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Antibacterial activity of *Eurya acuminata* DC. leaves ethanol extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract

Antibiotics as modern medicine in the treatment of infections have experienced resistance due to irrational use. Traditional medicinal plants have many chemical components that have been used to treat various diseases including skin diseases, one of them is *Eurya acuminata* DC. The study aimed to determine the optimum concentration of *E. acuminata* leaves extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This antibacterial test was carried out using the diffusion and dilution method. The concentrations of ethanol extract used were 20%, 40%, 60%, 80% and fresh extract. The highest antibacterial activity of *E. acuminata* leaves ethanol extract was by diffusion against *P. aeruginosa* and *S. aureus* were present at a concentration of 60% with an inhibition zone by 11.72 mm and 10.84 mm, respectively. The MIC and MBC values of the ethanol extract of *E. acuminata* leaves were not found. It was concluded that *E. acuminata* leaves ethanol extract might be an alternative antibacterial agent because it was able to inhibit *P. aeruginosa* and *S. aureus*.

Keywords: Antibacterial; Ethanol extract; *Eurya acuminata; Pseudomonas aeruginosa; Staphylococcus aureus*

1. Introduction

Infection is a state of entry of microorganisms into the body and multiply, causing disease. Wounds that are not properly cared for can also be a cause of infection or bleeding due to bacteria. Bacteria that cause infectious diseases in wounds include *Pseudomonas aeruginosa* and *Staphylococcus aureus* [1]. There are many cases where bacteria are resistant to several antibiotics, so it is necessary to look for alternative compounds derived from natural ingredients as antibacterials. One of them is by utilizing the leaves of the medicinal plant *Eurya acuminata*.

E. acuminata (*Pentaphylacaceae*) is a shrub or tree plant with a height of up to 15 m which lives in forests and shrubs on mountain slopes at an altitude of 700-3000 m. This plant is spread in Bhutan, India, Indonesia, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand, dan Vietnam [2, 3]. The leaves of *E. acuminata* have good properties in wound healing, dysentery/diarrhea, typhus, and sore throat [4]. The leaves also can be used as herbal medicine for mothers in childbirth [5], besides that the leaves also can be consumed directly or as a food ingredient [6].

Previous study by Malewska [7], showed the antibacterial activity of *E. acuminata* extract in inhibiting the growth *of S. aureus* (MIC 625 µg/mL) and *Salmonella typhimurium* (MIC 2,500 µg/mL). *E. acuminata* leaves extract contains active compounds, namely saponins and tannins that have an astringent taste and have antibacterial activity [5]. Faisal et al. [8] reported that *E. acuminata* leaves contain active phytol compounds (acyclic diterperne) and β-sitosterol which have antibacterial properties. *E. acuminata* also contains the compound hexatriacontan-1-ol which exhibits antimicrobial activity against *Candida albicans* [4].

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The content of antibacterial compounds in *E. acuminata* leaves can be an alternative to the use of antibiotics that have experienced resistance. Therefore, in this study various concentrations of ethanol extract were used to analyzed the antibacterial activity.

2. Material and methods

2.1. Sample collection

Leaves samples were obtained from the Biology Education and Research Forest Andalas University, Padang, Indonesia. Fresh leaf samples were cleaned and air-dried. Then the sample is mashed using a grinder. Then sieved using a 50 Mesh sieve [9].

2.2. Extraction

The extraction process was carried out using a modified method by Sulaiman et al. [9]. 1 kg of dried leaf powder was weighed into a maceration container, then extracted by the maceration method using a 96% ethanol solution. The maceration container is left for 3x24 hours at room temperature while stirring is repeated so that the active substance is completely extracted. After 3 days the extract was filtered using filter paper. Then the extract obtained was concentrated by vacuum distillation and rotary evaporator to separate the solvent from the active substance so that a thick extract was obtained.

2.2.1. Extract concentration

The ethanol extract obtained was then prepared at a concentration of 20%, 40%, 60%, 80%, by dilution and fresh extract. For a concentration of 80%, 20 grams ethanol extract was weighed and DMSO solvent was added until the volume 25 ml. For a concentration of 60%, 15 ml of a 80% concentration was take and added with DMSO until the volume becomes 20 ml. For a concentration of 40%, 10 ml of a 60%concentration was taken and added with DMSO until the volume becomes 15 ml. For a 20% concentration, 5 ml of a 40% concentration was taken and added with DMSO until the volume becomes 10 ml. For fresh extracts, 5 grams of fresh leaves crushed and added with 10 ml of distilled water [10].

