PHARMACIA

Pharmacia 70(3): 587–594
DOI 10.3897/pharmacia.70.e109333

Research Article

The water fraction of Cantigi (*Vaccinium varingiaefolium* Bl. Miq.) fruits demonstrate the highest antimetabolic syndrome properties on enzyme assay

Ana Yulyana[1,2], Chaidir Chaidir[2,3], Partomuan Simanjuntak[2,3], Lilik Sulastri[4], Syamsudin Abdillah[2]

1 *Institut Sains dan Teknologi Nasional (ISTN), Jakarta, Indonesia*
2 *Doctoral Program of Pharmaceutical Sciences, Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia*
3 *Research Center for Pharmaceutical Ingredient and Traditional Medicine, National Research and Innovation Agency (BRIN), KST Sukarno Cibinong 16911, Jakarta, Indonesia*
4 *Sekolah Tinggi Teknologi Industri dan Farmasi Bogor, Bogor, Indonesia*

Corresponding authors: Ana Yulyana (anaycantigi@gmail.com); Syamsudin Abdillah (syamsudin.abdillah@univpancasila.ac.id)

Received 11 July 2023 ♦ **Accepted** 25 July 2023 ♦ **Published** 9 August 2023

Citation: Yulyana A, Chaidir C, Simanjuntak P, Sulastri L, Abdillah S (2023) The water fraction of Cantigi (*Vaccinium varingiaefolium* Bl. Miq.) fruits demonstrate the highest antimetabolic syndrome properties on enzyme assay. Pharmacia 70(3): 587–594. https://doi.org/10.3897/pharmacia.70.e109333

Abstract

Vaccinium varingiaefolium, Cantigi (local name), is a member of the berries family that has various benefits to human health. It contains numerous biological compounds, including polyphenols, flavonoids, and anthocyanin. Although many studies reported the pharmacological activity of berries families, the pharmacological Cantigi's activity is still limited. The research aims to examine the antimetabolic, inhibition of lipase, alpha-glucosidase (αGIA), and angiotensin-converting enzyme (ACE) of Cantigi. The fractionation process originates from a 70% ethanol extract that underwent treatment with the addition of HCl until reaching a pH of 3. To comprehensively analyze the diverse array of compounds present in the Cantigi water fraction, we employ non-targeted screening through HPLC-Q-TOF-MS. Additionally, the brine shrimp lethality test (BSLT) was applied to check the acute toxicity. The results clearly indicate that the water fraction of Cantigi's extract exhibits the highest activity against Lipase, ACE, and αGIA with values of 27.74±3.66 µg/mL, 19.33±2.86 µg/mL, and 15.90±1.82 µg/mL, respectively. Furthermore, we found delphinidin was the major anthocyanin of the water fraction. Finally, the toxicity assay showed 196.56±14.8 mg/ml. In conclusion, the water fraction of Cantigi extract demonstrated the highest antimetabolic activity, and we propose that delphinidin contributes significantly to this observed activity as it serves as a major component.

Keywords

Cantigi, delphinidin, mass spectra analysis, metabolic syndrome

Introduction

Nutritional supplements, which include vitamins, minerals, herbs or other botanicals, and amino acids, are products meant to enrich the diet and bear or contain one or more of the dietary constituents (Chauhan et al. 2013). Around 80% of the world's population currently consumes conventional herbal remedies. Consumers

today place a premium on food that contains bioactive chemicals and minerals that improve health. Functional foods traditionally substitute natural plant ingredients for synthetic additions, which consumers no longer favor. One of the most popular categories of food components today is a herb that has been used for medical purposes and therapy for a long time (Wachtel-Galor 2011). They contribute to the flavor and aroma of food, its composition and frequently its bioavailability, and the bioactivity of its vital and active elements (Wójcik et al. 2021). Additionally, recent trends indicate that supplement products are increasingly being utilized to address various degenerative conditions, including metabolic syndrome.

