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CHARACTERIZATION OF ANTIBACTERIAL BIOACTIVE COMPOUNDS FROM KUSAMBI LEAF EXTRACT (*Schleichera oleosa* (L) Oken)

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ABSTRACT

Background: The kusambi plant is traditionally used by people to treat various diseases. This plant is thought to contain various secondary metabolites with various pharmacological activities, including antibacterial. **Objectives:** This study aims to determine the antibacterial activity of various extracts using n-hexane, ethyl acetate, and 96% methanol as solvents against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 bacteria and identify what groups of compounds are contained in the kusambi leaf extract. **Method:** Kusambi leaves were extracted with Soxhlet using n-hexane, ethyl acetate, and 96% methanol (1:4 w/v). Activity testing uses the agar diffusion method, and compound class testing uses the TLC (thin layer chromatography) method and spray reagents. **Results:** The research showed the highest yield was 22% for methanol extract, 12% for N-hexane extract, and 20% for ethyl acetate extract. Methanol extract activity test Kusambi leaves show moderate intensity inhibitory activity against bacteria *S.aureus* bacteria, while bacteria *E.coli* did not show inhibitory activity. The most significant inhibition zone diameter in the ethyl acetate extract was 9.22 mm with a concentration of 10µg/mL, the methanol extract had an inhibition zone diameter of 8.82 mm with a concentration of 10 µg/mL, and the leaf N-hexane extract had no inhibition zone. The identification results of the compound groups were flavonoids, alkaloids, phenols, steroids, and triterpenoids, as proven by the TLC method and spray reagents. **Conclusion:** Kusambi leaf extract using ethyl acetate solvent has inhibitory activity against bacteria *S.aureus* and contains flavonoids, phenols, steroids, alkaloids, and triterpenoids.

Keywords: Kusambi plant; antibacterial; secondary metabolite compounds.

INTRODUCTION

Indonesia's biodiversity is extraordinary, making it one of the countries rich in natural resources. Indonesia has 13,576 species of medicinal plants, 15,671 types of health herbs, and 1,183 types of traditional medicines. However, those that have been used as new traditional medicine range from 180 types to meet the needs of the drug and herbal industry. Indonesia's biodiversity is widespread throughout the island. In addition, other living organisms, such as microorganisms and fungi, have yet to be touched much by research. This biodiversity

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diversity is a source of biomolecules of organic compounds that are very abundant [1]. Various attempts have been made from time immemorial to the present day to find out the valuable activity of various types of plants for the treatment of diseases, killing bacteria and fungi. However, the purpose of development and preservation requires continuous research so that bioactive compounds and their properties can be obtained to treat these diseases [2].

One plant that has potential as a natural antibacterial candidate is kusambi, known as *Schleichera oleosa* L Oken. Kusambi plants from the tribe Sapindaceae are tree plants that are widespread in tropical regions of Asia, such as India, Nepal, and Malaysia. In Indonesia, Kusambi can be found throughout East Nusa Tenggara, Java, and Bali specifically [3,4]. The community has traditionally used Kusambi as an antibacterial for skin infections, scabies, scabs, and inflammation of the ear [5].

In the current trend of people's lifestyles, the use of herbal products made from nature is still in demand by the Indonesian people even though modern medicine is widely circulated; the reason the community still trusts herbal medicine is because there is still the belief of a particular group of people in knowledge derived from ancestors passed down from generation to generation using ingredients from nature or through the services of someone who is believed to be able to treat [6]. According to Fabricant and Farnsworth (2001), approaches that can be used in medicinal plant research include chemical compound screening and taxonomic approaches to find drugs and bioactive compounds [7].

According to research conducted by Simpson et al. (2010), the bioactivity of kusambi has been investigated, and it is used as an antioxidant, anti-inflammatory, and antidiabetic [8,9]. Its use in the community treats several diseases such as fever, skin pain, sprains, dysentery, anti-inflammatory, and wound healing. Therefore, research and empirical use in the community encourage researchers to prove that kusambi leaves have secondary metabolites that have the potential to be antibacterial drug candidates.

METHODS

1. Object of Research

This research uses Kusambi or *Schleichera oleosa* L Oken, which grows wild in North Central Timor Regency, East Nusa Tenggara Province. The diameter of the tree is 42-50 cm, and the part used in this research is the leaves. The positive control uses the antibiotic chloramphenicol, and the negative control uses a solvent adapted to each extract. *S. aureus* and *E. coli* were used as test bacteria.

