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Antibacterial Activity of Grape Seed Extract (*Vitis vinifera* L.) against *Enterococcus faecalis*

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Abstract

Background: Grape seeds are one part of the grape plant known to have antibacterial properties because they contain secondary metabolite compounds such as tannins and flavonoids. Previous studies have shown that grape seed extract has antibacterial activity against *Streptococcus pyogenes*, *Streptococcus mutans*, *Propionibacterium acnes*, and *Staphylococcus epidermidis*. However, testing for Enterococcus faecalis from 70% ethanol extract and n-hexane has not been found. This study aims to determine the antibacterial activity of n-hexane and 70% ethanol extract from grape seeds against *E. faecalis* bacteria. **Methods:** Extraction was carried out using the maceration method using two solvents: n-hexane and 70% ethanol. The antibacterial activity test used the disc diffusion method to determine the Diameter Inhibitory Power (DDH) value at extract concentrations of 5%, 10%, 20%, and 40%. **Results:** The results obtained showed that grape seed extract had antibacterial activity against *E. faecalis*, with the DDH value of n-hexane extract respectively being 9.70 mm, 10.36mm, 10.55mm, and 11.31 mm at concentrations of 5%, 10%, 20%, and 40%. The DDH value of the 70% ethanol extract was 11.20 mm, 12.34mm, 13.63mm, and 15.49 mm at concentrations of 5%, 10%, 20%, and 40%, respectively. **Conclusions:** These results indicate that n-hexane and 70% ethanol extract from grape seeds have antibacterial potential against *E. faecalis*.

Keywords: Antibacterial; *Enterococcus faecalis*; Ethanol; n-Hexane; *Vitis vinifera*

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Introduction

Grapes are a fruit that people often consume. Grapes can be directly used as fresh fruit or juice for various processed foods or drinks (FAO, 2016). The grapes usually only use flesh and skin, while the seeds are discarded. Grape seeds generally have a polyphenol content of around 60-70% (Shi et al., 2003; Godevac et al., 2010; Di Stefano et al., 2022). The polyphenolic compounds that grape seeds are known to contain are tannins and flavonoids (Syafriana et al., 2020a). This compound is essential in the health sector as an antimicrobial (Xia et al., 2010; Kanagarla et al., 2013).

Several studies have reported that grape seed ethanol extract can inhibit the growth of oral pathogenic bacteria, such as *Streptococcus pyogenes* (Syafriana et al., 2020a), *Streptococcus mutans* (Hamida et al., 2021), and *Aggregatibacter actinomycetemcomitans* (Mirkarimi et al., 2013) as well as skin bacteria such as *Propionibacterium acnes* and *Staphylococcus epidermidis* (Syafriana et al., 2020b). Grape seeds have also been reported to inhibit the growth of urinary tract pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus aureus* with methanol solvent (Ranjitha et al., 2014).

However, little research has been reported regarding the effects of grape seed extract on *Enterococcus faecalis* bacteria. The activity of grape seed extract against *E. faecalis* was reported by Baydar et al. (2004) from a mixed extract of ethyl acetate: methanol: water (60:30:10) and acetone: water: acetic acid (90:9.5; 0.5) using the soxhletation extraction method. This bacterium is known to be a pathogenic bacteria in humans and animals with a relatively high level of resistance (Syafriana et al., 2019).

Enterococcus faecalis is a Gram-positive bacterium, which is a facultative anaerobe. This commensal bacteria can be found in the digestive tract of babies or adult humans (Selleck et al., 2019). *E. faecalis* is known to be one of the leading causes of nosocomial infections, such as urinary tract infections, endocarditis, bacteremia, and intra-abdominal and intra-pelvic abscesses (Bhardwaj et al., 2013). This bacteria can also be found in the female reproductive organs or the oral cavity, although in smaller numbers than in the digestive tract (Stuart et al., 2006). Enterococcus genus bacteria are known to be resistant to various antibiotics, including beta-lactams, aminoglycosides, lincosamides, and streptogramins (Tyne et al., 2013; Selleck et al., 2019; Syafriana et al., 2019). The widespread resistance of Enterococcus to various antibiotics has triggered research to look for new sources of antibacterial agents from natural ingredients, such as grapes.

Based on the description above, research was carried out on antibacterials derived from natural ingredients, namely grape seed extract (*Vitis vinifera* L.), against one of the pathogenic bacteria, *E. faecalis*. This research aims to determine whether grape seed extract from ethanol and n-hexane solvents can inhibit the growth of *E. faecalis*.