Metabolic syndrome (MS) is one of the conditions most frequently linked to unhealthy lifestyle choices. Numerous health issues, such as central obesity, blood sugar issues, high blood pressure, inflammatory disorders, impaired glucose tolerance, hypertriglyceridemia, and hypercholesterolemia are associated with overeating (Refdanita Refdanita 2021). In general, type 2 diabetes, cardiovascular disease, stroke, and death are all made more likely by MS (Belete et al. 2021). Further, patients with MS are encouraged to adopt lifestyle modifications, incorporating increased physical activity and dietary changes, in addition to receiving medication treatment. Certain enzymes related to MS, such as pancreatic lipase, angiotensin-converting enzyme (ACE), α-amylase, and α-glucosidase, play crucial roles in the metabolic processes that contribute to the condition. To support their health, individuals with MS should include foods rich in these enzyme inhibitors in their diet. Plant-derived phenolic compounds have been extensively studied for their inhibitory effects on these enzymes and are considered beneficial complements to pharmaceutical therapies for managing MS (Tungmunnithum et al. 2018). Moreover, recent interest has been drawn to many berry fruits due to their actions in vitro or links in observational studies with lowered risk of several chronic diseases (Blumberg et al. 2013). One of the plants belonging to the berry group that grows in highlands in Indonesia is *Vaccinium varingiaefolium*.

Vaccinium varingiaefolium Bl. Miq, a native shrub related to blueberries (*V. corymbosum*) and bilberries (*V. myrtillus*), is a member of the Ericaceae family known as Cantigi in local languages, particularly purple Cantigi in some Indonesian places, such as West Java (Yulyana et al. 2016). This plant is well-known for withstanding a wide range of grips, most related to the development of traditional on-land craters. A prior study has reported the potency of Cantigi as an antioxidant due to its high polyphenol content (Yulyana et al. 2016). This study is in line with that reported on the berries family, which mentioned that it is rich in anthocyanin, a member of polyphenols (Pap et al. 2021). Moreover, one of the most important naturally occurring plant pigments for promoting good health, anthocyanins are classified as polyphenols and members of the flavonoids group due to the quantity and degree of methylation, position of the hydroxyl group, and the number of rings attached to the sugar moieties (Wu and Prior 2005).

Anthocyanins, which belong to the group of polyphenols, have a wide range of biological functions, including insulin-sensitizing actions, antioxidant and anti-inflammatory properties (Hämäläinen et al. 2007; Pandey and Rizvi 2009; Sodagari et al. 2015). Moreover, it has been suggested that natural remedies and herbal medicines, particularly those high in anthocyanins, may aid in managing MS. Thus, the primary objective of this study was to investigate the potential of anthocyanin-rich herbs, Cantigi, as prospective alternative medicines for MS management. Additionally, we sought to explore the potential mechanisms of action through which these herbs may operate, with a particular focus on the inhibition of lipase, alpha-glucosidase, and ACE.

Materials and methods

Research material

The research material used was fruits of Cantigi (*Vaccinium varingiaefolium* Bl. Miq) obtained from Tangkuban Perahu Mountain in West Java, Indonesia (Fig. 1). Before its processed, the plant was authenticated by National Research and Innovation Agency (BRIN): Research Centre for Biology, Cibinong Indonesia.

Cantigi's extraction

The preparation of the 70% ethanol extract of Cantigi fruit (EOC) involved the use of 1000 g of Cantigi fruits, which were subjected to the maceration method with the addition of 1% HCl in a quantity of 10 liters. Following this step, the mixture underwent stirring and filtering processes to obtain the filtrate. Subsequently, the filtrate was subjected to evaporation and concentration using a freeze-dryer, resulting in a thick extract. To further fractionate specific compounds, the extract was partitioned using ethyl acetate and water, leading to the formation of an ethyl acetate (EAC) fraction and water (WTC) fractions (Yulyana et al. 2016). This fraction is expected to contain high anthocyanin content derived from the Cantigi fruit, which could hold potential activity to combat MS.

Total Phenolics Content (TPC)

The total phenolic content of Cantigi seed extracts was determined using Folin-Reagent Ciocalteu's (FCR) and a modest modification (Mansouri et al. 2005). Methanol, FCR, and 5% $CaCO_3$ were added to the extract. At λ 725 nm, the absorbance of reaction mixtures was measured (Hitachi U-2000 spectrophotometer 1210002, Tokyo, Japan). TPC was measured in milligrams of (+)-gallic acid equivalents (GAE) per gram of extract.