2. Research Instruments

The tools used in this research were a rotary evaporator, oven, autoclave, microtube, sterile cotton bud, analytical balance, petri dish, bunsen lamp, test tube, Erlenmeyer 100 mL, test tube rack, aluminum foil, plastic wrap, blank disc paper diameter 10 mm, micropipette, centrifugator, Thin Layer Chromatography (TLC) F254 and laminar airflow.

The materials used in this research were 96% methanol, n-hexane, and ethyl acetate (technical). Spray FeCl reagent3, Anisaldehyde sulfuric acid (A.A.), L.B. (Liebermann-Burchard), Dragendorff (D.D.), Sitroborat (Sitro), McFarland 0.5 from the Department of Microbiology, Faculty of Medicine, Public Health and Nursing (FKKMK-UGM), physiological NaCl (sterile), sterile aqua, 70% alcohol, *Escherichia coli* ATCC 25922, and *S.aureus* ATCC 25923 from the Department of Microbiology, Faculty of Medicine, Public Health and Nursing (FKKMK-UGM); Chloramphenicol discs 30 µg from OXOID,

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nutrient broth (N.B.) and nutrient agar (N.A.) from OXOID.

3. Research Procedure

a. Simplisia preparation

Leaves are taken according to the results of the determination. Picked leaves are leaves that have fully bloomed, picked in the morning. A total of 5 kg of leaves are sorted first to separate dirt or other foreign material. The leaves are thoroughly washed with running water to remove dirt or sap attached to the leaves.

Clean leaves are reduced in size by chopping them into small pieces, which is done to facilitate the drying process. The drying method is dried for twenty-four hours in the oven at 50°C for 24 hours. After drying, the leaves are in a blender, sifted, and stored in a dry container [10].

b. Extract Manufacturing Process

Kusambi leaf powder, as much as 510 grams, was then extracted by the Soxhlet method using a solvent gradient of n-hexane, ethyl acetate, and 96% methanol with the amount of solvent used 1.5 liters. The soxhlet process is carried out during 27 circulations. The result is filtrate accommodated and evaporated with a rotary evaporator. Then, evaporate with a water bath to remove the remnants of solvent that are still mixed with the extract.

c. Sterilization Equipment

Tools and materials used in antibacterial tests are cleaned with soap in running water. Then, the equipment is dried and sterilized in an autoclave for 30 minutes at a temperature of 121 °C and a pressure of 1 atm [11].

d. Bacterial Growing Media

Nutrient broth media weighed 2.6 grams, and nutrient agar 3 grams on the analytical balance. They dissolved in 200 mL in Erlenmeyer (the amount of media weighing can be seen in terms of use stated on the material certificate). Stir using a stirring rod until the powder dissolves entirely while heating. The media was sterilized in an autoclave for 30 minutes at a temperature of 121°C pressure of 1 atm [12].

e. Medium Mc Monkey Bacteria

A total of one ose of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 bacteria were taken with sterile ose, scratched on an inclined agar surface containing 5 mL of media, and then incubated for 24 hours in an incubator at 37 °C [13].

f. Antibacterial Activity Test of Kusambi Leaves

Antibacterial activity test using agar diffusion method paper disk with nutrient agar media and broth. The working procedure is as follows: *Escherichia coli* and *Staphylococcus aureus* bacteria are taken by each bacterium in a colony of sterile use, then put into physiological NaCl and compared with the Mc-Farland 0.5 standard [14]. Next, add 10 mg/mL of kusambi leaf extract to 1 mL of organic solvent. They are using a micropipette taken 10 µg, 7.5 µg, 5.0 µg, and 2.5 µg then inserted into the surface of sterile disc paper. Leave for ±10 minutes until the solvent evaporates completely.