2.3. Antibacterial activity

2.3.1. Diffusion test

Pure cultures of *P. aeruginosa* and *S. aureus* were inoculated on Nutrient Agar (NA) medium and incubated for 24 hours at 37°C. Then, 1 ose of each bacteria was taken and suspended with 0.9% NaCl until turbidity was obtained which was equivalent to 0.5 McFarland. Paper discs with a diameter of 0.6 cm were dipped in each extract concentration and then placed on the surface of the media. Incubated at 37°C for 24 hours. Chloramphenicol used as positive control and DMSO as negative control. The microbial free area formed was measured using a caliper, then the average was taken [11].

2.3.2. Minimum Inhibitory Concentration (MIC) test

2 ml of Nutrient Broth (NB) medium was put into the first to 10^{th} tube, then 2 ml of extract was put into each tube and homogenized. Then, from the first tube pipetted as much as 2 ml and put into 2^{nd} tube, and so on until the 10^{th} tube. Next, 2 ml of 10^{th} tube was taken and discarded so that the volume of each tube was 2 ml. 1 ml of bacterial suspension put in each tube. For control, the 11^{th} tube contains 2 ml extract and 2 ml of NB medium, the 12^{th} tube contains 2 ml NB medium and 1 ml bacterial suspension, and the 13^{th} tube contains 2 ml extract and 1 ml bacterial suspension. Then incubated at 37°C for 24 hours. The final process of this test is the appearance of turbidity which indicates the growth of the tested bacteria. If there is the lowest concentration that inhibits bacterial growth, indicated by the absence of turbidity, the Minimum Inhibitory Concentration (MIC) is obtained [12].

2.3.3. Minimum Bactericidal Concentration (MBC) test

15-20 ml of NB medium is put into a petri dish, then the media is allowed to stand until solid. Next, a extract was added by streaking the plate on the media and then incubated for 18 hours at 37°C. Observations for MBC was characterized by the growth or absence of bacteria to determine the potential of plant extracts that can kill pathogenic bacteria [13].

3. Results and discussion

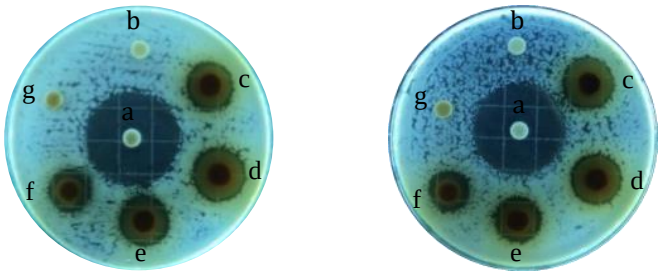
3.1. Antibacterial activity

3.1.1. Diffusion test

The ethanol extract of *E. acuminata* leaves produced antibacterial activity against *P. aeruginosa* and *S. aureus* as indicated by the presence of clear zones (Table 1, Figure 1). The ethanol extract of *E. acuminata* leaves at a concentration of 60% produced the largest inhibition zone against *P. aeruginosa* (11.72 mm.) Whereas for *S. aureus*, a large inhibition zone was produced by the ethanol extract of E. acuminata leaves at a concentration of 60% (10.84 mm).

Table 1 Inhibition zones of *E. acuminata* leaves ethanol extract of against *P. aeruginosa* and *S. aureus*

No	Extract concentration	Average inhibition zone diameter (mm)	
		Pseudomonas aeruginosa	***Staphylococcus aureus***
1.	80%	10.15	10.49
2.	60%	11.72	10.84
3.	40%	10.83	9.87
4.	20%	8.32	7.79
5.	Fresh extract	0	0
6.	Chloramphenicol	24.06	19.44
7.	DMSO	0	0



P. aeruginosa *S. aureus*

Figure 1 Antibacterial activity of *E. acuminata* leaves ethanol extract by diffusion test; (a) chloramphenicol, (b) DMSO, (c) concentration 80%, (d) concentration 60%, (e) concentration 40%, (f) concentration 20%, (g) fresh extract