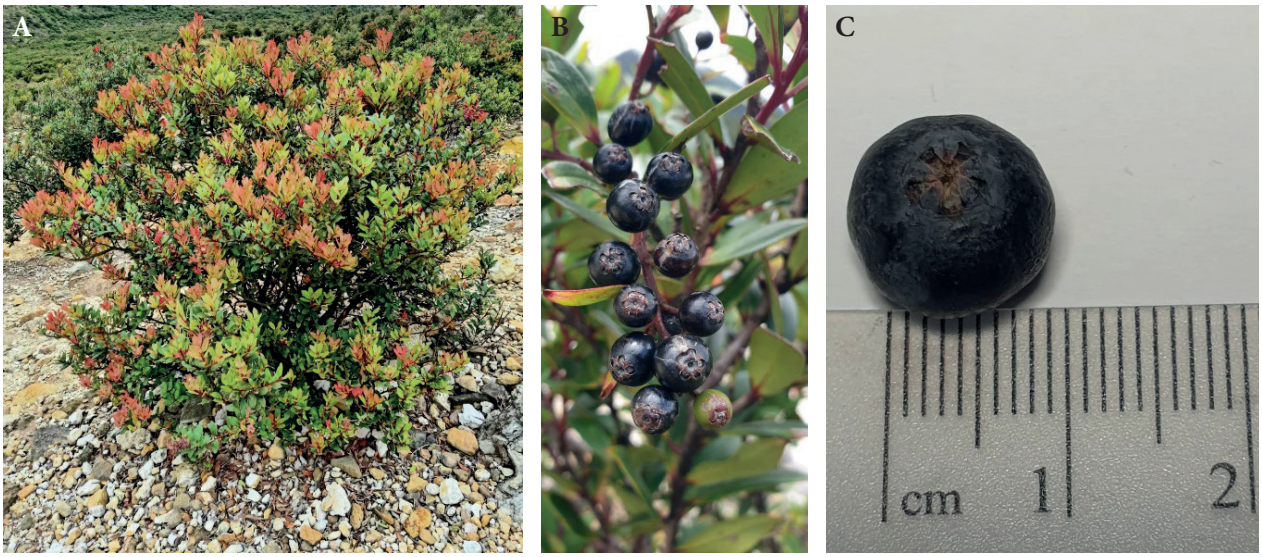

Figure 1. Image of intact Cantigi plant (**A**) and Cantigi fruit (**B**) harvested from Mount Tangkuban Perahu, Indonesia (**C**). The original size of Cantigi's fruit.

Total Flavonoids Content (TFC)

Cantigi extracts' total flavonoid content was determined using the method described by Samirana et al. (Samirana et al. 2016). The extract (0.25 mL, concentration 1–10 µg/mL depending on solvent) was mixed with 1.25 mL of distilled water and a 5% sodium nitrite solution (0.75 mL). After 6 minutes, the mixture was treated with 10% aluminum chloride (1.50 mL) and sodium hydroxide (1 M, 5.00 mL). Water was added right away to make the final volume of 10.00 mL, and after that, the mixture was properly mixed and let to stand for an additional 15 minutes. The absorbance was measured at λ 510 nm. The total flavonoid content (TFC) was estimated as mg quercetin equivalents (QE) per gram of extract.

Anthocyanin content

Using a previously established method by Nile et al., the total anthocyanin content was calculated (Nile et al. 2015). Each extract was diluted (5:95, v/v) in 1% HCl in methanol to attain an absorbance between 500 and 1.000 at λ 530 nm. A molar extinction coefficient of 27.900 was applied, and the values were represented as mg cyanidin-3-glucoside equivalents per 100 g fresh weight. All analyses were carried out in triplicate.

Pancreatic lipase inhibitory

Lipase inhibitory activity was determined using Lipase Activity Colorimetric Assay Kit (BioVision, Catalog #K722-100). The sample (Cantigi's extract) concentration was 25, 50, 100, 200, and 400 µg/L while the final volume of the reaction mixture was 100 µL (93 µL Assay Buffer, 2 µL OxiREd Probe in DMSO, Enzyme Mix 2 µL, 3 µL sample). Changes in absorbance at λ 570 nm were measured after 60 min using Synergy HTX Multi-Mode Reader (BioTek, Bad Friedrichshall, Germany).

Angiotensin-Converting Enzyme (ACE) Inhibitory Assay

The ACE inhibitory activity of the sample was determined using BioTek Microplate Readers and the spectrophotometric technique. An ACE1 Inhibitor Screening Kit (Catalog # K719-100) by BioVision was utilized. We set sample concentrations for 25, 50, 100, 200, and 400 µg/L. For the ACE activity assay, 25 µL of each (inhibitor control, buffer, and solvent control) was added to 40 µL ACE1 Enzyme Solution. Incubates the cocktail for 15 min at 37 °C (protected from light) and then adds 50 µL of the reaction mix containing ACE1 Assay Buffer 40 µl and 10 µl samples. Re-incubate the mixture and then measure the absorbance at λ 345 nm.

