 yo, female]

## Opportunity

As described under theme "behaviour", requests of natural medicines were infrequently received from doctor prescriptions. Paediatricians were reported to often prescribe natural medicines for their patients. Certain doctors had also been identified to frequently prescribe particular natural medicines.

"*When dengue fever season comes, we supply a large number of psidii capsules as three doctors around here usually prescribed the medicines. Sometimes they also prescribe natural medicines for patients with hemorrhoid or kidney stones. We never receive natural medicine requests from other doctors*" [#3, 34 yo, female]

The majority of participants reported that natural medicines were rarely requested. Individuals who visited a community pharmacy expected to receive medicines that give immediate effects, unlike responses given by natural medicines. Only certain groups, such as pregnant women, old people and those with complex diseases tended to ask natural medicines that were assumed to be safe. Despite that, when chemical medicines were not available yet for certain conditions, people were willing to accept participants' suggestions to consume natural medicines.

"*People around here rarely accepted our recommendation about natural medicines or asked for them because natural medicines take a long time to heal. People tend to think that chemical medicines give instant healing, so it would be useless if we recommend natural medicines, unless for immune booster*" [#4, 27 yo, female]

"*Not many people would like to take natural medicines, it's just about 50%. But if they are pregnant, old or patients with complex diseases, (they) are likely to ask for natural medicines so (they will) not get side effects … (unless) there are no conventional medicines available, for example for a light cold*" [#1, 37 yo, female]

### Motivation

Some participants showed motivation to increase natural medicines usage particularly due to the potential for Indonesian biodiversity. However, two themes mentioned above indicated that the majority of participants had little opportunity to receive requests either from doctors (based on prescriptions) or individuals (for self-medication) and limited capability to provide information about natural medicines. As a consequence, the participants revealed low motivation to supply a variety types of natural medicines and looked for detailed information about natural medicines.

"*I think we need to switch to use natural medicines because (Indonesia) has a large number of natural ingredients potential for medicines so not only depending on chemical medicines. (Unfortunately) research development on natural medicines (in Indonesia) is not as advanced as China. (Therefore), we have to participate to support the increased use of natural medicines … however, it's not easy when people have inaccurate perception about natural medicines*" [#4, 27 yo, female]

"*Natural medicines in Indonesia are expected to be more popular because we have the potential. We need to have more clinical studies. If the number of phytopharmaca increased, pharmacists would be happy to maximise the trade*" [#9, 24 yo, female]

## Discussion

The findings of this study indicated that capability and opportunity seemed to influence community pharmacists' motivation to the supply of, the delivery of, and the provision of information about natural medicines. Meanwhile the three factors could affect community pharmacist behaviour in the area of natural medicines. Such relationships supported the COM-B system as defined by Michie et al. [15].

The supply of, and delivery of natural medicines depended largely on doctor and individual requests. Only a few and certain doctors were reported prescribing natural medicines. It is understandable because natural medicines were less likely to be taught to medical students [16]. Moreover, the concept of healing with natural medicines differed from that of chemical ones [17]. When the former is known to implement a holistic therapy in which helping the body's own healing process, the latter is understood to relieve symptoms through scientific pathology. As a consequence of receiving insufficient knowledge about natural medicines during formal education, doctors were unlikely to recommend the use of natural medicines in their practice.

Little requests about natural medicines from individuals seemed contradictory to the literature reporting a high proportion of natural medicine users [2, 3, 5–9]. However, it should be noted that there was a great number of people who preferred to use traditionally-made natural medicines. Meanwhile, people who visited community pharmacists tended to expect chemical medicines that give immediate beneficial effects. The reason for this was because chemical medicines are designed to generate a specific reaction, so the effect can be easily identified. In contrast, natural medicines have synergistic actions on physiological systems, so the effect is non-specific and hard to be pointed out [17].

In terms of community pharmacists' capability to provide information about natural medicines, the findings of this study were consistent with others that limited knowledge and access to reliable information were the main barrier [1]. In fact, the Indonesian Drug and Food

Control Agency has just published an informatory book about Indonesian original modern drug, referring to OHT and FF products [18]. As the publication occurred during Covid-19 pandemic, pharmacists may have only been aware that the reference focuses on immune booster products. As a result, information about OHT and FF products for several chronic conditions, such as diabetes mellitus and hypertension, has not been known.

Interestingly, this study participants showed interest to support the trade of phytopharmaca, natural medicines with scientific and clinical evidence. Thus, suggestions that natural medicine manufacturers should offer reliable and accessible resources were prominent [19]. This indicates that natural medicines researchers attributed responsibility for the dissemination of scientific evidence. Finally, to help improve practicing community pharmacists' knowledge, pharmacy professional organisations need to offer seminars and continuing professional education programs in the area of natural medicines.

## Conclusions

Little opportunity for natural medicines requests either from doctors or the community as well as limited community pharmacists' capability to provide appropriate information due to scarcity of sources of reliable information contributed to poor motivation to supply natural medicines. It is clear that practicing community pharmacists require support not only from natural medicine manufacturers and researchers, but also from the government and even pharmacy professional organisations.

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**Author contributions:** Detailed contributions of each author in the study are as follows:

- HP Puspitasari: designed the study, verified data collected from all study sites, analysed data, drafted & finalised manuscript for publication.
- D Fatmaningrum: revised interview guidance; collected, verified, and analysed data from one of study sites (Kediri), provided final approval of manuscript for publication.
- S Zahro: revised interview guidance; collected, verified, and analysed data from one of study sites (Lamongan), provided final approval of manuscript for publication.
- S Salsabila: revised interview guidance; collected, verified, and analysed data from one of study sites (Surabaya), provided final approval of manuscript for publication.
- ZA Rizqulloh: revised interview guidance; collected, verified, and analysed data from one of study sites (Tulungagung), provided final approval of manuscript for publication.
- A Yuda: designed the study; verified and analysed data from one of study sites (Lamongan), provided final approval of manuscript for publication.
- Mufarrihah: designed the study; verified and analysed data from one of study sites (Surabaya), provided final approval of manuscript for publication.
- AI Sukorini: designed the study; verified and analysed data from one of study sites (Kediri), provided final approval of manuscript for publication.
- N Purwitasari: designed the study; verified and analysed data from one of study sites (Tulungagung), provided final approval of manuscript for publication.

All authors have provided agreement to be accountable for all aspects of the work.

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**Informed consent**: Informed consent was obtained from all individuals included in this study.

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Agnis P.R. Aditama, Burhan Ma'arif, Hening Laswati and Mangestuti Agil*

# *In vitro* and *in silico* analysis of phytochemical compounds of 96% ethanol extract of semanggi (*Marsilea crenata* Presl.) leaves as a bone formation agent

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**Abstract**

**Objectives:** Osteoporosis is the result of an imbalance in the rate of bone resorption and bone formation due to a decrease in estrogen. Phytoestrogens are plant compounds with structures and functions similar to estrogen. Phytoestrogens that bind to estrogen receptors in bone cells are able to modulate bone formation. Semanggi (*Marsilea crenata* Presl.) is a plant that contains phytoestrogens. The purpose of this study was to observe the expression of osteocalcin and predict the content of extract phytoestrogens through a computer simulation study to study the bone formation activity of the 96% ethanol extract of *M. crenata* leaves on hFOB 1.19 cells.

**Methods:** hFOB 1.19 cells were cultured in 24-well microplates, and 96% ethanol extract of *M. crenata* Presl. leaves was added at 62.5, 125 and 250 ppm. The expression of osteocalcin was analyzed using CLSM immunocytochemistry. Using PyRx 0.8 software and 1ERE protein for molecular docking, the compound was analyzed by computer.

**Results:** The 96% ethanol extract of *M. crenata* Presl. leaves can increase the expression of osteocalcin, the optimal dose is 125 ppm, and $p<0.05$ is 881.658 AU. *In silico* study was obtained six compounds that showed similar activity 17β-estradiol as ER-β agonists.

**Conclusions:** The 96% ethanol extract of *M. crenata* Presl. leaves contain six compounds that are thought to be phytoestrogens and ER-β agonists, and play a role in increasing bone formation activity and have the potential to be used as an oral drug.

**Keywords:** bone formation; hFOB 1.19 cell; *Marsilea crenata* Presl; osteocalcin; phytoestrogens.

***Corresponding author: Mangestuti Agil**, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia, Phone: +62 81331921251, E-mail: mmangestuti@yahoo.com

**Agnis P.R. Aditama,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia; and Department of Pharmacy, Pharmacy Academy of Jember, Jember, Indonesia

**Burhan Ma'arif,** Department of Pharmacy, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia

**Hening Laswati,** Department of Physical Medicine and Rehabilitation, Universitas Airlangga, Surabaya, Indonesia

## Introduction

Osteoporosis is the result of an imbalance in the rate of bone resorption and bone formation due to decreased estrogen [1, 2]. At any age, especially after menopause, bone loss is common after estrogen production stops [3].

Semanggi (*Marsilea crenata* Presl.) is a plant that grows in East Java, Indonesia and is used as a typical food in the Surabaya region [4]. Clinical testing of *M. crenata* Presl. by radioimmunoassay (RIA) method showing extract of *M.* crenata Presl. has a compound with similar activity to 17β-estradiol [5]. Addition of *n*-hexane of *M. crenata* Presl. on osteoblast MC3T3-E1 cells which gave the result of increased proliferation and differentiation of osteoblasts [6, 7]. UPLC-QToF-MS/MS analysis shows that the 96% ethanol extract of *M. crenata* Presl. leaves contains 75 compounds, namely known compounds (59 compounds) or unknown compounds (16 compounds) [8].

Research shows that *M. crenata* Presl. potentially can be a source of phytoestrogens. Phytoestrogens are plant compounds with structures and functions similar to estrogen [9, 10]. Phytoestrogens that bind to estrogen receptors-β (ER-β) in the bone can modulate bone formation, such as genistein [9]. Genistein is a phytoestrogen that can increase the production of osteocalcin in the classic bone formation markers, a product that is synthesized by osteoblasts [11]. Osteocalcin is released by osteoblasts during bone formation and binds to the mineralized bone matrix [12].

Based on the many studies that have been done on *Marsilea crenata* Presl. the results are limited to testing some activities and have not shown the compounds that play a role in the effect of these activities, so an *in vitro*

study should be carried out with the observation of increased osteocalcin as a marker, and ER-β agonist prediction of phytoestrogen compounds in *Marsilea crenata* Presl. through the *in silico* studies. The purpose of this study was to observe the expression of osteocalcin and predict the content of extract phytoestrogens through a computer simulation study to study the bone formation activity of the 96% ethanol extract of *Marsilea crenata* leaves on hFOB 1.19 cells.

# Materials and methods

## Materials

The leaves of *M. crenata* Presl. were collected in Benowo, Surabaya, Indonesia and identified in UPT Materia Medica in Batu. The specimen number is 1a17b-18a-1. The leaves are prepared to obtain dried *M. crenata* Presl. leaves powder. Human fetal osteoblasts hFOB 1.19 (CRL-11372) were purchased from American Type Cell Culture (ATCC). The 96% ethanol was purchased from Merck (Merck, Darmstadt, Germany). Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), paraformaldehyde (PFA), and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich. Anti-mouse osteocalcin (OCN) was purchased from Abcam. G418, Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin 1%, fetal bovine serum (FBS) were purchased from Aretha Laboratory, Bandung, Indonesia. Tween-80, PFA were purchased from Laboratorium Sentral Ilmu Hayati (LSIH) at Universitas Brawijaya, University of Malang, Indonesia.