Grape seed extract was obtained through a maceration process using two solvents with significant polarity differences: n-hexane and 70% ethanol. The maceration method was chosen because it is the simplest way to extract natural ingredients (Ministry of Health of the Republic of Indonesia, 1995). Antibacterial activity was tested using the disc diffusion method (Kirby-Bauer Disk Diffusion) by observing the clear zone formed around the disc as the Diameter of Inhibitory Power (DDH) (Hudzicki, 2009; Dafale et al., 2016). Determining antibacterial activity using the diffusion method is essential because the equipment is simple, is a standard method for testing microbes, and has better test results than other methods (Fadillah et al., 2017).

Methods

Tools

The equipment used in this research was a rotary evaporator (vacuum rotary evaporator), water bath, oven (Memmert), Laminar Air Flow (LAF), blender (Philips), analytical balance (Excellent), hot plate stirrer (B-One), aluminum foil (Klin Pak), blender (Phillips), caliper (Kenmaster), microscope (Olympus), slide, cover glass, autoclave (Hirayama), incubator (Memmert), stir bar, vortex (Barnstead), Petri dish, test tube (Pyrex), tube rack, Beaker glass (Iwaki), Erlenmeyer (Iwaki), volumetric flask (Iwaki), micropipette (VWR and Peqpette), tweezers (GOOI), tube needle, gauze, cotton, parchment paper, dropper pipette, vial, spirit burner, evaporator cup, measuring cup (Pyrex).

Materials

The test materials used were grape seeds, Mueller Hinton Agar (Oxoid), blank discs (Oxoid), Amoxycillin discs 25 µg/disc (Oxoid), 100% dimethyl sulfoxide (DMSO), 70% ethanol (Brataco), n-hexane, distilled water (Brataco), Bouchardat, Mayer and Dragendorff reagents, FeCl3 (Merck), ammonia (Merck), NaNO2 (Merck), anhydrous acetic acid (Merck), chloroform (Merck), HCl (Merck), ether, H2SO4 (Merck), 0.9% physiological NaCl solution, immersion oil (Gargille), crystal violet solution (Merck), iodine solution (Merck), safranin solution.

Test Material Processing

40 kg of grapes obtained from the Kramat Jati Main Market, East Jakarta, were separated from the seeds. The grape seeds obtained are then cleaned of any remaining skin or flesh and washed thoroughly with running water. Once clean, the seeds are air-dried for 4 days. The dried grape seeds are then sorted from rotting or moldy seeds. Seeds that meet the criteria are ground using a blender and sieved using a 60 mesh to make the particle size homogeneous (Syafriana et al., 2020b).

Preparation of Grape Seed Extract and Ethanol Free Test

Grape seeds that have been ground and sifted are extracted using the maceration method using n-hexane and 70% ethanol in a ratio of 1:10 for 1x24 hours. The maceration results (macerate) obtained are then filtered and stored in a new container. Next, maceration is carried out twice by soaking the filter residue with a new solvent until a clear macerate is obtained as a sign that all compounds are attracted. The filtrate obtained from maceration and remaceration was evaporated using a rotary evaporator and continued over a water bath to produce a thick extract (Syafriana et al., 2020b).

The thick extract obtained was subjected to ethanol examination to ensure no solvent remained in the extract. A total of 1 g of extract was added with 1 mL of 1 N sodium hydroxide, then left for 3 minutes, then 2 mL of 0.1 N Iodine was added. Positive results will be indicated by the appearance of the smell of iodoform and the formation of a yellow precipitate within 30 minutes. If the extract has no iodoform odor and no yellow precipitate is formed, the reaction is declared hostile, indicating that the extract is free from solvents (Sumiati, 2014).

Phytochemical Screening

Phytochemical screening was carried out to determine secondary metabolites from grape seed extract (*Vitis vinifera* L.). Secondary metabolites were tested qualitatively, including tests for alkaloids, flavonoids, saponins, tannins, and terpenoids (MOH RI, 1989; Syafriana et al., 2020a; Syafriana et al., 2020b).

Antibacterial Activity Test of Grape Seed Extract

Antibacterial activity testing was carried out using the disc diffusion method. The test bacteria used was Enterococcus faecalis, which was obtained from the Parasitology Laboratory, Faculty of Medicine, University of Indonesia. The bacterial culture of *E. faecalis,* which has been rejuvenated for 24 hours, is taken in one dose and then suspended in 5 mL of 0.9% NaCl solution. The turbidity is equivalent to Mc Farland 3, 9 x 108 CFU/mL. Then, 1 mL of the suspension is put into a test tube containing 9 mL of 0.9% NaCl, homogenized with a vortex (107 CFU/mL) (Rachmatiah et al., 2020).