Alpha-Glucosidase (αGIA) Inhibitory Assay

The αGIA was measured following the protocol from Sigma-Aldrich (**α**-Glucosidase Activity Assay Kit, Catalog Number MAK123). This assay is based on a kinetic reaction with **α**-NPG as a substrate. The prepared standard (25, 50, 100, 200, and 400 µg/L) were diluted in phosphate buffer, pH 7.0. Transfer 20 µL of water to two wells of a transparent 96-well plate. Add 200 µL of water into one of these wells and 200 µL of Calibrator to the other wells. Transfer 20 µL of each sample into separate wells of the plate. Transfer 200 µL of the Master Reaction containing Phosphate buffer 200 µL and **α**-NPG 8 µL into each of the sample wells. Incubate the samples at 37 °C and after 20 minutes, take the final absorbance at λ 405 nm.

Acute toxicity assay

The brine shrimp lethality method was used to test the acute toxicity. In a transparent tank filled with seawater, *Artemia salina* eggs began to hatch. To keep the temperature between 25 °C and 30 °C during the hatching process,

a light bulb with a power supply of 40–60 watts was given. A blower was used to provide oxygen. In the toxicity test, larvae with a 48-hour life cycle were utilized. A vial was filled with 10 shrimp, 5 ml of seawater, and extracts at 10, 100, and 1000 ppm concentrations. Observations of brine shrimp cytotoxicity were made following a 24-hour incubation period at room temperature. The number of shrimps that survived was counted. A percentage of cytotoxicity was calculated (Choudhary 1999).

Non-targeted analysis

Analytes were separated using high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-Q–TOF-MS) using a Phenomenex C18 column (Aqua 5 m, 200 m, and 250 m i.d.) to characterize the water fraction of Cantigi's extract. A mobile phase system consists of two solvents A: 0.1 % formic acid in water and solvent B: 0.1% formic acid in acetonitrile. The elution procedure was a gradient; 0–15 min (A:95% & B:5%), 15–17 min (A:65% & B:35%), 17–24 min (A:25% & B:75%), 24–30 min (A:95% & B 5%) was used for the elution. The Mass HPLC-Q–TOF-MS (Agilent Technologies, 6520) fitted with twin electrospray ionization sources was used to separate the analytes that can be identified from *m/z* 100–1000. (ESI). The following instrument parameters were used to identify the analytes: gas flow, 8 L/min; gas temperature, 250 °C; nebulizer, 35 psig; and capillary voltage, 2750 V. Using ChemDraw Pro 8.0, the structures of the discovered compounds were shown.

Data analysis

Thermo Scientific's Compound Discoverer software (Thermo Scientific, USA) was used to process the ionic chromatogram. The data were filtered according to the compound name, best match to Mz Cloud, and MS2 of DDA for preferred ions.

Statistical analysis

All measurements were made in triplicate. STATISTICA 13.1 software was used for statistical analysis, which included a mean comparison using ANOVA and a posthoc Tukey's honestly significant difference (HSD) test at a significance level of = 0.05.

Result

Phytochemical screening was carried out to determine the secondary metabolic content of the samples, as shown in Suppl. material 1.

Phenolic content of Cantigi's extract

One of the most important groups in Cantigi's fruits is phenolic compounds. In our study, we determined the phenolic content in three solvents. As shown in Table 1, Ethanol (EOC) has the highest content for all groups of compounds, indicating that the polarity within the samples was between EAC and WTC (Galanakis et al. 2011).

Table 1. Total phenolic compounds in the samples.

Sample	Compounds		
	Phenol*	Flavonoid**	Anthocyanin***
EOC	392.24±0.5$^{\text{T}}$	160.39±0.8$^{\text{I}}$	40.89±0.6§
EAC	8.33±0.5$^{\text{T}}$	51.39±0.1$^{\text{I}}$	0.44±0.1§
WTC	37.38±0.1$^{\text{T}}$	100.86±1.7$^{\text{I}}$	4.07±0.1§

$^{\text{T}}$, $^{\text{I}}$, § indicating significantly different at $p<0.05$.
*mg gallic acid eq./100 g extract.
**mg quercetine eq./100 g extract.
***mg cyanidin 3-glucoside eq./100 g extract.