## Methods

**Extraction:** The ultrasonic-assisted extraction method (Soltec Sonica 5300EP S3, Italy) used on *M. crenata* Presl. The leaf powder was treated with 96% ethanol. The process was repeated and the supernatant was collected, and then the solvent was evaporated using a rotary evaporator (Heidolph Hei-VAP G3, Germany) to obtain a 96% ethanol extract.

**Cell culture:** The hFOB 1.19 cells were put in a 75 mL flask and cultured in a complete medium prepared from a mixture of DMEM, G418, 1% penicillin–streptomycin, 10% FBS, and incubated for 6 days at 37 °C in a 5% CO2 incubator. During the process, cell development was observed for 24 h, and the culture medium was replaced at any time if color is changed due to the depletion of nutrients. After reaching 80–90% of confluency, cells were seeded on a 24-well microplate.

**Osteocalcin measurement:** The solution of 96% ethanol extract with concentrations of 62.5, 125, and 250 ppm [7, 10, 13] obtained from 50 mg of each concentration mixed with 0.5% DMSO and 0.5% Tween-80 in a volumetric flask produce 5,000 ppm, then sterilized using a 0.22 µm millipore filtration method. Well containing medium (DMEM, G418, 1% Penicillin-streptomycin, 10% FBS) and hFOB 1.19 cells were used as negative controls. All solutions mixed with hFOB 1.19 cell with six repetitions in a 24-well microplate, and incubate for 48 h at 37 °C in a 5% CO2 incubator. Then 4% PFA was added and mixed, followed by 10% BSA. PBS was used to rinse the cells in every step of the treatment. The anti-mouse osteocalcin was added to microplates and incubated at 4 °C for 24 h. Microplates rinsed with PBS, and anti-mouse rhodamine was added, then incubate in a dark room at 37 °C for 1 h. Immunofluorescence analysis was conducted using CLSM (fluoview Olympus FV1000) at wavelengths of 543 nm. The immunofluorescence of markers was analyzed using Olympus Fluoview Ver.4.2a. software to determine the value of osteocalcin expression.

***In silico* study:** The ER-β receptor structure used in this study was obtained from the protein database (http://www.rcsb.org) with the code 1ERE [14]. Preliminary preparations were made using 2016 Biovia Discovery Studio Visualizer to separate the 17β-estradiol as an internal ligand from the protein to understand the appropriate application to use and prepare the target protein for the next tethering molecule. Using SwissADME and boiled-EGG web applications, 59 secondary metabolites in 96% ethanol extract of *M. crenata* Presl. were prepared to predict the phytochemical composition and determine the permeability of these compounds [8, 15, 16]. One of the efforts that can be done is related to the structure of the compound to Lipinski's rule of five, which is related to the properties of the compound that needs to be further developed because it has potential in treatment [17]. Lipinski's five criteria: molecular size less than 500 molecular weight, 10 hydrogen bond acceptors, five hydrogen bond donors, five solubility log P, 10 rotatable bonds molecular flexibility [18]. Use Avogrado 1.90.0 to determine internal ligands and compounds, and use MMF94s method for energy optimization. Then use PyRx 0.8 and Vina Wizard for molecular docking simulation [19, 20]. Use 2016 Biovia Discovery Studio Visualizer to visualize receptor–ligand complexes from simulated docking.

# Results

## Osteocalcin measurement

The 1.6 kg of *M. crenata* Presl. leaves powder was extracted with 96% ethanol to obtain 70 g of extract. Increasing of osteocalcin expression was performed using the immunocytochemical (ICC) as a result of the test, compared with the negative control group, all treatment groups with 96% ethanol extract *M. crenata* Presl. dose can increase the expression of osteocalcin, $p<0.05$. Visualization of osteocalcin expression in hFOB cells (Figures 1 and 2), while the results of the analysis of osteocalcin expression (Table 1). A dose of 62.5 ppm could increase the expression of osteocalcin by 215.607 AU, a dose of 125 ppm of 881.658 AU, and a dose of 250 ppm of 502.086 AU. From these data, it is known that a dose of 125 ppm produces the highest expression of osteocalcin and indicates a non-monotonic dose response (NMDR) profile which is shown by different slope values on the graph at several points within the given concentration range.

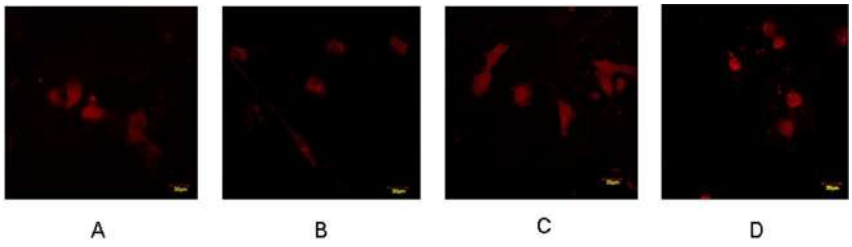


**Figure 1:** Osteocalcin expression, (A) negative control, (B) 62.5 ppm, (C) 125 ppm, (D) 250 ppm.

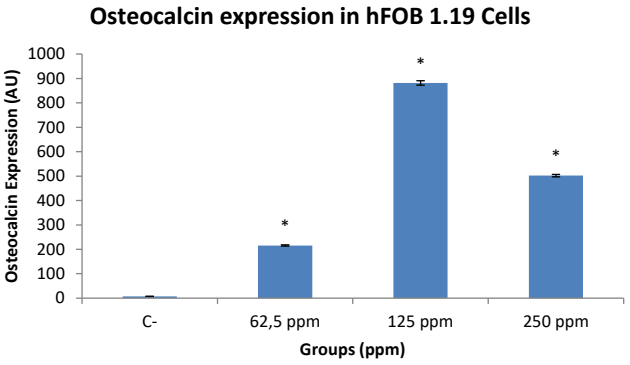


**Figure 2:** Osteocalcin expression in hFOB 1.19 cells. (*) Significantly different from the negative control.

**Table 1:** Osteocalcin expression in hFOB 1.19 cells.

| Treatment dose | Osteocalcin expression (AU) ± SD |
|---|---|
| Negative control | 7.316 ± 0.039 |
| 62.5 ppm | 215.607 ± 1.797 |
| 125 ppm | 881.658 ± 6.549 |
| 250 ppm | 502.086 ± 0.706 |

### *In silico* study

The result of molecular docking internal ligan 17β-estradiol with 1ERE protein (Figure 3). *In silico* test was carried out with physicochemical parameters and six compounds whose ER-β agonists (Table 2). A compound is said to be an agonist if it must be able to interact with amino acids HIS (D: 524), ARG (D: 394), and GLU (D: 353).

## Discussion

Osteocalcin is the main and most common non-collagen protein from adult human bones which is a γ-carboxyglutamic acid (GLA) residue, which is produced by osteoblasts and secreted into the bone microenvironment. Osteocalcin has a high affinity for calcium which then undergoes a change in the alpha-helix conformation depending on calcium, where osteocalcin binds to calcium in hydroxyapatite (Ha) and forms Ha crystals and encourages Ha absorption in the bone matrix. This process is a mechanism that allows the presence of osteocalcin as a marker to initiate the formation of Ha crystals. The Ha crystals are arranged uniaxially along the collagen fibers, and the c-axis is parallel to the longitudinal direction of the bone. The position and highly regular orientation of the very small Ha-nanocrystals located within the collagen fibers also significantly contribute to the structural rigidity and strength of bones. In this way bone mineralization and bone formation processes occur. Osteocalcin is one of the markers in the bone formation process produced by osteoblasts so that the increased expression of osteocalcin indicates an increase in the bone formation process [11, 12, 21–24]. Osteocalcin is very representative when it is used as a marker of increased osteoblast activity. In this study, *M. crenata* Presl. leaves extract at various concentrations can increase the bone formation process which is shown to increase expression of osteocalcin in hFOB 1.19 cells and is suspected due to the presence of phytoestrogens.

The mechanism of estrogen and phytoestrogens increases bone formation through receptors (dependent) and without through receptors (independent). The dependent pathway is through binding to the estrogen receptor-α (ER-α) or estrogen receptor-β (ER-β) contained in the cell. The binding of estrogen and its receptor will activate the estrogen receptor. Activation of estrogen receptors will bind to estrogen response elements (ERE) which can stimulate the transcription factors Runx2 and osterix and synthesize certain proteins such as osteocalcin [10, 25].

In this study, the activity of increasing bone formation was indicated by an increase in osteocalcin expression. But the increased dose of the extract was not accompanied by an increase in osteocalcin expression, this phenomenon is called a non-monotonic dose-response effect (NMDR) (seen in Tabel 1). It is known that a dose of 125 ppm produces the highest expression of osteocalcin and indicates a NMDR profile which is shown by different slope values on the graph at several points within the given concentration range. The cause of the non-linear activity response can occur due to several factors, namely cytotoxicity, receptor selectivity, and receptor downregulation. One possible