After 10 mL of sterile N.B. media into a petri dish, add 100 µL of bacterial suspension. Paper discs given to test samples are placed with a clamp to the surface of the media in a petri dish that has been inoculated with bacteria. Then, the disc paper is placed in a circle and marked for each concentration [15]. Organic solvents were used as negative controls and 30 µg chloramphenicol as positive controls. Petri dishes are left for a few minutes so the paper disk adheres perfectly to the agar surface. They were then incubated at 37°C for 24 hours. Observations of the inhibitory zone around the paper disk

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are qualitative; the size of the inhibitory zone indicates the strength of antibacterial activity; the more significant the inhibition zone, the stronger the antibacterial activity [16].

g. Resistance Measurement

Observation of the incubation results in the presence or absence of bacterial colonies growing on the media. Instructions for bacterial sensitivity to antibacterial materials used as test materials are seen from the clear zone around the paper disc, expressed by the area of the inhibitory zone, and measured using a caliper to determine the vertical diameter in mm units [17].

h. Phytochemical Test

This study's phytochemical tests included flavonoids, phenols, steroids, alkaloids, and triterpenoids. TLC methods and spray reagents will be used to identify chemical compounds. The testing process is as follows: the first was extracted on the TLC plate, the second TLC was eluted with a mixture of chloroform: methanol (95:0.5 v/v), the third visualized using UV 254 and 365 nm, and the fourth used a spray reagent. Flavonoid test: The sample was toggled on the silica gel F 254 TLC plate. The mobile phase is a mixture of chloroform and methanol (95:05 v / v). Flavonoid Yellow spots indicate the presence of flavonoid content after spraying with citrate and blue color when observed in a 254 nm U.V. detector[18]. Alkaloid test: The sample is tolerated on the TLC plate. The mobile phase is a mixture of chloroform and methanol (95:05 v / v). After spraying with Dragendorff and heating at 105°C, orange spots indicate the presence of alkaloid content. Several journals reported on the discovery of phenols, steroids, and triterpenoids but did not specify exactly what method was used to prove the findings, so the author conveyed only the findings based on observations in journals that were used as references[19].

RESULTS

This research conducted at the Laboratory of the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, was experimentally conducted in the laboratory in vitro to test the antibacterial activity of kusambi leaf extract (*Schleichera oleosa* (L) Oken) against *Escherichia coli* bacteria (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and characterization of compounds. The stages of this research are carried out sequentially, starting from the extraction process, antibacterial activity tests, phytochemical tests, and determination of compound groups.

The extraction results were based on the Soxhlet method with a solvent polarity gradient of n-hexane, ethyl acetate, and 96% methanol. The results of 510 grams of dry kusambi leaf simplisia and the yield of each extract obtained are presented in Table 1. The yield results were obtained from each solvent where the 96% methanol solvent had the highest yield value, namely methanol at 22%, ethyl acetate at 20%, and n-hexane at 12%. This research also uses hot extraction because the advantage of the soxhletation method is that it uses the hot method so that extensive extract results are obtained with less solvent used and in a faster time. With assistance provided repeatedly, it will have an impact on increasing the solvent's ability to extract insoluble compounds at room temperature conditions, as well as maximizing the withdrawal of compounds, thereby increasing yield.

According to research conducted by Setiawan et al. (2023), yield is one of the extraction parameters [20]. The yield value can be influenced by the type of solvent and extraction method used. The difference in yield obtained from the extraction process is caused by differences in the ability of the solvent to filter, extraction time to obtain the active substance in the simplisia, and the

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solubility of the active substance in different solvents. The effectiveness of the extraction process is influenced by the type of solvent used as the filter fluid, the size of the slurry, the extraction method, and time. The yield results show the efficiency and effectiveness of the extraction process. A high yield value indicates that the extract produced is high.

The extraction results were then used to test antibacterial activity using the paper disk diffusion method to determine the activity of kusambi leaf extract against the test bacteria. The most significant inhibitory diameter value was owned by the ethyl acetate extract at 9.22 mm with a kusambi leaf extract concentration of 10 µg/mL, followed by the methanol extract at 8.82 mm with a kusambi leaf extract concentration of 10 µg/mL in the test bacteria *S.aureus*. In contrast, bacteria *E.coli*, the negative control, showed no activity. More detailed results are shown in Tables 2, 3, and Figure 1.

The results of the kusambi leaf extract with the most significant resistance value were then characterized. In this research, the compound was characterized only on the leaf ethyl acetate extract and not on the N-hexane extract and methanol extract. Phytochemical characterization was carried out only on extracts inhibited by the test bacteria characterization using TLC detection and spray reagents. The results of phytochemical characterization using TLC detection and anisaldehyde spray reagent formed blackish-purple spots on a red background. This leads to the triterpenoid group of compounds. The appearance of TLC spots was detected using citroborate spray reagent, which was strongly identified as a class of flavonoid compounds, as evidenced by yellow spots on the TLC. The detection of steroids with spray reagents showed an orange stain on the TLC, which was identified as evidence of being identical to alkaloid class compounds. Detection of phenolic group compounds showed the presence of blue-black spots on TLC, which were identified as polyphenols. The research results on phytochemical tests for compound groups can be seen in Table 4.