In vitro anti-metabolic assay (lipase, ACE, and αGIA)

High blood pressure and obesity are symptoms of metabolic syndrome. These two problems are caused by bad eating habits, which increase the activity of enzymes like ACE and lipase (Franklin 2006; Litwin and Kułaga 2021). Blood glucose abnormalities are one of the metabolic syndrome illnesses. Large fluctuations in blood glucose levels are extremely harmful to patients. In addition to medication for high blood glucose levels and insulin resistance, it is critical to inhibit glucose release from food (Succurro et al. 2022). In this circumstance, it is critical to reduce the activity of enzymes involved in polysaccharide hydrolysis. Here we utilized IC_{50} to express the inhibition activity of samples. The inhibitory concentration (IC_{50}) needed to lower an enzymatic reaction's rate by 50%. The highest *in vitro* antimetabolic activity was obtained by WTC, 27.74±3.66 µg/mL; 19.33±2.86 µg/mL; and 15.90±1.82 µg/mL for Lipase; ACE; and αGIA, respectively. It should be noted that higher numbers mean the sample relatively has low activity, as shown in Table 2.

Table 2. Antimetabolic activity of the samples.

Sample	Inhibition activity (IC_{50}) µg/mL		
	Lipase	ACE	αGIA
EOC	110.48±2.13*	27.32±1.24$^{\text{I}}$	53.72±1.98§
EAC	158.79±2.84*	62.83±2.31$^{\text{I}}$	83.62±1.01§
WTC	27.74±3.66*	19.33±2.86$^{\text{I}}$	15.90±1.82§

*, $^{\text{I}}$, § indicating significantly different at $p<0.05$.

Chemical components

HPLC-MS is a fundamental analytical technique for a variety of applications and areas. With typical electron ionization (EI) ion sources, the use of 70 eV EI libraries in -MS enables rapid sample identification with names and structures at the isomer level (Medeiros 2018). The LC-MS spectrum confirmed the presence of multiple compounds with varied retention durations, as shown in Fig. 2. The data demonstrated that delphinidin, an anthocyanin compound, was the major component observed in WTC.