The extract is first diluted according to the test concentration (5%, 10%, 20%, and 40%) using 100% DMSO solvent (Syafriana et al., 2020a). To obtain an extract with a concentration of 5%, 5 g of the thick extract was weighed and then dissolved with 100% DMSO until a final solution volume of 100 mL was obtained. The same thing is done for other concentration variations.

1 mL of the test bacterial suspension was pipetted, put into a petri dish containing solid media, and spread using an L rod. After that, a sterile paper disc on which 20 µL of the test solution had been dripped was placed on top of the media containing the bacterial suspension. This study used a positive control of the antibiotic amoxicillin 25 µg/disc, while the negative control was 100% dimethyl sulfoxide (DMSO) (Syafriana et al., 2020a).

After all the discs were inoculated into the test medium, the plates were incubated at 37^{o}C for 24 hours in an incubator. After incubation, the bacterial growth was observed by observing whether or not a clear zone formed around the disc that had been filled with the test solution. The clear zone formed is measured using a caliper by taking measurements on three sides of the clear zone horizontally, vertically, and obliquely. The

measurement results are then averaged and expressed as Diameter Inhibitory Power (DDH) values in millimeters (mm) to indicate the magnitude of the inhibitory activity of the extract against the test bacteria (Hudzicki, 2009; Dafale, 2016).

Data Analysis

The DDH values obtained were averaged using Microsoft Excel and analyzed descriptively.

Result

Test Material Processing

As a result of processing grape seeds from 40 kg of fruit, 370 g of fresh seeds were obtained, and 192 g of dry seeds were obtained. These dry seeds were then ground into simplicia powder and sieved with a mesh fineness of 60. The resulting fine powder was 91 g.

Ethanol-free Extraction and Test

The results of grape seed extraction are shown in Table 1.

Table 1. Results of percentage yield of grape seed extract (*Vitis vinifera* L.)

Solvent	Weight of Simplicity (g)	Weight of condensed Extract (g)	Yield (%)
n-heksan	30	2,14	7,13
Etanol 70%	40	22,60	56,50

The results of the ethanol-free test showed that grape seed extract gave negative results in the ethanol-free test. This was indicated by the absence of the smell of iodoform and the absence of yellow precipitate formed.

Phytochemical Screening

The results of the phytochemical screening of grape seed simple powder, 70% ethanol extract, and n-hexane extract can be seen in Table 2.

Table 2. Results of phytochemical screening of grape seed powder and extract

Compound	Phytochemical Screening Results		
	Powder	Ethanol Extract 70%	n-hexane extract
Alkaloids	(-)	(-)	(-)
Saponins	(+)	(+)	(+)
Flavonoids	(+)	(+)	(-)
Tannins	(+)	(+)	(-)
Terpenoids	(+)	(+)	(+)

Description:
(+): Contains Chemical Content or Compounds
(–): Does Not Contain Chemical Content or Compounds

Phytochemical Screening

The results showed antibacterial activity at each test concentration of both grape seed extracts against *E. faecalis*. The values of the Inhibitory Power Diameter (DDH) of both extracts against *E. faecalis* are shown in Table 3.

Table 3. Average Inhibitory Power Diameter (DDH) of Grape Seed Extract against Enterococcus faecalis

Extract	Diameter Resistance Value (DDH) (mm)					
	Extract concentration				Positive control	Negative control
	5%	10%	20%	40%	Amoksisilin 25 µg/disk	DMSO 100%
n-heksan	9,70	10,36	10,55	11,31	24,25	-
etanol 70%	11,20	12,34	13,63	15,49	27,05	-

Description:

- : No clear zone is formed around the disc.

Discussion

The drying process of grape seeds is carried out using the air-dry method. This process is chosen to maintain the compound content in grape seeds so that they are not easily damaged by exposure to sunlight. After drying, the seeds are ground to a fineness of 60 mesh. Sieving is done so that the powder has a homogeneous size for efficiency in extraction. Homogeneity and small particle size will increase solvent penetration and solute diffusion, making the extraction process more efficient (Zhang et al., 2018; Syafriana et al., 2020b). The extraction results with n-hexane solvent were obtained as much as 2.14 g with a yield of 7.13%, while with 70% ethanol, 22.6 g of thick extract was obtained with a yield percentage of 56.5% (Table 1). From the data obtained, the results with 70% ethanol solvent produced a higher extract yield compared to n-hexane solvent. This shows that the compounds contained in grape seeds are more suitable to be extracted with 70% ethanol than n-hexane. These results are also by previous studies that showed that the yield of 70% ethanol extract was greater than that of n-hexane extract (Syafriana et al., 2020b).