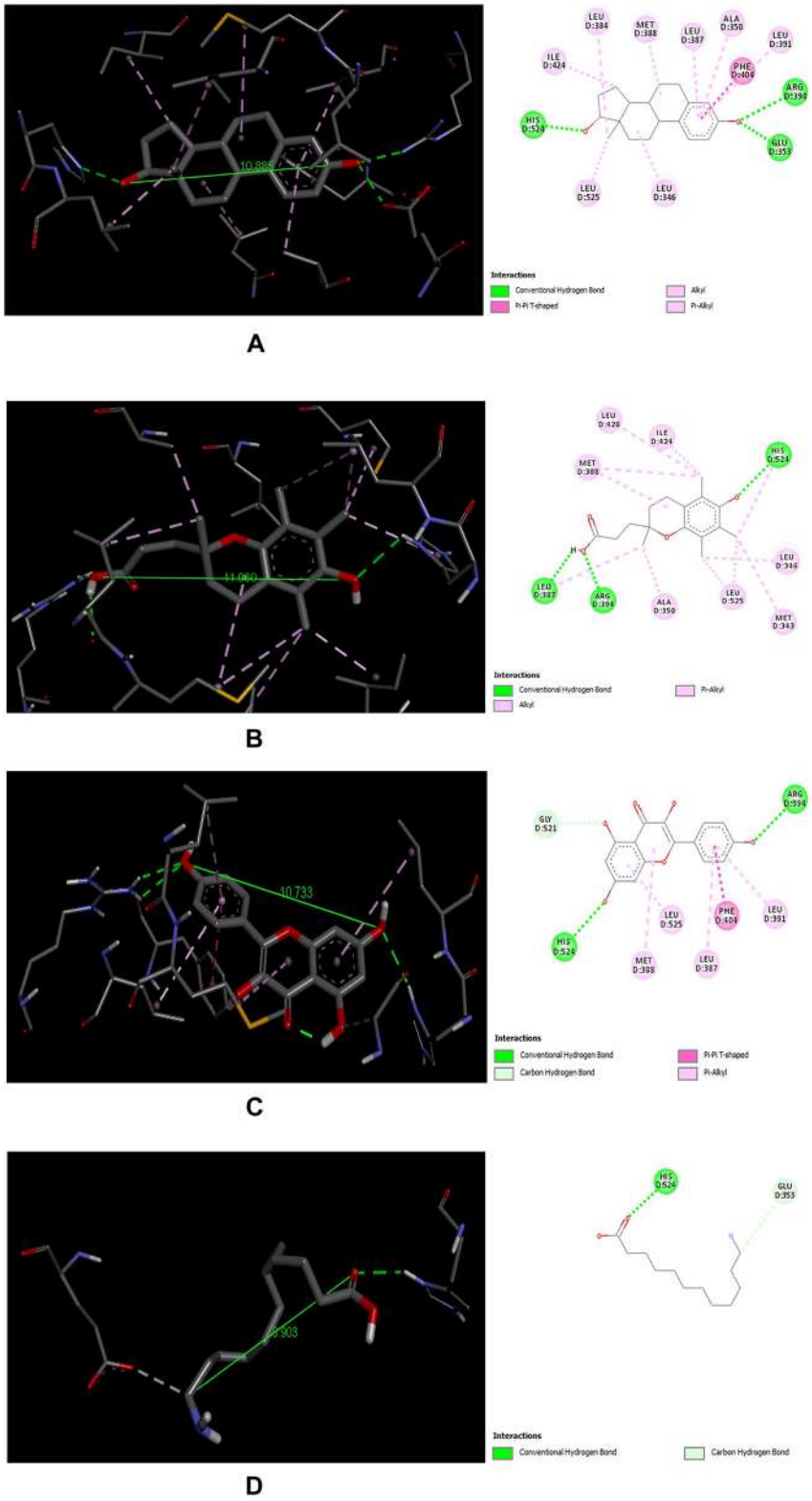


**Figure 3:** The interaction of internal ligand (A) (17β-estradiol) with 1ERE protein, (B) 3-(6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-chromen-2-yl)propanoic acid, (C) kaemferol, (D) 3. 12-aminododecanoic acid, (E) cyclazocine, (F) morin, (G) (3R,4R)-3-{[(2-Hydroxyethyl)(methyl)amino]methyl}-4-(hydroxymethyl)-N-isopropyl-N-methyl-1-pyrrolidinesulfonamide.

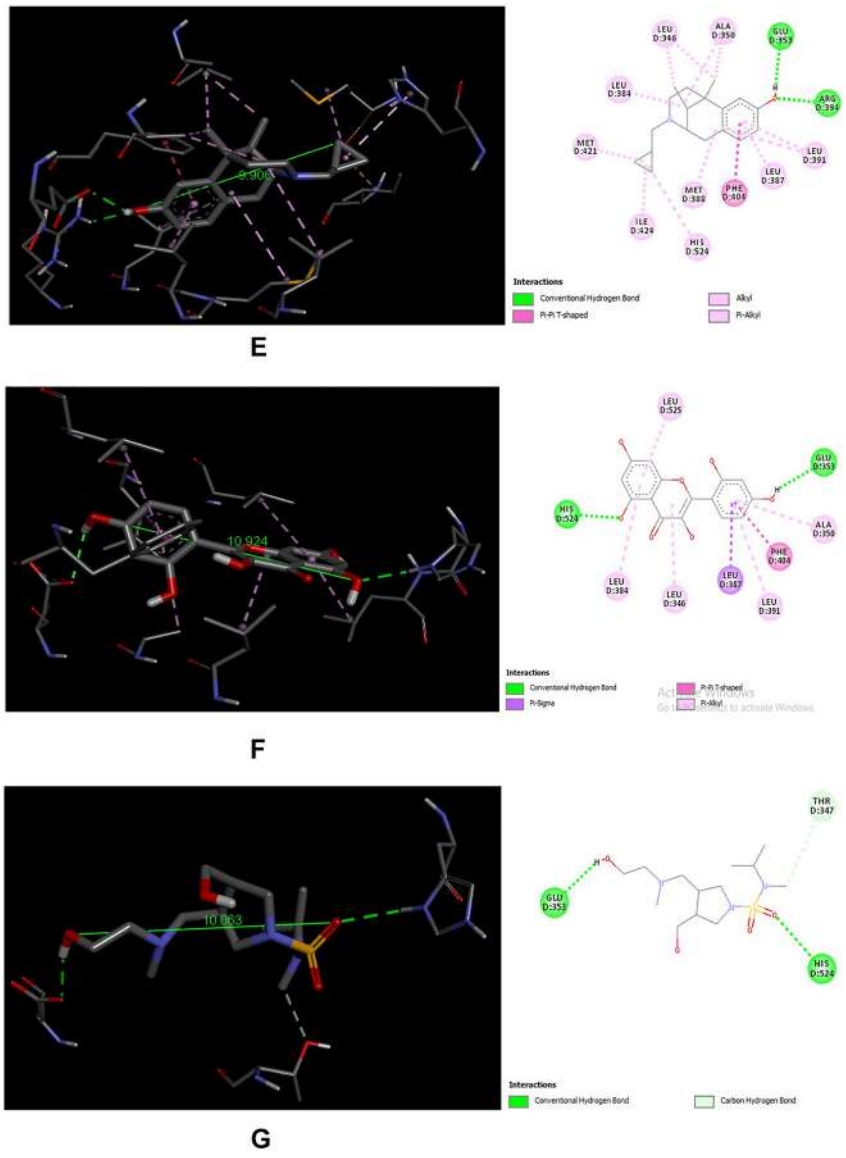


**Figure 3:** Continued.

explanation for this was related to the NMDR effect, in which non-linear activity responses in increased concentrations often occurs *in vitro* activity tests, as well as in studies with several hormones or hormone replacement compounds. Differences in affinity levels between hormones and hormone replacement compounds to target proteins such as receptors, cause responses to be difficult to predict despite increased doses of treatments [13]. This happens because increasing the concentration of the sample can cause the bound compound to make ER insensitive to bind to the compound. But, when increasing the concentration continuously can increase the amount of ER that is degraded and is not proportional to the ER produced, so that it can cause cells to produce ER massively and can increase the bonding of compounds with ER and activity response [26–29].

Based on the study of Ma'arif et al. [8], the 96% ethanol extract of *M. crenata* Presl. contains 75 compounds, either known compounds (59 compounds) or unknown compounds (16 compounds). Known compounds are compounds that exist in the ChemSpider database. Unknown compounds may be considered impure compounds that can still be detected by the instrument, or they may be new compounds that cannot be detected in the ChemSpider database, especially unknown compounds at high concentrations. If the compound can bind with at least two amino acids HIS (D: 524), ARG (D: 394), and GLU (D: 353) then it is an ER agonist,

**Table 2:** Agonist compounds of 96% ethanol extract of *M. crenata* Presl.

| No. | Compound name | Binding affinity | % Area [8] | Amino acid and bond type | Pharmacophore distance | Lipinski's rule of five |
|---|---|---|---|---|---|---|
| 1 | 3-(6-hydroxy-2,5,7,8-tetramethyl-3, 4-dihydro-2H-chromen-2-yl)propanoic acid | −8.3 | 0.1695 | His 524 (Hydrogen); Arg 394 (Hydrogen) | 11.060 | Yes |
| 2 | Kaempferol | −5.3 | 0.1725 | His 524 (Hydrogen); Arg 394 (Hydrogen) | 10.733 | Yes |
| 3 | 12-Aminododecanoic acid | −5.9 | 0.3283 | His 524(Hydrogen); Glu 353 (Carbon) | 8.903 | Yes |
| 4 | Cyclazocine | −8.2 | 1.7700 | His 524 (Pi-Alkyl); Glu 353 (Hydrogen); Arg 394 (Hydrogen) | 9.906 | Yes |
| 5 | Morin | −7.3 | 1.3135 | His 524 (Hydrogen); Glu 353 (Hydrogen); | 10.924 | Yes |
| 6 | (3R, 4R)-3-{[(2-hydroxyethyl)(methyl) amino]methyl}-4-(hydroxymethyl)-N-isopropyl-N-methyl-1-pyrrolidinesulfonamide | −6.2 | 0.1188 | His 524 (Hydrogen); Glu 353 (Hydrogen) | 10.063 | Yes |

and the smaller the binding affinity value, the more stable the compound and gives the same effect as 17β-estradiol [18, 20, 30], then tested the compounds against the Lipinski's rule criteria. Lipinski's rule of five is a rule of thumb used to assess the similarity of drugs or whether a compound with a certain pharmacological or biological activity has the characteristics that make it an orally active drug for humans [18]. The 96% ethanol extract of *M. crenata* Presl. leaves contains six compounds, which fully meets Lipinski's five rules, so it is recommended as a safe oral active drug.

From the *in vitro* results, it is well known that the 96% ethanol extract of *M. crenata* Presl. The leaves have a strong potential to increase bone formation by increasing osteocalcin. This activity is thought to be due to the presence of phytoestrogen compounds, the predicted compounds for phytoestrogens are 3-(6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydro-2H-chromen-2-yl) propanoic acid, kaempferol, 12-aminodecanoic acid, cyclazocine, morin, (3R, 4R)-3-([(2-hydroxyethyl) (methyl) amino] methyl)-4-(hydroxymethyl)-*N*-isopropyl-*N*-methyl-1-pyrrolidinesulfonamide.

## Conclusions

The 96% ethanol extract of *M. crenata* Presl. leaves have a bone formation activity through the increased osteocalcin expression with optimum dose at 125 ppm by 881.658 AU. There are six compounds predicted as ER-β agonists and they have an *in silico* activity similar to 17β-estradiol as a bone formation agent. It gives an indication that the content of phytoestrogens in the 96% ethanol extract of *M. crenata* Presl. leaves may be related to bone formation, so it is recommended as a safe oral active medicine.

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Yudi Purnomo*, Juliah Makdasari and Faiqoh Inayah Fatahillah

# Inhibitory activity of *Urena lobata* leaf extract on alpha-amylase and alpha-glucosidase: *in vitro* and *in silico* approach

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**Abstract**

**Objectives:** In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzymes that are responsible to convert a carbohydrate into glucose. Inhibition of both enzyme activities can prolong absorption of glucose in intestine and reduce post-prandial increase of blood glucose concentration, thus, it is beneficial for type-2 diabetes treatment. Traditionally, *Urena lobata* (*U. lobata*) has been used to manage diabetes, but the scientific proof of this claim remains scarce. Therefore, the objective of this study to examine the anti-diabetic potential of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.

**Methods:** *U. lobata* leaf extract was obtained through extraction process using ethanol and the chemical compounds in the extract were analyzed by liquid chromatography–mass spectra (LC–MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase was evaluated by *in silico* using docking server, whereas *in vitro* enzymatic assays were using *para*-nitrophenyl-α-D-glucopyranoside (α-NPG) and starch as substrates. The data were presented as mean ± SD and the $IC_{50}$ value was calculated using SPSS.

**Results:** *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the $IC_{50}$ value was 43.73 and 83.73 μg/mL, respectively, meanwhile, acarbose as standard has $IC_{50}$ value at 1.14 and 0.08 μg/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on inhibition constant (Ki) value.

**Conclusions:** Ethanolic extract of *U. lobata* showed inhibition activity on α-glucosidase stronger than on α-amylase as antidiabetic.