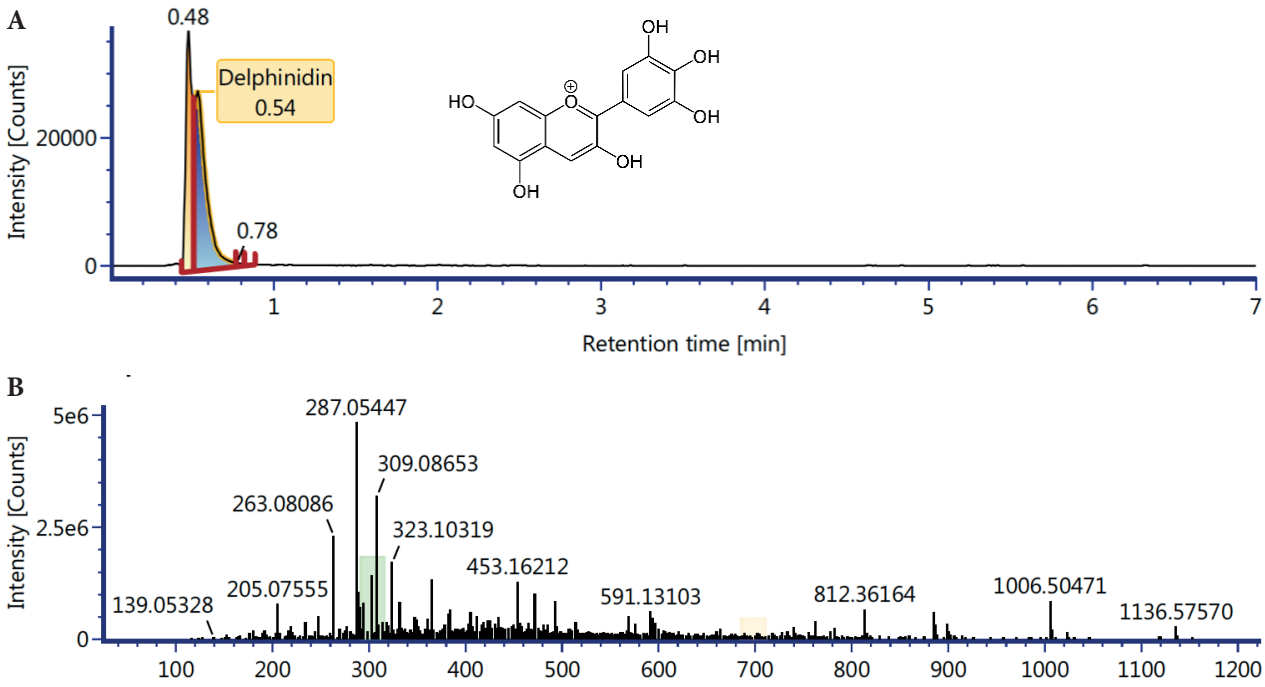

Figure 2. **A**) HPLC chromatogram of individual anthocyanins in the WTC and **B**) Ionic fragmentation of delphinidin in WTC of Cantigi.

Brine shrimps cytotoxicity of ethanol extract

The results of Brine shrimp's cytotoxicity assays are summarized in Fig. 3. The LC_{50} is 196.56±14.8 mg/ml (n = 3) for WTC.

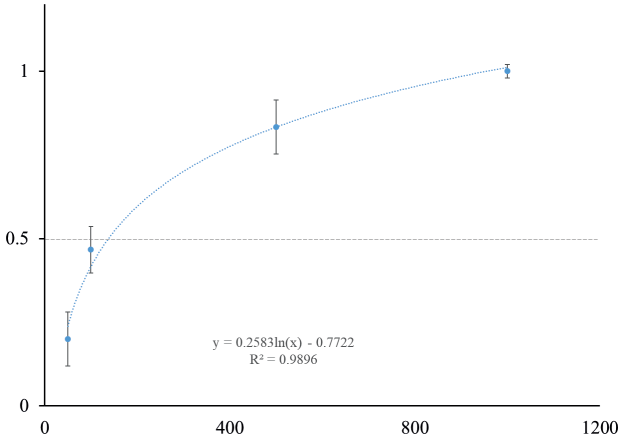

Figure 3. Brine shrimp cytotoxicity of WTC of *Vaccinium Varingiaefolium* Bl. Miq.

Discussions

Vaccinium's genus is well known for its health advantages, allegedly because of its high phenolic component concentrations or specific, potent polyphenolic chemicals that may interact (additively or synergistically) to improve human health conditions (Seeram et al. 2004). Blackberries rank high among fruits in terms of antioxidant strength due to their high quantities of phenolic components such as gallic acid, tannins, ellagitannins, quercetin, ellagic acid, cyanidins, and anthocyanins. (Hager et al. 2008). The water fraction (WTC) of ethanol extract shows higher phenol, flavonoid, and anthocyanin content than EAC (Table 1). This result is in agreement with what was previously reported by Sergio et al. (Vilas Boas 2017). Additionally, the phenolic and anthocyanin content of WTC is fivefold more than EAC. It is suggested to use water solvent for further isolation. Furthermore, according to the result, Cantigi has a lower anthocyanin concentration than blackberries (89–211 mg/100g) and Vaccinium's genus (84–430 mg/100g). It is due to that anthocyanin contents vary greatly among cultivars and plant species, even those belonging to the same genus (Daniela et al. 2017).

It is reported that anthocyanin was found to reduce body fat accumulation and obesity in mice significantly (Prior et al. 2008). Our result demonstrated that WTC has higher lipase inhibitor activity than EAC (Table 2), which is in line with a study by Simona et al. (Fabroni et al. 2016). Interestingly, the anthocyanin content in WTC is higher than in EAC (Table 1), indicating phenol compounds, anthocyanin, taking play in lipase inhibition; Other studies confirm this finding (Fabroni et al. 2016; Shirai 2017; Xie et al. 2020). Further, the *in vitro* ACE inhibition activity test of samples was measured using ACE Kit (Biovision) because of the fast, accurate, and specific process. WTC had the highest inhibition of samples, followed by EOC and EAC, confirming that phenolic chemicals play an important role in inhibitory activity. Like lipase and ACE inhibition, αGIA inhibition activity also demonstrated the highest activity to WTC. This finding is in line with previous studies about the role of phenolic compounds in various pharmacological activities, including αGIA (Zeng et al. 2006; Lipinski 2011; Niki 2011). In general, phenolic compounds derived from natural sources have been recognized as the primary phytochemicals with antioxidant

effects. They function by lowering the formation of reactive oxygen species (ROS) in diabetic complications (Alam et al. 2017).