S.aureus antibacterial activity test	*E.coli* antibacterial activity test

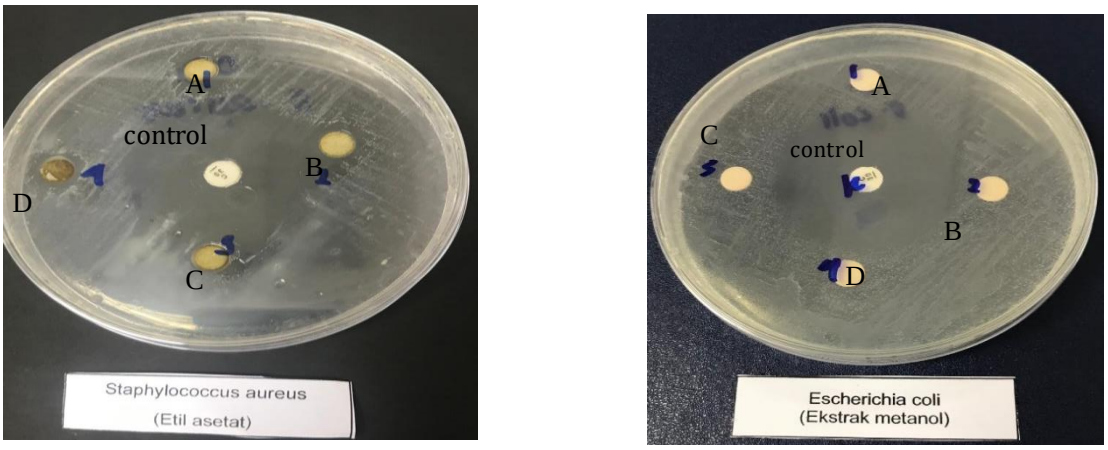

Figure 1. Results of the antibacterial activity test of the ethyl acetate extract against bacteria *S.aureus* concentration (A) 2.5 µg/mL, (B) 5.0 µg/mL, (C) 7.5 µg/mL, (D) 10 µg/mL, positive control chloramphenicol 30 µg.

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Table 1. Results of Kusambi Leaf Extract

Dry weight (g)	N-hexane		Ethyl Acetate		Methanol 96%	
	Extract Weight (g)	Yield (%)	Extract Weight (g)	Yield (%)	Extract Weight (g)	Yield (%)
510	63.67	12	102.82	20	115.19	22

*Dry weight is Kusambi leaf simplicia powder; extract weight is the weight of viscous extract evaporated with a rotary evaporator.

Table 2. Test results of antibacterial activity of Kusambi extracts against *E.coli* bacteria by agar diffusion method

Extract levels (µg)	Inhibition zone diameter (mm)* *E.coli*		
	N-hexane	Ethyl Acetate	Methanol
2.5	-	-	-
5.0	-	-	-
7.5	-	-	-
10	-	-	-
(-)	-	-	-
(+)	15.00	15.00	15.00

Test of antibacterial activity of n-hexane, ethyl acetate, and methanol extracts against *E. coli* bacteria. Each extract showed no inhibitory activity against *E. coli* bacteria. In contrast, chloramphenicol 30 µg antibiotics showed inhibitory activity against *S. aureus* bacteria.

Table 3. Test results of antibacterial activity of Kusambi leaf fraction against *S.aureus* bacteria by agar diffusion method

Extract levels (µg)	Inhibition zone diameter (mm)* *S.aureus*		
	N-hexane	Ethyl Acetate	Methanol
2.5	-	8.41	6.32
5.0	-	8.62	7.48
7.5	-	9.02	8.72
10	-	9.22	8.82
(-)	-	-	-
(+)	15.00	15.00	15.00

Test of antibacterial activity of n-hexane extract, ethyl acetate, and methanol against *S.aureus* bacteria. The negative control was the solvent of each fraction (n-hexane, ethyl acetate, methanol). In contrast, the positive control was the 30 µg chloramphenicol.