**Keywords:** anti-diabetic; enzyme; metabolism; molecular docking; polysaccharides.

***Corresponding author: Yudi Purnomo**, Faculty of Medicine, University of Islamic Malang, Malang, Indonesia, E-mail: y_purnomo92@yahoo.com
**Juliah Makdasari and Faiqoh Inayah Fatahillah,** Faculty of Medicine, University of Islamic Malang, Malang, Indonesia

## Introduction

Carbohydrate metabolism is regulated by enzymes that break polysaccharides into monosaccharides. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzymes that are responsible for the conversion of a starch complex into a simple starch [1]. α-Amylase is produced by the salivary glands and pancreatic glands, which metabolizes starch into maltosa, dextrin, and maltotriosa. These products are then delivered in to the small intestinal mucosa, where they are then hydrolyzed by α-glucosidase and results in glucose which are absorbed into the blood [2]. It contributes to an increase in blood glucose level post-prandial, which needs to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduce carbohydrate metabolism and glucose absorption, and thus, preventing an increase of blood glucose level, beneficial especially in patients with diabetes mellitus [1, 2]. Herbal remedies are one of the choices to suppress activity of both α-amylase and α-glucosidase in the therapy of diabetes mellitus. Some benefit of herbal remedies includes less adverse reaction and is relatively cheaper. Most antidiabetic herbal remedies work through inhibitory activity of α-amylase and α-glucosidase [3].

Pulutan (*Urena lobata* [*U. lobata*]) is an herbal remedy used by Nigerian people to manage many diseases, including diabetes mellitus [4]. This plant has a bitter taste, which is the basis of the intuition that it may be able to treat diabetes. Traditional healers used *U. lobata* in both in single and in combination with other herbs to manage diabetes. Research indicates that the administration of *U. lobata* leaf and roots extracts has hypoglycemic activity on rats induced with streptozotocin [4, 5]. This is due to several active compounds in the herbs, such as sterol groups, alkaloids, and flavonoids [6, 7]. Antidiabetic

activity of *U. lobata* leaf extracts has not been evaluated, especially regarding its α-amylase and α-glucosidase inhibitory activity. The objective of the study was to evaluate antidiabetic potency of *U. lobata* through inhibitory activity of α-glucosidase and α-amylase.

# Materials and methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase, meanwhile *para*-nitrophenyl-α-D-glucopyranoside (α-NPG), starch substrate, dinitrosalicylic acid (DNSA), Gly-pro-*p*-nitroanilide (GPPN), and Tris-HCl buffer were purchased from Sigma Aldrich, meanwhile ethanol was from Merck (pro analysis grade).

### Sample preparation

Leaves of *U. lobata* were obtained from Balai Materia Medika Batu, Malang, Indonesia, with a certificate number of 074/027/101.8/2015. About 50 g of the sample materials were extracted in 250 mL ethanol 80% for 4 h using water bath shaker. The extraction was repeated for another two times using fresh solvent. The extracts were evaporated using a rotary evaporator to produce a paste form (weight) and diluted with solvent according to the designated concentration.

### Identification of active substances

Ethanolic extract of *U. lobata* leaf was subjected to a qualitative analysis using liquid chromatography–mass spectra (LC–MS) Accela 1250 pump. Mobile phase contains 0.1% formic acid in methanol and water combination. The identification included the 10 active substances, considered based on previous study compounds including phytosterol, flavonoid, and alkaloid groups.

### α-Amylase inhibitory assay

About 100 µL of sample or standard solution was added to 150 µL of 5 unit/mL α-amylase and 20 mM of phosphate buffer pH 6.9. This mixture was incubated at 37 °C for 30 min followed by addition of 250 µL of 1% starch in distilled water. The mixture was incubated again for 10 min at 37 °C. Then, 50 µL of 1% DNSA was added and the mixture was heated on a water bath. The solution was cooled down to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as standard.

### α-Glucosidase inhibitory assay

Various concentrations of test material or standard were mixed with 320 µL 100 mM phosphate buffer pH 6.8 and 50 µL of 10 mM α-NPG. This mixture was incubated at 30 °C for 5 min, followed by addition of 20 µL of α-glucosidase in phosphate buffer. The mixture was incubated for another 5 min at 30 °C and the reaction was stopped by adding 3 mL of 50 mM sodium hydroxide. The sample absorbance was measured at 410 nm with a microplate reader. Acarbose was used as a standard.

### Molecular docking study

Activity of identified substances in *U. lobata* leaf extracts both on *α-amylase* (P0DTE8) and *α-glucosidase* (O43451) was evaluated by *in silico* approach using a web-based software application (www.dockingserver.com). The structures of identified compounds were obtained from PubChem database and protein target (enzymes) from Uniprot database. Molecular docking was performed by uploading both chemical compound and protein target on software. The prediction value of parameters include inhibitory constant (Ki), free energy of binding and surface interactions.

### Statistical analysis

The $IC_{50}$ value was calculated by linear regression curve fit using SPPS version 16.0 and the data are presented as the mean ± SD.

# Results

## Identification of active substances in *U. lobata* leaf extracts

The active compounds from ethanolic extract of *U. lobata* leaf can be seen in Figure 1 and Table 1.

The qualitative analysis using LC–MS indicated that the most abundant secondary metabolites in *U. lobata* leaf extract was gossypetin and stigmasterol. Other compounds such as β-sitosterol, chrysoeriol, and mangiferin were also identified in the extracts of *U. lobata*; however, the concentration was low.

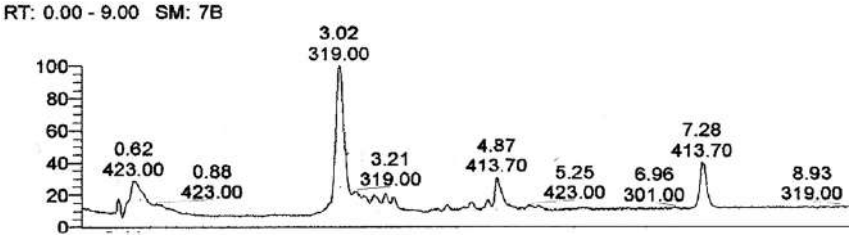


**Figure 1:** Chromatogram of *U. lobata* leaf extract identified by LC–MS [1–29].

**Table 1:** Active compounds in *U. lobata* leaf extracts.

| No | Active compounds | Molecule weight | Ethanolic extract |
|---|---|---|---|
| 1 | Stigmasterol | 413 | (++) |
| 2 | β-Sitosterol | 415 | (+) |
| 3 | Mangiferin | 423 | (+) |
| 4 | Quercetine | 303 | (–) |
| 5 | Kaempferol | 286 | (–) |
| 6 | Hypolaetin | 302 | (–) |
| 7 | Gossypetin | 318 | (+++) |
| 8 | Luteolin | 286 | (–) |
| 9 | Apigenin | 270 | (–) |
| 10 | Chrysoeriol | 300 | (+) |

(–): negative, (+): weak, (++): moderate, (+++): strong.

## Molecular docking of *U. lobata* on α-amylase and α-glucosidase

The *in silico* inhibitory activity of the identified compounds in *U. lobata* leaf extract on α-amylase and α-glucosidase results can be seen at Tables 2 and 3.

Molecular docking studies indicated that stigmasterol, β-sitosterol, and mangiferin have low inhibition constant and free energy of binding; however, the surface interaction showed high value. Meanwhile, gossypetin and chrysoeriol have higher values on binding free energy and inhibition constant as compared to stigmasterol, β-sitosterol, and mangiferin. The differences in each parameter value signified distinct inhibitory activity both of on α-glucosidase and α-amylase activities. Based on inhibition constant, stigmasterol and β-sitosterol showed higher inhibition activity toward both α-glucosidase and α-amylase. These activities are more prominent than acarbose, especially on α-amylase.

**Table 2:** Molecular docking of active substances in *U. lobata* leaf extracts with *α-amylase*.

| No | Active compounds | Estimation free energy of binding, Kcal/mol | Estimation of inhibition constant Ki, μM | Interaction surface |
|---|---|---|---|---|
| 1 | Stigmasterol | –9.63 | 0.0875 | 892.16 |
| 2 | β-Sitosterol | –8.66 | 0.4500 | 793.55 |
| 3 | Mangiferin | –7.84 | 1.80 | 702.30 |
| 4 | Gossypetin | –6.40 | 20.36 | 621.77 |
| 5 | Chrysoeriol | –6.50 | 17.13 | 653.91 |
| 6 | Acarbose | –8.78 | 0.3470 | 1,087.32 |

**Table 3:** Molecular docking of active substances in *U. lobata* leaf extracts with *α-glucosidase*.

| No | Active compounds | Estimation free energy of binding, Kcal/mol | Estimation of inhibition constant Ki, μM | Interaction surface |
|---|---|---|---|---|
| 1 | Stigmasterol | –10.20 | 0.0331 | 891.01 |
| 2 | β-Sitosterol | –9.59 | 0.0931 | 894.65 |
| 3 | Mangiferin | –7.98 | 1.4100 | 764.01 |
| 4 | Gossypetin | –7.20 | 5.2400 | 687.04 |
| 5 | Chrysoeriol | –6.70 | 12.190 | 705.30 |
| 6 | Acarbose | –0.3.98 | 0.0012 | 457.77 |

## α-Glucosidase inhibitory activity and α-amylase of *U. lobata* leaf extract

The inhibitory activity of ethanolic extract of *U. lobata* leaf on α-amylase and α-glucosidase were evaluated *in vitro* using enzymatic assay and the results are shown in Tables 4 and 5.

Based on these results, the inhibition activity of the ethanolic extract of *U. lobata* on α-glucosidase was two times stronger than α-amylase. However, the inhibitory activities are lower than acarbose.

# Discussion

## Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves have been reported to contain non nutritional substances having pharmacological effects [8, 9]. These include flavone or flavonol compounds, such as gossypetin, which was shown to be an effective antioxidant and possess antimicrobial, anti

**Table 4:** *α-Amylase inhibitory* activity of *U. lobata* leaf extracts and acarbose.

| Group | Sample | n | Concentration, μg/mL | % inhibition | $IC_{50}$, μg/mL |
|---|---|---|---|---|---|
| 1 | Ethanolic | 3 | 6.25 | 20.62 ± 3.79 | 83.73 |
| | extract of | 3 | 12.50 | 22.36 ± 1.24 | |
| | *U. lobata* | 3 | 25.00 | 27.33 ± 1.24 | |
| | | 3 | 50.00 | 40.99 ± 4.35 | |
| | | 3 | 100.00 | 54.65 ± 8.14 | |
| 2 | Acarbose | 3 | 0.05 | 47.77 ± 0.99 | 0.08 |
| | | 3 | 0.10 | 53.40 ± 3.05 | |
| | | 3 | 0.15 | 58.74 ± 0.44 | |
| | | 3 | 0.20 | 61.04 ± 2.48 | |
| | | 3 | 0.25 | 61.47 ± 4.12 | |

**Table 5:** *α-Glucosidase inhibitory* activity of *U. lobata* leaf extracts and acarbose.

| Group | Sample | n | Concentration, µg/mL | % inhibition | $IC_{50}$, µg/mL |
|---|---|---|---|---|---|
| 1 | Ethanolic | 3 | 6.25 | 20.00 ± 0.00 | 43.73 |
| | extract of | 3 | 12.5 | 30.00 ± 0.00 | |
| | *U. lobata* | 3 | 25.0 | 45.00 ± 0.00 | |
| | | 3 | 50.0 | 60.00 ± 3.85 | |
| | | 3 | 100.0 | 80.00 ± 3.85 | |
| 2 | Acarbose | 3 | 0.25 | 16.37 ± 11.52 | 1.14 |
| | | 3 | 0.50 | 29.09 ± 0.11 | |
| | | 3 | 0.75 | 31.98 ± 6.85 | |
| | | 3 | 1.00 | 44.74 ± 1.69 | |
| | | 3 | 1.25 | 52.02 ± 12.44 | |

atherosclerotic, and antimutagenic properties [10]. A flavon, chrysoeriol, was also reported in these extracts, and was shown to be having antihistamine and anti inflammatory activities [11]. Gossypetin is known to be very soluble in nonpolar eluents (such as chloroform and benzene), moderately soluble in semipolar eluents (in this case, ether and ethanol), and insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a plant sterol commonly found in many medicinal herbs in plant fats or oils [12, 13], was also reported to be found in the extract. This compound was insoluble in water (similar to other sterols), and soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol showed to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [13, 14]. Other hydrophobic phytosterol, β-sitosterol [15], showed anticholesterol activity and could act as an immunomodulator [16]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol, and methanol was found to be antimicrobial, antiglycemic, and an antioxidant [17, 18].