The concentration of 60% was the effective concentration of the ethanol extract of *E. acuminata* leaves in inhibiting the growth of *P. aeruginosa* and *S. aureus* and included in the strong antibacterial category. According to Davis and Stout [14], the antibacterial power consists of four categories; the inhibition area of 20 mm or more is very strong, the inhibition area of 10-20 mm is the strong category, the inhibition area of 5-10 mm is the medium category, and the inhibition area is 5 or less included in the weak category. Based on Rastina et al. [15], the effective concentration is the concentration whose antibacterial power can form the largest inhibition zone. Irianto [16] stated that if the concentration of antibacterial chemical compounds exceeds a certain concentration, the increase in disinfection power will decrease. However, Malewska [7] report that the ethanol extract of *E. acuminata* leaves tested by diffusion did not show antibacterial activity against *S. aureus*, MRSA, MDRSA and *Escherichia coli*. This could be happened because the amount of ethanol extract of *E. acuminata* leaves used in the disc was only 2 mg which caused the extract not to diffuse properly.

Another factor that can affect the bacterial inhibition zone is the ability of the extract to diffuse into the disc paper. In this study, the extract obtained was thick and diluted to obtain each concentration. This causes the diffusion speed of each extract concentration to be different. Zeniusa et al. [17] stated that the diffusion process of the extract can be affected by the dilution factor. The higher concentration of the extract, the lower the solubility (thickens like a gel), so

this can slow down the diffusion of the active ingredients of the extract into the media and can ultimately reduce the ability of extracts with high concentrations to inhibit bacterial growth. Tambunan [18] states that the diameter of the inhibition zone depends on the diffusion speed of the antibacterial compound in the agar medium. The speed of diffusion can be affected by the ratio of the amount of solvent and solute. In certain circumstances, antibacterial can work optimally at low concentrations. At low concentrations, the amount of solvent is greater than the solute, making it easier to diffuse.

The highest inhibition zone at 60% concentration was still smaller when compared to the positive control of chloramphenicol. Chloramphenicol is a pure compound with a mechanism of interfering with protein synthesis from bacteria so that it can result in bacterial death. Katzung [19] stated that chloramphenicol is a broad-spectrum antibiotic that is active against Gram-positive and Gram-negative bacteria. Dewi et al. [20] stated that the mechanism of chloramphenicol in inhibiting bacteria is by joining the ribosomal subunits thereby preventing the joining of amino acids into proteins. This causes the synthesis of amino acids to be disrupted or not even take place which eventually results in the death of the bacterial cell.

From the results of the study it was also known that the fresh extract treatment of both *P. aeruginosa* and *S. aureus* did not show antibacterial activity. This can happened because of the type of solvent used, namely distilled water. Aquadest has a low polarity level compared to ethanol so it is not able to bind the active ingredients optimally. Ethanol binds polar compounds contained in *E. acuminata* leaves such as saponins, tannins, acyclic diterpenes and β-sitosterol. In addition, a concentrated solvent will attract the active substances in the material so that it can produce greater maceration results. This is related to Harborne's explanation [21] that a compound will dissolve in solvents that have the same polarity.

3.2. MIC and MBC Values

MIC test needs to be done to determine the strength of an antibacterial substance in inhibiting bacterial growth. Then proceed with the MBC test to find out whether an antibacterial substance can kill bacteria or only inhibit its growth. Based on Purnamasari [22], to assess MIC, turbidity in each test tube was observed and compared to positive controls and negative controls. The positive control is the turbidity limit because there is no antibacterial substance that inhibits the growth of the bacteria given. Conversely, the negative control is the limit of clarity because there are no test bacteria or contaminating organisms that can produce turbidity. While the growth control is an antibacterial substance that inhibits the growth of bacteria.

Table 2 MIC values of *E. acuminata* leaves ethanol extract against *P. aeruginosa* and *S. aureus*

Extract concentration	**Turbidity in broth**	
	P. aeruginosa	**S. *aureus***
50%	+	+
25%	+	+
12.5%	+	+
6.25%	+	+
3.125%	+	+
1.562%	+	+
0.781%	+	+
0.39%	+	+
0.195%	+	+
0.097%	+	+

