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Antibacterial and Antibiofilm Activity of Katuk Leaf Fraction (*Sauropus androgynus* (L.) Merr.) against Bacteria *Staphylococcus Aureus* ATCC 25923

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A B S T R A C T

This study aims to determine the antibacterial and antibiofilm activity of extracts and fractions from katuk leaves against *Staphylococcus aureus*. Katuk leaves were extracted using the maceration process and fractionated using the liquid-liquid method with solvents such as n-hexane, ethyl acetate, and water. Diffusion and dilution methods were used to measure the antibacterial activity, and crystal violet staining at a wavelength of 595 nm was used to measure the inhibition and biofilm destruction activities. The antibacterial and antibiofilm test results obtained were analyzed using ANOVA statistical test. The results of the diameter of the inhibition zone with a concentration of 75 mg/ml showed that the extract was 11.3 ± 1.02 mm, the ethyl acetate fraction was 18.1 ± 2.46 while the water and n-hexane fractions were 0.0 ± 0.0 mm. The antibiofilm test results showed inhibitory activity against *Staphylococcus aureus* bacteria with the average IC_{50} values obtained from extracts, n-hexane, ethyl acetate, and water fractions were 10.248±0.405; 11.799±0.355; 8.332±0.585; and 11.372±0.557. While the average EC_{50} values obtained from the extract, n-hexane, ethyl acetate, and water fractions were 9.343±0.586; 10.530±0.483; 7.695±0.491; and 8.490±0.954.

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INTRODUCTION

Infectious diseases have increased in recent years in general bacteria, viruses, fungi or parasites are microorganisms that often cause infectious diseases in humans. One of these disease-causing microorganisms is *Staphylococcus aureus*. *Staphylococcus aureus* is known to have the ability to form biofilms. Biofilm is a collection of cells or aggregates of microorganisms (protozoa, bacteria, algae or fungi/fungi) covered by *Extracellular Polymeric Substances* (EPS) that can be attached to biotic or abiotic surfaces. Biofilms are composed of several structures including bacterial cells, exopolysaccharides, enzymes, water, proteins, DNA and RNA (Rabin et al., 2015). When biofilms form, the bacteria in them will become less susceptible to antibiotics and other chemicals than other bacteria that do not have biofilms. Thus, this can also lead to increased resistance of biofilm-producing bacterial cells to antimicrobial agents and reduce the efficacy of biofilm-related treatments (Adnan et al., 2020).

Katuk leaves are traditionally known as a vegetable that has various health benefits, including increasing breast milk production and containing antioxidants. This plant contains active compounds that have the potential to be used as alternative medicine (Selvi and Basker, 2012). Several previous studies have shown that katuk leaf extract has antibacterial activity against various types of pathogenic bacteria. In research (Ramadheni *et al*., 2017) stated that the antibacterial activity of ethanol extracts of katuk leaves with concentrations of 5%, 10%, 20%, 40%, and 80% showed antibacterial activity against Staphylococcus aureus bacteria. In another study, the antibacterial activity of katuk leaf extract with concentrations of 50 mg/ml and 100 mg/ml, obtained inhibition zones of 50 mg/ml (10mm) and 100 mg/ml (16 mm) with an average of 18 mm (Palaksha, 2019).

This study aims to explore and evaluate the antibacterial activity of the active fraction of katuk leaves and identify the chemical content that plays a role in inhibiting the growth of *Staphylococcus aureus* and its ability to prevent and destroy biofilms formed by these bacteria.

LITERATURE REVIEW

Katuk Leaf

Katuk leaves or in the Latin name Sauropus androgynus have attracted attention as a potential source of alternative medicine because of their rich nutritional and phytochemical content. Some chemical compounds found in katuk plants are also known to be utilized as drugs (Suhaillah et al., 2017). The content of chemical compounds contained in the ethanol extract of katuk leaves includes alkaloids, tannins, flavonoids, saponins, triterpenoids (Syahadat *et al*., 2020). It also contains polyphenols, steroids, quinones, monoterpenoids, and sesquiterpenoids (Nurdianti *et al*., 2017). The flavonoid content of katuk g leaves plays a role in disrupting the integrity of bacterial cell membrane components. In addition, flavonoids contained in katuk leaves work by forming complex compounds in extracellular proteins in the bacterial cell membrane, the existence of these bonds causes an imbalance of membrane components until bacterial cell membrane lysis occurs (Suhaillah *et al*., 2017). Other studies

have also found tannin, saponin, flavonoid, and alkaloid compounds that can be utilized as antibacterials (Zukhri *et al*., 2018).