Phenolic substances, such as flavonoids (flavonols and anthocyanins), are thought to be biologically active non-nutrient substances that contribute to defining the antioxidant potential of fruits. Antioxidant activity in blueberries corresponds strongly with anthocyanin and total phenolic content (Prior et al. 1998; Ehlenfeldt and Prior 2001). Here we found that delphinidin is an anthocyanin component of WTC. There are only six anthocyanidins that are widely distributed and essential for food, including delphinidin, cyanidin, malvidin, pelargonidin, peonidin, and petunidin (Tanaka et al. 2008). Prior reports mentioned the various delphinidin biological activity such as anti-inflammation (Sogo et al. 2015), anti-microorganism (Santos et al. 2011; Hariri et al. 2016), antioxidant (Nam et al. 2016; Goszcz et al. 2017), anti-cancer (Keravis et al. 2015; Kim et al. 2017), and anti-metabolism (Szotáková et al. 2013; Srovnalova et al. 2014). Due to the broad range pharmacological activity of delphinidin, we proposed that delphinidin contributes to lipase, ACE, and glucosidase inhibition activity. Furthermore, our result shows delphinidin as a diphenyl propane-based polyphenolic ring structure. However, other secondary modifications of delphinidin exist. Its modifications contribute to its stability, bioavailability, and transformation (Sakaguchi et al. 2013).

Incorporating natural ingredients into processed meals and beverages is critical for boosting customer acceptance of these items. Anthocyanins and many other dietary bioactive substances do not currently have dietary reference intakes in the United States, Canada, or the European Union. Currently, China has set a precise suggested limit of 50 mg/d for anthocyanins (Wallace and Giusti 2015). The BSLT test shows the LC_{50} of WTC is 196.56±14.8. For examining bioactive substances found in nature, BSLT was a well-recognized toxicity evaluation method that finally led to the measurement of the IC_{50} of a poisonous component of the plant extract (Hamidi et al. 2014). Meyer's toxicity index classifies extracts as dangerous or non-toxic according to their LC_{50} value, which ranges from 1000 g/ml to > 1000 g/ml (Meyer et al. 1982). Although other studies indicate that anthocyanin toxicity ranges from low to no, our result demonstrated taking precautions for anthocyanin toxicity. Furthermore, the dietary intake of anthocyanins for people over the age of 20 has been calculated to be 11.6 1.1 mg/d according to data from the 2007–2008 NHANES (Wallace and Giusti 2015). Furthermore, the toxicity of delphinidin has been reported to occur at concentrations above ~10 μM. This finding may shed light on the reason why Katrzyne et al. are predominantly excluded in the gut and why plasma concentrations are kept below ~10 μM (Goszcz et al. 2017). The observed toxicity threshold indicates that higher concentrations of delphinidin could have adverse effects on the body, leading to a selective exclusion mechanism in the gut and strict regulation of plasma levels to maintain safety.

Conclusion

The present study suggests that anthocyanin strongly correlates with the inhibition of lipase, ACE, and αGIA**.** We suggested these activities due to the phenolic compounds, including anthocyanins, in the water fraction of ethanolic extract. The HPLC-Q–TOF-MS data demonstrated that the major anthocyanin of WTC is delphinidin; thus, we guessed that it contributes to the pharmacological assays.

Acknowledgments

Firstly, we would like to express our sincere gratitude to the Doctoral Program of Pharmaceutical Sciences in the Faculty of Pharmacy at Pancasila University for their support and guidance throughout the course of this study. Secondly, we highly appreciate to National University (UNAS) as an awardee for collaboration with Rutgers University, The Center for Botanicals and Chronic Diseases (CBCD) and the research reported in this publication was supported by the Fogarty International Center of the National Institutes of Health under Award Number D43TW009672. We are incredibly grateful to Research Center for Pharmaceutical Ingredient and Traditional Medicine of National Research and Innovation Agency (BRIN) for the facilities and technical support.

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Supplementary material 1

Phytochemical screening of Cantigi extract

Authors: Ana Yulyana, Chaidir Chaidir, Partomuan Simanjuntak, Lilik Sulastri, Syamsudin Abdillah

Data type: .docx

Link: https://doi.org/10.3897/pharmacia.70.e109333.suppl1