The 70% ethanol extract of grape seeds obtained was then subjected to an ethanol-free test. This test is carried out to determine whether the extract still contains ethanol. This is done because of the nature of ethanol, which can kill microbes, so it is feared to affect the antibacterial activity of the ethanol extract of grape seeds (Syafriana et al., 2020a). The results showed that the grape seed extract gave negative results in the ethanol-free test. This was indicated by the absence of an iodoform odor and the absence of a yellow precipitate formed. Based on these results, the extract can be continued for antibacterial testing because there is no residual ethanol, so the results will not be confused.

Phytochemical screening was carried out to determine the secondary metabolites contained in grape seeds. Based on the results of phytochemical screening (Table 2), it was found that the powder and ethanol extract of grape seeds contained saponins, flavonoids, tannins, and terpenoids. In contrast, the n-hexane extract only contained saponins and terpenoids. These results follow the literature stating that 70% ethanol as a polar solvent can attract more secondary metabolite compounds than nonpolar n-hexane (Syafriana et al., 2020a). These results are also in line with the law of like dissolves like, which shows that the nonpolar solvent n-hexane can attract nonpolar terpenoid and saponin compounds, while the polar solvent 70% ethanol can attract phenolic compounds (Tiwari et al., 2011; Hepsibah et al., 2017; Zhang et al., 2018; Putra et al., 2019; Syafriana et al., 2020a). Phenolic compounds, such as tannins and flavonoids, are secondary metabolites found in all types of plants (Crozier et al., 2006; Tiwari et al., 2011). Based on the literature, phenolic compounds are the most significant component in grapes after carbohydrates and fruit acids (Shi et al., 2003). Alcohol is known to be effective in attracting phenolic compounds, so this solvent is very suitable for extracting grape seeds, which are rich in these compounds (Syafriana et al., 2020a).

Antibacterial activity was carried out using the disc diffusion method. Observations were made by observing the clear zone formed around the disc, indicating that the

extract could inhibit the growth of the test bacteria (Dafale et al., 2016). The test results showed antibacterial activity at each test concentration of the two grape seed extracts against *E. faecalis*. The Inhibitory Power Diameter (DDH) values in n-hexane extracts with concentrations of 5%, 10%, 20%, and 40% were 9.70 mm, 10.36 mm, 10.55 mm, and 11.31 mm. The results of measuring the DDH value of 70% ethanol extract of grape seeds at concentrations of 5%, 10%, 20%, and 40% were 11.20 mm, 12.34 mm, 13.63 mm, and 15.49 mm, respectively. The results of measuring the DDH value obtained from the n-hexane and ethanol extracts were still below the DDH value of the positive control, Amoxicillin 25 µg/disk, with DDH values of 24.25 mm and 27.05 mm (Table 3). The overall antibacterial activity test results showed that the higher the extract concentration value, the greater the inhibitory power formed. This is likely due to the higher content of active substances so the antimicrobial activity will be more significant (Dewi et al., 2019; Syafriana et al., 2020b). This pattern is consistent with previous studies on different microorganisms (Fadillah et al., 2017; Syafriana et al., 2020a; Syafriana et al., 2020b; Syafriana et al., 2020c; Hamida et al., 2021). This shows that grape seed extract with n-hexane and 70% ethanol solvents has the potential as an antibacterial agent for *E. faecalis*.

The data in Table 3 also shows that the DDH value in 70% ethanol extract is greater than that of n-hexane extract. This is likely because 70% ethanol extract contains more compounds that act as antimicrobials than n-hexane extract. The more active substances extracted, the greater the chance of attracting antimicrobial substances, so the resulting inhibitory value is more significant (Lingga et al., 2016; Syafriana et al., 2020a; Hamida et al., 2021). The inhibition of bacterial growth by grape seed extract cannot be separated from the work of the chemical compounds contained therein. Although n-hexane extract only shows the presence of two compounds, saponins, and terpenoids, both can disrupt bacterial cell stability. Saponins have a detergent-like structure to dissolve bacterial cell membranes, while terpenoids can disrupt the peptidoglycan component of bacterial cell walls (Cowan, 1999; Ibrahim & Kuncoro, 2012). In addition, other compounds were found in the 70% ethanol extract of grape seeds, namely tannins and flavonoids. Flavonoids are known to cause bacterial proteins to denature so that they will interfere with bacterial metabolism, while tannins are thought to shrink cell walls and interfere with the work of DNA gyrase in the DNA replication process (Cowan, 1999; Ibrahim & Kuncoro, 2012; Górniak et al., 2019; Khameneh et al., 2019). If these compounds work synergistically, they can cause bacterial cell death.

Conclusions

Grape seed extract with n-hexane and 70% ethanol solvent inhibited the growth of Enterococcus faecalis bacteria at each test concentration.

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Declaration statement

The authors reported no potential conflict of interest.

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