The identified compounds in the extract of *U. lobata* leaf were found to be influenced by the polarity of the solvents used in the extraction. Nonpolar solvents, such as acetone, diethyl ether, and hexane, would extract nonpolar compounds, such as alkaloids, terpenoids, and steroids, while polar solvents, such as water and methanol, would extract flavonoids, phenols, and glycosides [19, 20]. The solubility of polar substances in polar solvents, and *vice versa*, concurs with the basic determinate solubility theory ("like dissolves like") [20].

Herbal medicine, or herbal extract, is considered as an antidiabetic due to its potency in reducing blood glucose levels, which is often resulted from the phytochemical compounds, such as terpenoid, steroid, alkaloid, and flavonoid classes through different mechanisms of action [21]. Some may act by increasing insulin secretion or insulin sensitivity, and others may act by inhibiting α-glucosidase and DPP-4 [3, 5]. Furthermore, since an extract could contain many compounds, the activity of the extract may result in synergistic or even antagonistic interactions of some compounds existing in the extract [22].

## Molecular docking of *U. lobata* on α-glucosidase and α-amylase

In the pharmaceutical field, molecular docking is often used to screen and predict the potential candidates the drug target of ligands with a known structure, based on its free energy binding, inhibition constant, and surface interaction. Free energy binding would inversely correspond to the binding affinity of a ligand to a target molecule (a lower free energy binding value would indicate higher binding affinity) [23]. The inhibition constant (Ki) is used in *in silico* studies to predict the inhibitory activity of a ligand to a drug target. Similarly, this score also operates inversely, in which a lower Ki score would indicate a higher inhibition activity of a protein target. Lastly, surface interaction represents the surface area and molecular recognition between a ligand and a binding pocket in a protein target. A higher surface interaction would indicate a higher number of interactions between a ligand and a molecule target [24].

Based on the molecular docking in this study, stigmasterol and β-sitosterol were found to have the lowest inhibition constant when tested against α-glucosidase and α-amylase. Free energy binding score showed that stigmasterol, followed by β-sitosterol and mangiferin, had the lowest free energy binding, respectively, and a high surface interaction in the same order. Both scores indicate a stronger binding with the drug target and may indicate a strong biological activity [23, 24] and, therefore, indicating the inhibitory activity of *U. lobata* leaf extracts on α-glucosidase and α-amylase.

Based on those categories, stigmasterol has the lowest score of inhibition constant and followed by β-sitosterol either on α-glucosidase and α-amylase. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol has the highest score of surface interaction followed by β-sitosterol and mangiferin, respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol has the lowest score in

the free energy of binding, meanwhile β-sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of their biology activity [23, 24]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U. lobata* leaf extract, both on *α-glucosidase* and *α-amylase*.

Molecular docking research is widely used to predict the potential candidates of drugs in the pharmaceutical fields. Binding orientation of these active substances to their molecule targets reveals their activity and affinity as possible candidates of drugs [24].

### α-Glucosidase and α-amylase inhibitory activity of *U. lobata* leaf extract by *in vitro*

Our results showed that inhibitory activity of *U. lobata* leaf extract on α-glucosidase was stronger than on α-amylase. It may be due to the different chemical composition of the extracts, which interacts with α-glucosidase and α-amylase. *U. lobata* contains compounds such as phenol, tanin, stigmasterol, beta sitosterol, mangiferin, quercetin, and also some flavon compounds such as gossypetin dan chrysoeriol, with total phenol content of 25%. Active compounds found to inhibit α-glucosidase activity are those from the flavonoid, alkaloid and tanin group [25]. However, this depends on other factors, such as isoflavone level which is contained in herbs, molecule size and structural variation of tanin. Meanwhile, molecular configuration on binding site of α-glucosidase active sites also contributes to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-glucosidase [26]. Isoflavones are active compounds having an inhibition activity of α-glucosidase stronger than flavone compounds [25].

Flavonoid inhibits α-glucosidase by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against α-glucosidase. Inhibition of α-glucosidase retains carbohydrate hydrolysis into maltose; therefore, it decreases glucose level postprandially [1]. *U. lobata* contains stigmasterol and β-sterol from phytosterol group and also mangiferin from xanthone glucosidase group. Based on previous *in silico* study, *U. lobata* is able to suppress α-glucosidase activity controlled by active compounds mentioned above.

Previous study showed antidiabetic of *U. lobata* leaf extract through inhibitory activity of dipeptidyl peptidase-4 (DPP-4) using *in vitro* and *in vivo* approach [5, 6]. Other study indicated both aqueous and methanolic extract of *U. lobata* leaf have antihyperglycemic or hypoglycemic effect on rats and alkaloids, flavonoids, saponins, and tannins present in the extracts may be responsible for the effects [4, 12]. Study in rabbits showed *U. lobata* aqueous extract of roots significantly reduced fasting glucose and body weight [8].

Other active substances inhibiting α-glucosidase are sterol and xanthonoid [26, 27]. Enzyme α-glucosidase is a glucoamylase enzyme that hydrolyzes polysaccharides, disaccharides, and oligosaccharides on the brush border of the microvilli in epitel intestine into glucose monomers. Activity of α-glucosidase is influenced by temperature and acidity level. Optimum temperature and acidity level for enzyme are 37 °C and 6.8, respectively. This condition is suitable with reactions occurring in the human body [28]. Active substances found to be able to suppress α-amylase are flavonoid, fenol, alkaloid, miscellaneous, terpenoid, xanthone, glucosidase, and sterol [26, 27, 29].

## Conclusions

Ethanolic extract of *U. lobata* leaf has inhibition activity stronger on α-glucosidase than on α-amylase as antidiabetic.

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Case Report

Niswah N. Qonita and Hanik B. Hidayati*

# Effect of hydrocortisone on hypocorticolism caused by pituitary adenoma

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**Abstract**

**Objectives:** Pituitary adenoma is a tumor that can cause hormonal secretion problems, including hypocortisolism. Hypocortisolism may result in negative impacts such as an increase in proinflammatory cytokine and immune system activation. Hypocortisolism therapy is performed by giving high-dose hydrocortisone. This case report presented a hypocortisolism therapy using hydrocortisone in a patient with pituitary adenoma.

**Case presentation:** A 17-year-old boy was admitted to a hospital due to right-eye vision loss, headache, and swallowing difficulty. During the treatment at the hospital, the patient had light depression. The brain Magnetic Resonance Imaging (MRI) scanning with contrast showed there was a supratentorial axial lesion enlarged from the intrasellar to the suprasellar. The anamnesis and physical examination, as well as laboratory and supporting examinations, showed that the patient was diagnosed to suffer from pituitary macroadenoma. The laboratory examination showed that the size of hypocortisolism was at <0.5 μg/dL (reference value ranges from 4.30–22.40 μg/dL). The patient was treated with hydrocortisone IV therapy at 100 mg/dose administered in the morning and evening for 4 days. Then, the dose tapering off of 100 mg/dose was administered in the morning for 4 days. After that, the patient received hydrocortisone of 20 mg/dose peroral administration in the morning and evening until the patient was discharged from the hospital. Tapering off was performed to prevent the side effects of high-dose hydrocortisone. Besides, the patient was alsounder the Endoscopic Endonasal Transsphenoidal Hypophysectomy (EETH). The cortisol level in the pretreatment was at <0.5 and 5.3 μg/dL during the treatment. There were no side effects of the treatment when the patients were hospitalized.

**Conclusions:** The hydrocortisone IV therapy with 100 mg/do was administered in the morning and evening for 4 days, and then the dose tapering off of 100 mg/dose was done in the morning for 4 days. Then, the hydrocortisone therapy of 20 mg/dose peroral administration to the patient with pituitary macroadenoma in the morning and evening to improve the cortisol level. The cortisol level in the pretreatment was at 0.5 and 5.3 μg/dL in the post-treatment.

**Keywords:** hydrocortisone; hypocorticolism; pituitary adenoma.

## Introduction

A pituitary adenoma is a hypophysis gland tumor found in the hypophysis anterior [1]. Pituitary adenoma emerges in one of the five hypophysis anterior cells, such as lactotrophs, gonadotrophs, somatotrophs, corticotropes, and thyrotropes. Pituitary adenoma rarely emerges in the combination of this anterior cells [2]. Based on its size, the pituitary adenoma is classified into three: microadenoma (<10 mm), macroadenoma (>10 mm), and giant adenoma (>40 mm) [3]. Based on the absence or presence of clinical syndrome due to hormonal hypersecretion, the pituitary adenoma is also classified into two: functioning pituitary adenoma and nonfunctioning pituitary adenoma [4].

The prevalence of pituitary adenoma was estimated to be at 0.2%, and the incidence reached two cases per 100,000 population [5]. The rate of microadenoma or macroadenoma rarely occurs in child and adolescent populations at the prevalence of 1:1,000,000 [6]. Pituitary adenoma in child and adolescent populations mostly suffer from functioning pituitary adenoma (80–97%) [7].

Clinical symptoms because of hypophysis adenoma consist of three manifestations: hypersecretion or hormonal deficiency, neurological manifestation because of the mass effect that develops to the gland, or incidental

***Corresponding author: Hanik B. Hidayati**, Department of Neurology, Universitas Airlangga, Dr. Soetomo General Hospital, Surabaya, Indonesia, Phone: +62 082131035699,
E-mail: hanikhidayati@fk.unair.ac.id
**Niswah N. Qonita,** Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

discoveries in the imaging performed. A hormonal manifestation that commonly occurs involves hyperprolactinemia, acromegaly, and Cushing syndrome. Other hormonal manifestations include partial or full hypopituitarism [2]. The most common hypopituitarism types include growth hormone deficiency and hypogonadism, while hypercortisolism rarely occurs [8].

Pituitary adenoma therapy aims to normalize prolactin serum, perform a tumor surgery, or lessen tumor size [8]. A therapy on hypercortisolism was done through glucocorticoid replacement. Decisions about the number of doses are also based on patient preference, differences in daily activities, and patient experience. There is no reliable biochemical marker to assess the appropriateness of dose in glucocorticoid replacement treatment, and so dose modification is guided by clinical judgment and subjective perception of symptoms and signs of glucocorticoid underreplacement and over-replacement [9].