Antibacterial

Antibacterials are substances that can kill or inhibit the activity of microorganisms in various ways. Antibacterial compounds consist of several groups based on their working mechanism or the purpose of their use. Antibacterial activity that can be observed directly is their reproduction. Therefore, microbes are said to be dead if they cannot reproduce. Some antibacterial properties are:

a. Bacteristatic.That is, inhibiting or stopping the growth of microorganisms such as stopping the growth of fungi, cytostatics against cancer. In such circumstances the number of microorganisms becomes stationary, for example sulfonamides, tetracycline, chloramphenicol, and erythromycin.

b. Bacteriocide.Namely bacteria that are capable of killing microorganisms. In this case the number of microorganisms will decrease and run out, unable to multiply or reproduce, for example penicillin, cephalosporin and neomycin.

A microorganism exhibits selective toxicity, where the drug is more toxic to the microorganism than to the host cell. This can occur because the drug's effect is selective to the microorganism or because the drug's effect on important biochemical reactions in the parasite cell is superior to its effect on the host (Natsir Djide and Sartini, 2008).

Antibiofilm

Biofilm is a collection of cells or aggregates of microorganisms such as protozoa, bacteria, algae or fungi/mushrooms covered by extracellular polymeric substances (EPS). This substance can adhere to biotic and abiotic surfaces. The structure of biofilm is composed of bacterial cells, exopolysaccharides, enzymes, water, proteins, DNA and RNA.(Rabin et al., 2015). Bacteria in biofilms are able to withstand antibiotics, disinfectants, and are able to withstand the host's immune system. In general, bacteria that can form biofilms are *Pseudomonas aeruginosa, Staphylococcus epidermidis, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus viridans, Escherichia coli, and Proteus mirabilis* (Nadar *et al*., 2022).

Biofilm formation

Adhesion, Aggregation, Maturation, and Dispersion are the four steps that make up the creation of biofilms. Planktonic cells adhere to the surface via surface-associated proteins during the initial stage of biofilm development. The interactions of hydrophobic and hydrophilic bacteria with biotic or abiotic surfaces affect the bacterial adhesion to a surface.

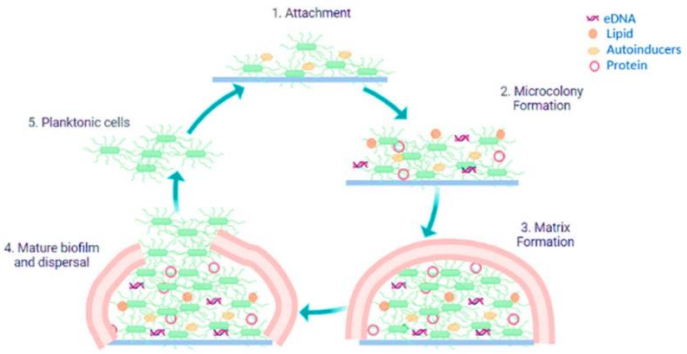

Figure 1. Biofilm formation process

Antibiotics

Antibiotics are antimicrobial substances obtained or formed and produced by microorganisms which are generally fungi or other synthetic substances and these substances in small amounts have the power to inhibit other microorganisms. Some antibiotics have a broad spectrum which means they are effective for many species of bacteria, both cocci, bacilli, and spirals (Anugrah, 2015).

METHODOLOGY

Research Design

The research to be conducted is an experimental research by extracting katuk leaves using 96% ethanol followed by fractionation with water, ethyl acetate and n-hexane fractions. Then, continued with antibacterial testing using the diffusion and dilution methods to see the MIC and MBC values and antibiofilm tests to see the inhibition and degradation of biofilms.

Tool

The tools used in the extraction and fractionation process are glass vessels, measuring cups, separating funnels, rotary evaporators, freeze dryers, glass stirrers and glass containers. The tools for microbiological testing are test tubes, petri dishes, Erlenmeyer flasks, glass funnels, droppers, water baths, incubators, tweezers, Bunsen burners, incubators, UV lamps, loop needles, measuring cups (Pyrex®), 96-well flat bottom microplates, vortexes, micropipettes, yellow tips, and loops.