+ = Growth, - = No Growth

Based on Table 2. the results showed that all test solutions extract showed bacterial growth as indicated by turbidity. The results obtained were different from Malewska [7] in that *E. acuminata* leaves had activity against *S. aureus* with MIC values reaching 625µg/mL. *P. aeruginosa* is one of the bacteria that is classified as resistant because in addition to being able to produce beta-lactamase enzymes that can hydrolyze beta-lactam rings (antibiotics) it also has the ability

to remove antibiotics from the cell by means of an efflux pump so that the bacteria can be resistant to several classes of antibiotics [23]. Some of the mechanisms of resistance to B-lactams are the expression of AmpC, OprD and efflux pumps [24]. Whereas *S. aureus* changed to a methicillin resistant strain (MRSA) because it received a large DNA element insertion between 20-100 kb called *Staphylococcal cassette chromosome mec* (SCCmec) and a change in the Penicillin Binding Protein (PBP). Penicillin Binding Proteins are a group of proteins involved in peptidoglycan biosynthesis, namely catalyzing transpeptidation reactions (formation of peptide bundles). This peptidoglycan is the target of beta-lactam antimicrobials [25].

The study continued with the MBC test by scraping the MIC results on NA medium (Table 3). The culture results for the negative control consisting of ethanol extract of *E. acuminata* leaves and NB media were sterile. None of the colonies grew on NA. This indicates that there is no contamination in the process so that there are no contaminant factors that affect the results. In the positive control consisting of bacterial suspension and NB media, there was growth of bacterial colonies. This showed the test bacteria can grow. In the growth control which consisted of the ethanol extract *of E. acuminata* leaves and bacterial suspension, there was also the growth of bacterial colonies which indicated that the bacteria were still alive in the ethanol extract. The culture results for first to 10th tubes showed the growth of bacterial colonies.

Table 3 MBC values of the ethanol extract of *E. acuminata* leaves against *P. aeruginosa* and *S. aureus*

Extract concentration	**Colony**	
	P. aeruginosa	**S. *aureus***
50%	+	+
25%	+	+
12.5%	+	+
6.25%	+	+
3.125%	+	+
1.562%	+	+
0.781%	+	+
0.39%	+	+
0.195%	+	+
0.097%	+	+

+ = Growth, - = No Growth

There are several factors that affect the results of the MBC test, namely the bacterial suspension, the method of scratching and the shape of the scratches. Different ways of scraping and the shape of the strokes also transfer different amounts of inoculum. These three factors cannot be made exactly the same in every work [22]. The antibacterial ability of the ethanol extract of *E. acuminata* leaves in inhibiting the growth of *P. aeruginosa* and *S. aureus* bacteria is strongly influenced by several secondary metabolite compounds that act as antibacterial compounds. These compounds include tannins, saponins, acylicditerpenes and β-sitosterol. According to Harborne [21], tannins are polyphenolic compounds that can precipitate proteins. If there is protein deposition in the cell wall and cytoplasm, the growth of the bacterial cell will be disrupted and result in cell death. Damage to the cell walls and cytoplasmic membranes caused by active substances *in E. acuminata* leaves, including foreign substances that are not wanted to enter the bacterial cells. Compounds that pass through the cytoplasmic membrane will enter and affect other cell organelles such as membrane proteins and mitochondria.

Saponins work as an antibacterial which disrupts the stability of the bacterial cell membrane which results in the release of important components from inside the bacterial cell, namely proteins, nucleic acids and nucleotides so that the process of forming the bacterial cell wall is incomplete [26]. Acylicditerpenes are phytol compounds, belonging to the class of diterpenoid and triterpenoid compounds which are thought to have antibacterial activity [27]. Based on Fadli [28], terpenoids have a great affinity for the cell membrane so that they have a high potential to penetrate the bacterial cell wall. Fessenden and Fessenden [29] stated that β-sitosterol (steroid) could inhibit the growth of Gram-negative bacteria. The mechanism of steroid action as an antibacterial is related to the lipid membrane and its sensitivity to steroid components which cause leakage in liposomes [30].

4. Conclusion

Ethanol extract of *E. acuminata* leaves produce the highest antibacterial activity against *P. aeruginosa* at a concentration of 60% with an inhibition zone of 11.72 mm, followed by concentrations of 40% (10.83 mm), 80% (10.15 mm), 20% (8.32mm). The highest antibacterial activity against *S. aureus* at a concentration of 60% (10.84 mm), followed by concentrations of 80% (10.49 mm), 40% (9.87), 20% (7.79 mm). While the fresh extract did not show antibacterial activity. There were no MIC and MBC values of the ethanol extract of *E. acuminata* leaves against *P. aeruginosa* and *S. aureus*. The ethanol extract of *E. acuminata* leaves had the potential to be developed as a new antibacterial agent drug.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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