## Case presentation

A 17-year-old boy visited a hospital and complained that he experienced headache, swallowing difficulty, vomiting in the morning, and vision problems in the right-eye. Specifically, the patient experienced blurry eyes and double vision in the last 5 months before being hospitalized. The patient lost right-eye vision ability a week before being hospitalized. He was diagnosed to suffer from hypophysis macroadenoma in August 2019.

In the next anamnesis, he experienced slight depression and had a blood pressure of 110/80 mmHg, a pulse of 80 times per minutes, breathing frequency of 20 times per minutes, body temperature at 36.8 °C, and 98% of saturated oxygen through the administration of oxygen mask at 6 L per minutes. The neurological treatment in the Glasgow scale of E4V5M6 showed the patient has a negative meningeal sign, and negative facial palsy. The results of funduscopic and confrontation visual tests showed optic atrophy and blindness in the right-eye. The patient's sense was in the normal range, and the motoric condition was generally weak.

The brain Magnetic Resonance Imaging (MRI) scanning with contrast showed an extra supratentorial axial lesion enlarged from the intrasellar to suprasellar tumors. The irregular clean edge margin was 1.3 × 2.1 × 2.31 cm in size which stresses dextral optic nerves and optic chiasm and causes dextral optic nervous edema, which made the image of hypophysis macroadenoma cleaner (Figure 1).

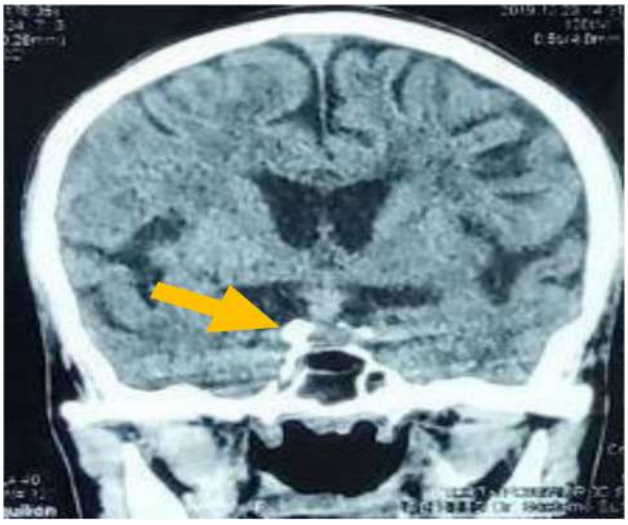

**Figure 1:** MRI results before the EETA procedure.
The brain MRI scanning with contrast showed an extra supratentorial axial lesion enlarged from the intrasellar to suprasellar tumors.

The laboratory examination in the presurgery on 20th December 2019 presented that the hypocortisolism level was at <0.5 μg/dL (reference value ranges from 4.30–22.40 μg/dL). On 23rd December 2019, a surgery using Endoscopic Endonasal Transsphenoidal Approach (EETA) was performed. The anatomical pathology test observed layers of tumor tissues. A tumor contains cells with rounded nuclear, relatively monotonous shape, smooth chromatin, eosinophilic cytoplasm, absence of mitotic, and absence of cancerous signs. The anatomical pathology test is presented in Figure 2. The patient received the hydrocortisone therapy IV with oral administration of 100 mg/dose in the morning and evening for 4 days, and then the dose tapering off of 100 mg/dose was administered in the morning for 4 days. Then, hydrocortisone was administered orally at 20 mg in the morning and evening until the patient was discharged from the hospital.

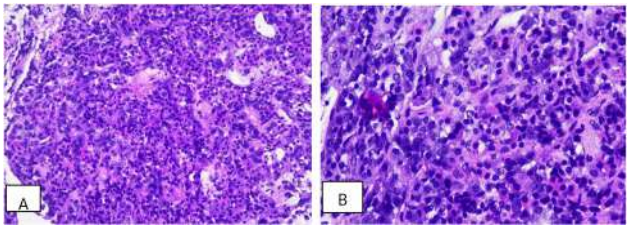


**Figure 2:** Tumor histopathological examination results (A) original objective 20×. (B) original objective 40×.
The anatomical pathology showed a tumor contains cells with rounded nuclear, relatively monotonous shape, smooth chromatin, eosinophilic cytoplasm, absence of mitotic, and absence of cancerous signs.

## Discussion

A pituitary adenoma may cause hormonal problems as a clinical manifestation. Hormonal problems could be in the forms of hormonal excess or deficit [5]. Hypopituitarism is one hormonal deficit or more produced in the anterior pituitary or posterior pituitary. The most common hypopituitarism is hormonal growth deficiency and hypogonadism, while hypocortisolism rarely occurs [8]. Hypopituitarism is one of the causes of high mortality rate, and its main risk factor is cortisol deficiency due to hormonal adrenocorticotropic deficiency (ACTH) [10].

Symptoms and signs experienced by the patient with hypo cortisol involve nausea, vomiting, constipation/diarrhea, stomach pain, asthenia, anorexia, weakness, headache, fasting hypoglycemia, weight loss, and hypotension. However, almost all the symptoms are not specific [11, 12]. The present patient experienced headache, swallowing difficulty, morning vomiting, and vision problems in the right-eye. The next anamnesis showed that the patient had slight depression. Besides, the laboratory test showed the patient's cortisol level was at <0.5 μg/dL (reference value ranges from 4.30–22.40 μg/dL). To identify a hypopituitarism diagnosis, it was recommended to measure cortisol level serum at 8–9 AM, and the cortisol level under 3 g/dL was an indication of hypo cortisol [13]. In acute illness possibly attributable to hypocortisolism, a different diagnostic strategy should be applied as an immediate therapeutic intervention is required even before the diagnosis is formally confirmed [14].

The daily physiological production of cortisol is about 5–6 mg/m$^2$ body surface area. The recommended hormone replacement therapy for hypocortisolism is administering hydrocortisone of 15–25 mg usually in 2–3 doses per day and 50–66% given in the morning right after the patient is awake. If given two times a day, the second dose is usually administered 6–8 h after the morning administration. If given three times per day, the second dose is given 4–6 h after the early morning administration, and the third dose is given 4–6 h after this. Some clinicians recommend weight-adjusted dosing to reduce intervals of excess in cortisol concentrations during the day and decrease variability of cortisol profiles [9].

Hydrocortisone is short-action glucocorticoid, with an elimination half-life of 1–2 h. Hydrocortisone is primarily bound to corticosteroid-binding globulin (transcortin). When transcortin binding sites are saturated, hydrocortisone binds to albumin. Only 5–10% is unbound and biologically active. Hydrocortisone is metabolized in the tissues and the liver to biologically inactive compounds, including glucuronides and sulfates. Hydrocortisone is excreted in the renal, and less than 1% of hydrocortisone is excreted as the unchanged drug in the urine [15].

Side effects correlated with glucocorticoid may be in the forms of endocrine, neuropsychiatric, gastrointestinal, musculoskeletal, cardiovascular, dermatology, ocular, or natural immunology. Different side effects may occur due to the use of glucocorticoids in a long-term of more than 60 days. These side effects may result from various doses and administration patterns. A patient administered with a low dose (≤7.5 mg/day) might experience these side effects as well [16]. The side effects due to the use of corticosteroids are also associated with therapy duration and the use of glucocorticoids in a long-term period. glucocorticoid therapy duration is classified into short-term duration (<10 days), middle-term duration (10–30 days), and long-term duration (>30 days). The use of corticosteroids for long period can stress the HPA axis, and thus the glucocorticoid therapy should be stopped in the right manner. Inappropriate stopping will stimulate the adrenal crisis due to the persistent HPA axis stress. Therefore, dose tapering off is required [17].

Apart from experiencing hypocortisolism, the patient also experienced hyperprolactinemia and would receive dopamine agonist therapy, namely bromocriptine and also EETA. Hypocortisolism therapy modalities in patients undergoing pituitary surgery are recommended using stress doses of the steroids before surgery and tapered doses after surgery before the testing repetition. Patients in the same condition will receive hydrocortisone therapy before surgery [13]. The present patient received hydrocortisone of 100 mg IV in the morning and evening for 4 days from 21st–24th December 2019, and then the dose tapering off of 100 mg/day was performed on 25th–28th December 2019. The patient received hydrocortisone of 20 mg per oral administration in the morning and evening until he was discharged from the hospital on 4th January 2020 and received hydrocortisone of 20 mg in the morning and evening afterward. Tapering off was done to avoid the side effects of giving high-doses of hydrocortisone. The EETA was carried out on 23 December 2019. There was an increase in pretreatment cortisol <0.5 and 5.3 μg/dL after the patient received hydrocortisone therapy. There was no side effect while the patient was hospitalized.

Decisions about the number of doses are also based on patient preference, differences in daily activities, and patient experience. There is no reliable biochemical marker

to assess the appropriateness of dose in glucocorticoid replacement treatment, and so dose modification is guided by clinical judgment and subjective perception of symptoms and signs of glucocorticoid under-replacement and over-replacement. The goal is to achieve the best clinical results with the lowest possible daily dose of steroids. Cortisol day curves are of little value in routine monitoring [9].

## Conclusions

The hydrocortisone IV therapy was performed through the administration of 100 mg/dose in the morning and evening for 4 days, and then dose tapering off of 100 mg/dose was administered in the morning only for 4 days. After that, hydrocortisone was administered orally each at 20 mg/dose in the morning and evening to the patient with hypophysis macroadenoma and hypercortisolism to increase the cortisol level. The cortisol level in the pretreatment was at <0.5 and 5.3 μg/dL in the post-treatment.

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# Turnitin test Maja Fruit rv

*by* c.teodhora 1

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**Teodhora*, Munawarohthus Sholikha, Asniatul Ania, Ika Maruya Kusuma**

# Secondary Metabolite and Antipyretic Effects of Maja (*Crescentia cujete* L.) in Fever-Induced Mice

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**Abstract**

**Objectives**: Fever is a condition when the body experiences an increase above average body temperatures above normal. Maja fruit (*Crescentia cujete* L.) contains chemical compounds including alkaloid, flavonoid, saponin, and terpenoid, suspected as potential antipyretics.
**Methods**: The study aimed to determine the antipyretic activity of ethanol extract of Maja fruit. A total of twenty-five male white mice of the DDY strain (20-30 g) healthy were divided into five groups; each contained five mice. These treatments divided into three groups with a dose extract of 125 mg/kg BW, 250 mg/kg BW, 500 mg/kg BW, standard groups of Ibuprofen 400 mg/kg BW, and control groups of CMC-Na 1%. Mice were injected Intraperitoneally with 0.1 cc of DPT Vaccine-Induced after basal rectal temperature assessment. Observations were made by measurement the rectal temperatures of mice using a digital thermometer before DPT vaccine injected or average temperatures, at 0-minutes (after DPT vaccine injected), 60, 120, 180, and 240 minutes after administered the test material. The differences between the positive control group, test group, and the negative control group were compared using statistical analysis using one-way variance analysis (ANOVA) if the data were normally distributed and homogeneous, followed by a Tukey's test. The results were considered statistically when the value is ($P<0.05$).
**Results**: The above phytochemical screening results showed that alkaloids, flavonoids, and saponins were present in the Maja fruit powder and extract (*Crescentia cujete* L.). Based on the results of the statistical analysis obtained, i.e., Group II was not significantly different from Group III and Group IV ($p = < 0.05$) and was significantly different from Group I and Group V. Group I was significantly different from Group II, Group III and Group IV and was not significantly different from Group V ($p = > 0.05$). It could also be inferred that the results on the decrease of fever temperature in the mice revealed that there was a substantial difference between Group II, Group III, and Group IV on the reduce of fever temperature; on the other hand, Group I and Group V did not have a significant difference in the reduce of fever. This suggests that group V had the best dose compared to group III and IV as the median initiation and duration of action of the medicinal product were close to those of group V.
**Conclusions**: The study showed that Maja fruit mice's antipyretic behaviour at doses of 125 mg/kg BW, 250 mg/kg BW and 500 mg/kg BW was confirmed as a result in reducing the body temperature of male mice. The 500 mg/kg BW dosage of Maja fruit extract (*Crescentia cujete* L) effectively reduced fever.