Table 4. Phytochemical test results of ethyl acetate fraction determination of compound class by spray reagent. AA (Anisaldehid), Citro (cytoborate), LB (Lieberman Burchard), DD (Dragendorff).

Prediction Of compound	*hRf*	Reagent	Visible	UV 254	UV 366	Result
Phenol	0	$FeCl_3$	Blackish blue	Damper	Blue fluorescence	+
Triterpenoids	0.12	Anisaldehid	Reddish purple	Damper	White fluorescence	+
Flavonoids	0.18	Sitroborat	Yellow	Damper	White fluorescence	+
Alkaloids	0.87	Dragendorff	Orange	Damper	-	+
Steroids	0.31	Liebermann Burchard	Blackish blue	Damper	fluorescence	+
Chlorophyll	1	-	Green	Blackish green	Red	+

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DISCUSSION

The extraction process is a stage in which active substance components are extracted from a material that contains the target active component. All secondary metabolite compounds extracted from samples or plants are called yields. The yield value shows the amount of secondary metabolite content extracted from the extract in a solvent [21]. A high yield value also shows that more active compounds are obtained from the extract [22]. A high yield value indicates that more raw materials can be used[23].

This research uses heat extraction because the advantage of the Soxhlet method is that it uses heat so that significant extract results are obtained with less solvent and in a faster time. This is due to the assistance of repeated heating treatments, which increase the solvent's ability to extract insoluble compounds at room temperature conditions, as well as the maximum withdrawal of compounds by the solvent, which continues to circulate in the process of contact with the simplicia, resulting in increased yields. Table 1 shows that the kusambi leaf extract extracted using the soxhlet method using 96% methanol solvent had a yield value of 22%, and the ethyl acetate solvent had a yield value of 20%. The N-hexane solvent has a yield value of 12%. The yield results of the three different extracts were due to differences in the polarity of the liquid, which was filtered continuously.

Therefore, this affects the yield of the extracted extract. In this study, an antibacterial activity test was performed, and ethyl acetate extract had a high inhibitory value. This is because ethyl acetate extract, according to its polarity properties, is semi-polar to nonpolar, so its ability to attract nonpolar chemical compounds will be more remarkable; it is said that compounds with nonpolar constituent structures can penetrate the defenses of bacteria, which results in weak bacterial defenses and will experience death [24]. The antibacterial activity of ethyl acetate extract against the growth of S. aureus and E. coli bacteria is suspected to be caused by the presence of active compounds in the extract. Active compounds such as polar flavonoids and polyphenols penetrate the more polar peptidoglycan layer of *S. aureus* bacteria more quickly than the lipid layer of *E. coli* bacteria[25].

This is in line with research testing the phytochemical content of kusambi leaf extract, which was carried out (2017). Kusambi leaves contain secondary metabolites in the form of alkaloids, phenolics, terpenoids, flavonoids, and tannins. Flavonoids are reported to have the most components in leaves. This test is based on screening to determine antioxidant activity [26,27].

Phytochemical tests are carried out to determine the chemical compound components in the test sample. Based on the data in Table 4, the results of the phytochemical test using the TLC method for the ethyl acetate fraction against the spray reagent show that the ethyl acetate extract is positive for containing phenolic compounds with hRf = 0 or at the initial spot, which is marked by a blue-black spot when treated with FeCl3 reagent; triterpenoids with positive hRf = 0.12 showed a color change to reddish-purple when treated with anisaldehyde sulfuric acid reagent; flavonoids with positive hRf = 0.18 showed a color change from yellow to more orange when treated with citroborate spray reagent; Alkaloid test results with a positive hRf = 0.87 are shown by the presence of orange-brown spots on the TLC surface when Dragendorff reagent is applied, Steroids with a positive hRf = 0.31 are shown by the presence of blackish-brown spots on the TLC plate, and chlorophyll with a positive hRf = 1 is shown in red when detected at UV 366 nm.

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CONCLUSION

Solvent polarity influences the yield and antibacterial activity of kusambi leaf extract. Kusambi leaf ethyl acetate extract has antibacterial activity against S.*aureus* bacteria with an inhibition zone of 9.22 mm at a 10 µg/mL concentration. Ethyl acetate extract also contains flavonoids, phenols, steroids, alkaloids, and triterpenoids.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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