**Keywords**: Antipyretics, *Crescentia cujete* (L.), *Diphtheria-pertussis-tetanus* vaccine, Fever

## Introduction

The globe is facing the Covid-19 pandemic, which significantly affects people's mobility to carry out activities. As one of the afflicted nations, Indonesia imposes large-scale social sanctions to break the chain of transmission. The transmission is caused by microorganisms, including viruses that invade the body, impair organ functions, and cause a risk of death if not treated properly. Medical effects that are developed due to microorganism infection can send signs, including fever, to the body. A stimulation of cells that are responsible for the immune system will increase the body temperature [1], namely the production of cytokinesis (IL-1, IL-6, IFN-a). By growing the PGE-2 synthesis pathway, leading to a rise in body temperature, this activation stimulates the hypothalamus to improve cytokines. If the state of the body decreases, microorganisms will defeat the immune system within the body [2]. When body temperature increases above normal levels, people take paracetamol and ibuprofen as antipyretic drugs which function by inhibiting prostaglandin production. Drugs, however, have many side effects and might potentially damage tissues, such as tissues in the liver and kidneys, when taken for a long term [3,4].

Several researchers in Indonesia are searching for medicines during the pandemic to maintain patients' endurance and avoid consequences of microorganisms within patients that have been infected or exposed to the virus. Many Indonesian researchers are devising a treatment therapy to strengthen the immune system to avoid the exposure to microorganisms. Starting from evolving medicines that have been circulating to looking for new drugs from different varieties of plants that are predicted to produce compounds used as drug action targets that can pharmacologically improve the immune system [5]. Many plants have pharmacological activities from various studies that had been conducted, including Maja (*Crescentia cujete* L), which reportedly provides pharmacological activity in overcoming bronchitis, influenza, asthma, pain, diarrhea [6], anti-inflammation [7], stimulating insulin production [8], lowering blood pressure [8], and increasing antioxidants [9] and antimicrobials [10]. There are fewer side effects from the use of alternative medicine [11].

Maja fruit has a lot of identified pharmacological activities. Fortunately, the efficacy of Maja fruit compounds as an antipyretic has not been documented. Therefore, if Maja's fruit ability to relieve fever is demonstrated, various previous studies on its pharmacological action linked to the immune system, such as antioxidants, analgesics, anti-inflammatory, and antimicrobials, will be sponsored. This study aims to include knowledge about its pharmacological ability to dramatically improve the body's immune system by creating new medicines.

## Materials and methods

### Materials

Ethanol 70% ($C_2H_5OH$), Sodium Hydroxide (NaOH) 1 N (Merck), *Difteri, Pertussis, Tetanus* (DPT) Vaccine (Pentabio), Alumunium Chloride ($AlCl_3$) 1% (Merck), HydrochloricAcid (HCl) 2N (Merck), Mayer reagent, Dragendorf reagent, Ferrous (III) Chloride ($FeCl_3$) 1% (Merck), Sodium Carboxymethylcellulose (CMC-Na) 1% (Merck), Acetate Anhydrase ($C_4H_6O_3$) (Merck), Sulfuric Acid ($H_2SO_4$) (Merck).

### Preparation, extraction of plant materials

The Maja fruit was collected from the South Jakarta National Institute of Science and Technology, Jakarta. The determination of the desired species of *Crescentia cujete* L. was confirmed by the Indonesian Institute of Science, Bogor, Biology Research Center, West of Java. The confirmed desired species are *Crescentia cute* L. and the Bignoniaceae tribe (Certificate Number of 2108/IPH.1.01/1f.07/XI/2019). 5 kg of the fruit was washed under running water. Then, the clean fruit was peeled

***Teodhora,** Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia, E-mail: c.teodhora@istn.ac.id
**Munawarohthus Sholikha :** Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia
**Asniatul Ania :** Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia
**Ika Maruya Kusuma :** Department of Pharmacy, National Institute of Science and Technology, Jakarta, Indonesia

and separated from the peel, sliced thinly, and dried at 60°C for 48 hours. 650 g of fine Maja fruit powder was filtered with a mesh with a size of 40, resulting in 2 kg of dried fruit powder, which was then pollinated. The dried Maja fruit powder was macerated in ethanol 70% at a room temperature for three days and shielded from the sunlight. The extract was filtered and concentrated under vacuum using a rotary evaporator until the ethanol in the extract evaporated.

## Preliminary Phytochemical Test

The preliminary phytochemical screening went through the identification of alkaloid, flavonoid, saponin, and tannin using the extract. The screening aimed to establish pharmacognosy standards.

**Alkaloids,** 1 g of Maja fruit powder and extract were moistened in a beaker glass with 5 ml of 25% ammonia ($NH_3$). 20 ml of chloroform were applied until the substance immersed, then stirred, heated, and filtered over a water bath. The filtrate was then halfway evaporated. The residue was poured into a test tube and added with 1 ml of 2 N hydrochloric acids (HCl), then shaken and left to form 2 layers; the formed transparent layer was taken and equally placed in 3 test tubes. The Mayer reagent was attached to the first tube, the second tube with the Bouchard reagent, and the third tube with the Dragendorff reagent. The deposition of white deposits in the Mayer reagent, brown deposits in the Bouchard at reagent, and red sediments in the Dragenddorff reaction suggest alkaloids. Alkaloid screening can be declared as positive if at least two experiments with the reagents form deposits [12].

**Tannin,** 1 g of Maja fruit powder and extract were separated and purified in 100 ml of hot water. 5 ml of filtrate was inserted into a test tube and a few drops of 1% ferric chloride ($FeCl_3$) solution were applied. If a green-purple or black color is produced, the test substance involves tannins [12].

**Flavonoids,** 1 g of Maja fruit powder and extract were filtered in 100 ml of hot water. In the test tube, 5 ml of filtrate was applied, then 1 ml of 5% sodium nitrite solution ($NaNO_3$) and 1 ml of 10% ammonium chloride solution ($AlCl_3$) were added and shaken, and 2 ml of 1 N hydroxide solution was applied. If the test substance contains flavonoids, the color will turn red or orange [12].

**Saponin,** 1 g of Maja fruit powder and extract were extracted and purified in 100 ml of hot water. In the test tube, 10 ml of filtrate was mounted and shaken vertically for 10 seconds. The production of a steady foam with the height of 1 to 10 cm suggests saponin. If the test substance includes saponin with the addition of 1 drop of 2 N hydrochloric acids (HCl), a stable ± 1 cm foam will be formed [12].

Male DDY mice weighing 20-30 g, between 2-3 months, were collected from the IPB University Faculty of Animal Science, Indonesia. The UPNVJ Health Research Ethics Committee (Letter Number B/2378/1/2020/KEPK) has accepted all experimental protocols for pharmacological trials to support researchers in the preservation of research ethics and attempts to protect human rights and the welfare of animals as research objects. The mice were previously acclimatized for one week, which helped the animals adapt with the laboratory environment. Before checking the antipyretic activity of the ethanol extract of the Maja fruit, an experimental test was done in advance.

This experimental test helped decide what needs to be done in the final test to achieve more reliable and precise data. The preliminary test included the administration of a dosage of DPT and a dosage of Maja fruit extract. In this test, there were four groups consisted of 2 mice for each group; a wireless thermometer was used to measure the original rectal temperature before the DPT vaccine was triggered. The DPT vaccine with separate volumes of 0.1 cc and 0.2 cc and the Intraperitoneal (IP) routes and Intramuscular (IM) administration as fever inducers were then administered to each mouse in each group; the temperature was again determined after 30 minutes of the induction of the DPT vaccine. A fever was identified by the initial increase of body temperature. The second preliminary test was carried out to evaluate the initial dosage of the test substance to be used on the mice for the antipyretic behaviour test. The initial dosage of 250 mg/kg BW and 500 mg/kg BW of Maja fruit extract (*Crescentia cujete* L.) were used along with the oral administration route in this preliminary research. BW, 300 mg/kg BW, and 600 mg/kg BW had an antipyretic effect with the highest antipyretic effect obtained at a dosage of 600 mg/kg BW [13] and Maja fruit (*Aegle marmelos* L. Corr.) anti-inflammatory test with doses of 100 mg/kg BW, 200 mg/kg BW, and 400 mg/kg BW [14]. The initial dose of 250 mg/kg BW and 500 mg/kg BW, based on the preliminary examination, indicated that antipyretic activity occurred in the form of a decrease in the mice's body temperature following a fever with the DPT vaccine induction. The additional dose of 125 mg/kg BW is the lowest dose among the original doses intended to perform antipyretic action.

The mice consisted of randomly chosen healthy 5 male mice. Fever using DPT vaccine 0.1 cc was induced Intraperitoneally for 1 hour to achieve a fever.

## Evaluation of Antipyretic Potential

Based on the preliminary test, the amount and route of administering the DPT vaccine used in the antipyretic activity test were 0.1 cc. (IP) as a fever induction dose, and 250 mg/kg BW and 500 mg/kg BW as the initial dose of Maja fruit extract (*Crescentia cujete* L.). The five research groups in this test are as follows:

| No. | Groups | Treatment |
|---|---|---|
| 1 | I | 0.5% CMC-Na (Control Groups), orally |
| 2 | II | Ibuprofen 400 mg/kg BW (Standard Drug), Orally |
| 3 | III | Extract 125 mg/kg BW suspended in 0.5% CMC-Na, Orally |
| 4 | IV | Extract 250 mg/kg BW suspended in 0.5% CMC-Na, Orally |
| 5 | V | Extract 500 mg/kg BW suspended in 0.5% CMC-Na, Orally |

The rectal temperature was measured by a digital thermometer for 240 minutes for every 60 minutes after the mice was treated. Both mice with various temperature changes, varying from 1.3 to 1.8°C, were infected by the DPT vaccine fever.

## Data Analysis

The data obtained from this research is quantitative. A decline in fever temperature is a quantitative data. The results were then examined statistically. The differences between the positive control group, test group, and the negative control group were compared using statistical analysis using one-way variance analysis (ANOVA) if the data were normally distributed and homogeneous, followed by a Tukey's test. The results were considered statistically when the value is ($P<0.05$).

# Results

This study's test material was the volleyball-shaped fruit of Maja (*Crescentia cujete* L.) with a diameter of 25 cm, with smooth and shiny green skin that was hard and woody, and soft white and fragrant flesh. The dried Maja fruit was collected and sorted to be separated from impurities that were left in the dried fruit and unwanted plant components. The above phytochemical screening results showed that alkaloids, flavonoids, and saponins were present in the Maja fruit powder and extract (*Crescentia cujete* L.). This shows that the Maja fruit extract (*Crescentia cujete* L.) contains secondary metabolites thought to have pharmacological effects as antipyretics. As shown in table 1.

The Intraperitoneally route had a higher absorption rate than intramuscular as the route of administration directly penetrated the abdomen and had a large absorption surface. The medication could quickly enter the systemic bloodstream,

providing a faster response. The DPT vaccine, which functioned as a pyrogen, induced the release of endogenous pyrogens (IL-1 and TNF-α) released by polymorphonuclear cells by increasing the temperature in mice. These pyrogens released arachidonic acid in the anterior hypothalamus's chemoreceptive region, which activated prostaglandin production and affected the increase of body temperature/fever [15]. Compared with positive and negative control therapies, the mice treated with oral administration of Maja fruit extract (*Crescentia cujete* L.) showed numerous temperature changes as shown in Table 2.

Based on the effects of the average rectal temperature calculation data on mice, the average rectal temperature decrease of mice was determined to assess the potential of treatments I, III, IV, and V to decrease the mice's rectal temperature. The average decrease in temperature indicates the test substance's antipyretic role in reducing the body temperature of mice, obtained by measuring the temperature 60 minutes after the induction of the DPT vaccine minus the temperature at a certain point after treatment. Table 2 shows the effects of the average decrease in the mice's rectal temperature. The findings of the average decrease in mice's rectal temperature, based on Table 2, demonstrate a difference in the temperature drop in 240 minutes per 60 minutes of each group. The drop in temperature after the treatment was not the same for each mouse, even in the same treatment category. The antipyretic activity of the Maja (*Crescentia cujete* L.) fruit extract has increased in quantity depending on the dosage. There was a greater antipyretic effect in group V compared to group I, II, III, and IV. However, antipyretic behavior was not substantially different from the statistical findings obtained from the control groups, I groups and V groups.

Based on the results of the statistical analysis obtained, i.e., Group II was not significantly different from Group III and Group IV ($p = < 0.05$) and was significantly different from Group I and Group V. Group I was significantly different from Group II, Group III and Group IV and was not significantly different from Group V ($p = > 0.05$). It could also be inferred that the results on the decrease of fever temperature in the mice revealed that there was a substantial difference between Group II, Group III, and Group IV on the reduce of fever temperature; on the other hand, Group I and Group V did not have a significant difference in the reduce of fever.

The next step was to observe the medication's average onset of action and the average duration of drug action obtained from the beginning of antipyretic therapy and the amount of time needed by the treatment to give therapeutic activity, as shown in Table 3.

It can be seen in Table 3 that group III, IV, and V have the same average onset of action at minute 60. Duration of drug action was different; group V had an overall duration of drug action that varied. Compared to group III with a duration of drug action of 120 minutes and treatment group IV with a duration of drug action of 180 minutes shorter than treatment group I and V. This suggests that group V had the best dose compared to group III and IV as the median initiation and duration of action of the medicinal product were close to those of group V.

## Discussion

Several active compounds were believed to be responsible for the antipyretic activity caused by the Maja fruit ethanol extract (*Crescentia cujete* L.), namely flavonoids, tannins, and saponins. Based on the antipyretic test findings, Maja fruit extract (*Crescentia cujete* L.) produced antipyretic activity in DPT vaccine-induced mice. It also reported that Alkaloid [10,16,17,18,19] saponin [16,17,20], tanin [16,17,18,21], flavonoid [16,17,18,21], steroid [18,21], antrakuinon [16,22], fenol [10,16], cardenolides [16,20], fitosterol [17], glikosida resin [18], apigenin and nephtoquinon [21].

The results of this study were also supported by previous research, which proved that plants containing flavonoids (a compound of the phenolic group) could inhibit cyclooxygenase. The flavonoid mechanism functioned by inhibiting eicosanoids, which could induce cyclooxygenase pathway blockade to disrupt changes in endoperoxide arachidonic acid, contributing to the production of prostaglandin E2 in peripheral tissues that could not directly directly with the brain, resulting in a reduction in the set point in the hypothalamus [23]. Tannins may have antipyretic properties by inhibiting arachidonic acid in prostaglandin biosynthesis [24].

Antipyretic activity existed due to secondary alkaloid metabolites, which suppressed the COX of the enzyme [25], thereby disrupting the synthesis of prostaglandin. Alkaloids of the matrine form were thought to suppress dopamine release or block dopaminergic receptors to interact with prostaglandin synthesis [26], revealed that the boldine alkaloid mechanism indicated that alkaloid was an important prostaglandin biosynthesis inhibitor. The study revealed that punarnavine, an alkaloid isolated from B *Diffusa*, decreased the rise in mouse levels of lipopolysaccharide-induced proinflammatory cytokines such as TNF-, IL-1, and IL-6. Prostaglandin E2, which was the primary mediator of fever, and was enhanced by releasing inflammatory mediators such as IL-1 and IL-6 in the body [27]. The mechanism of lowering the rectal temperature in fever-induced laboratory mice, the quality of flavonoids, hormones, tannins, and saponins was thought to function synergistically [28].

The use of vaccine-induced volumes of 0.1 cc and 0.2 cc DPT increased mice's body temperature within the 0.7-1.6◦C range [13,14]. With the mice's temperature, the DPT pyrogen-induced vaccine could facilitate endogenous pyrogen production (IL-1 and TNF alpha) produced by polymorphonuclear cells. These pyrogens functioned to release arachidonic acid in the anterior hypothalamus's chemoreceptive region, inducing prostaglandin synthesis, which influenced the increase in body temperature [15]. Based on previous antipyretic, the amount and route of administration of 0.1 cc and 0.2 cc DPT vaccines were used. The use of DPT vaccine volumes of 0.1 cc and 0.2 cc allowed the increase in mice's body temperature within the range of 0.7-1.6◦C [29]. The DPT vaccine volume was 0.1 cc and 0.2 cc i.p, depending on these test results, with a temperature rise of 1.2-1.7◦C [28].

The drop in rectal temperature in each mouse after the treatment was not the same, even in one treatment category. Variables such as hormones, climate, and gastric condition were responsible for this decrease. Psychological causes, such as tension endured due to repetitive measurements in the rectum of mice, might have also induced it [30]. The dose-antipyretic activity of Maja fruit extract (*Crescentia cujete* L.) improved as the dose increased. Group V showed more substantial antipyretic effects than group I, II, III, and IV. However, there was no substantial difference in statistical findings between treatment group I and V that provided antipyretic activity.

The isolation of alkaloid from *B. Diffusa* decreased lipopolysaccharide-induced elevated levels of TNF-1, IL-1, and IL-6 pro-inflammatory cytokines in mice [27]. The release of inflammatory mediators such as IL-1 and IL-6 within the body facilitated the prostaglandin E2 synthesis, the primary fever mediator [31]. Alkaloids such as bolidine could minimize rising temperatures by inhibiting the synthesis of prostaglandin E2 [32]. Cyclooxygenase and 5-lipoxygenase inhibition pathways and eicosanoid biosynthesis inhibition, along with their ability to block neutrophil degradation [33]. The inhibition of arachidonic acid peroxidation was also seen by flavonoids and their corresponding compounds, resulting in a decrease in prostaglandin levels, thereby minimizing fever and pain [34]. Saponin was involved in the mechanism of the opioid receptor [35] using antagonistic activity [36] by binding the sensory nerve terminals. Flavonoids, steroids, tannins, and saponin were the most

important bioactive compounds of plants and were suspected to function synergistically in the process of rectal temperature reduction in fever-induced laboratory mice [37].

These compounds had a wide variety of pharmacological activities, such as anthelmintic [38], antidepressant and anti-anxiety [39], anti-inflammatory [7,40], antibacterial [10,41], anti-fertility [42], anti-arthritis [43], and antidiarrheal [6,44]. The 500 mg/kg BW dosage of Maja fruit extract (*Crescentia cujete* L) reduced fever. These results can be added to the study on Maja fruit's pharmacological activity and continued to be formulated as a novel drug, particularly as an antipyretic via the pharmacological mechanisms of the secondary metabolites present in Maja. However, for further research, the active components of the Maja fruit shall remain through the compound isolation stage to improve research results.

## Conclusions

The study showed that Maja fruit mice's antipyretic behaviour at doses of 125 mg/kg BW, 250 mg/kg BW and 500 mg/kg BW was confirmed as a result in reducing the body temperature of male mice. The 500 mg/kg BW dosage of Maja fruit extract (*Crescentia cujete* L) effectively reduced fever. However, for further research, the active components of the Maja fruit shall remain through the compound isolation stage to improve research results.


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## Author contributions

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## Competing interests

Authors state no conflict of interest.

## Informed consent

Informed consent was obtained from all individuals included in this study.

## Ethical approval

The local Institutional Review Board deemed the study exempt from review.

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**Table 1:** Result for Phytochemical Constituents of Maja Fruit

| Constituents | Theory[12] | Test | Result | |
|---|---|---|---|---|
| | | | Extract | Powder |
| Alkaloid | Mayer : Formed white deposits | White deposits | (+) | (+) |
| | Bouchardat : formed brown deposits | Brown deposits | (+) | (+) |
| | Dragendorff : formed red brick deposits | Redbrick deposits | (+) | (+) |
| Flavonoid | Reddish orange | Reddish orange color | (+) | (+) |
| Saponin | Formed froth ±1 cm stable | Stable ±1 cm froth | (+) | (+) |
| Tannin | Formed greenish-black color | Greenish-black color | (+) | (+) |

**Table 2:** Effect of Plant Extracts on Maja Fruit Induced Pyrexia in Mice Male

| Groups | Treatment-dose (mg/kg *p.o*) | Temperature at 1 h after induction | Rectal temperature (°C) at different hours after the treatment with the extract | | | | | P-Value Tukey's test |
|---|---|---|---|---|---|---|---|---|
| | | | +1 h | +2 h | +3 h | +4 h | Average | |
| I | Control-Ibuprofen 400 | 38,54±1.40 | 37,42±1.12 | 37,04±0.38 | 36,54±0.5 | 36,84±0.30 | 36,96±0.42* | 0.424* |
| II | Standard-Na CMC 1% | 38,76±2.02 | 38,64±0.12 | 38,54±0.10 | 38,32±0.22 | 38,60±0.28 | 38,52±0.04 | 0.038 |
| III | Extract-125 | 38,26±1.42 | 37,50±0.76 | 37,92±0.42 | 38,08±0.16 | 38,00±0.08 | 37,87±0.06 | 0.062 |
| IV | Extract-250 | 38,50±1.36 | 38,02±0.48 | 37,08±0.94 | 38,08±1 | 38,02±0.06 | 37,80±0.12 | 0.118 |
| V | Extract-500 | 39,06±1.86 | 38,30±0.76 | 37,78±0.52 | 37,54±0.24 | 36,90±0.64 | 37,63±0.54* | 0.538* |

*Significantly different when compared to Group II (Standard); III (Extract 125 mg/kg); IV (Extract 250 mg/kg). Statistically significant $p<0.05$ analyzed by *one-way* ANOVA followed by the Tukey test.

**Table 3:** Onset and Duration of Action Extract of Maja Fruit Induced Pyrexia

| Groups | Treatment-dose (mg/kg p.o) | Onset (min) | Durasi (min) |
|---|---|---|---|
| I | Control-Ibuprofen 400 | 60' | 240' |
| II | Extract-125 | 60' | 120' |
| III | Extract-250 | 60' | 180' |
| IV | Extract-500 | 60' | 240' |

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