Material

The materials used as samples arekatuk leaves. The solvents used for extraction and fractionation were 96% ethanol, n-hexane, ethyl acetate, water, and sterile distilled water. The materials used in the antibacterial and antibiofilm testing were *Staphylococcus aureus* ATCC 25923 test bacteria obtained from the Microbiology Laboratory of the University of Setia Budi Faculty of Pharmacy. The bacterial test media used in this study were *Nutrient Agar* (NA), *Mueller Hinton Agar* (MHA),*Vogel Johnson Agar(VJA), Brain Heart Infusion*(BHI), DMSO, and crystal violet.

The Course of Research

Making Katuk Leaf Powder

The process of turning katuk leaves into dry powder involves washing the leaves under running water to get rid of any dirt that may have attached itself to them. The cleaned katuk leaves are then dried and ground using a blender then sieved using sieve no. 60. The powder is then stored in a closed container and the drying loss is determined.

Making Katuk Leaf Extract

Katuk leaf powder is taken as much as 1000 grams soaked with 96% ethanol solvent (1:10) in a glass container covered with aluminum foil. Maceration is carried out for 24 hours at room temperature with occasional stirring. Next, the macerate is separated by filtering through Whatman paper. The same solvent is utilized for at least one filtering cycle, with half as much solvent as was used for the first extraction. The extract is then concentrated with a Vacuum Rotary Evaporator at a temperature of 70°C to produce a thick extract. The percentage of yield obtained is calculated using the following formula:

$$\% \text{ Yield} = \frac{\text{extract weight}}{\text{powder until}}$$

Making Katuk Leaf Fraction

Using n-hexane, ethyl acetate, and water as solvents, fractionation was performed on a 96% ethanol extract of katuk leaves using the liquid-liquid extraction method. Ten grams of the 96% ethanol extract from katuk leaves were obtained, somewhat diluted in hot water, and then divided into a separating funnel with fifty milliliters of water and fifty milliliters of n-hexane solvent. This process was repeated three times. The filtrate above is the n-hexane fraction, and the filtrate below is the water fraction. A rotary evaporator is used to separate the n-hexane fraction from the water fraction, collect it, and concentrate it at a temperature of 500C for the water bath. Then, using a separating funnel and 50 ml of ethyl acetate solvent, the leftover water fraction from the n-hexane fraction is re-fractionated. Three times is this technique is repeated. Above is the filtrate, or ethyl acetate fraction, and below is the water fraction. A rotary evaporator is used to concentrate the ethyl acetate fraction after separating it from the water fraction at a temperature of 500C for the water bath. The water fraction, which is the leftover filtrate after the ethyl acetate fractionation, is thickened in a water bath until it reaches a thick consistency.

Phytochemical Screening Test

Phytochemical screening tests are conducted to determine the content of secondary metabolite compounds in the plants to be tested. Testing is carried out qualitatively to determine the alkaloid, flavonoid, steroid, tannin and saponin compounds contained in the test plants.

a. Identification of alkaloids. Extracts and fractions were weighed 1 mg and added 5 ml of 2N HCl. The solution obtained was divided into 3 parts. The first tube was usedAs a blank, 3 drops of Dragendroff's reagent were added

to the second tube and 3 drops of Mayer's reagent were added to the third tube. Positive results of the extract and fraction containing alkaloids were indicated by the formation of orange deposits in the second tube and white to yellowish deposits in the third tube.

b. ***Identification of flavonoids.*** Each fraction and extract was weighed as much as 2 mg, added with 10 ml of hot water and heated for 5 minutes.Identificationdone by adding a little Mg metal powder, 1 ml of concentrated HCl, and 1 ml of amyl alcohol into 5 ml of filtrate, then shaken. If a red/yellow/orange color appears on the amyl alcohol layer, it indicates the presence of flavonoids.
c. ***Identification of tannins***. As much as 0.1gr of extract and fraction of katuk leaves were added with 5 mL of distilled water then boiled for 5 minutes. This solution was filtered and the filtrate was added with 5 drops of 1% FeCl. If a blue or blackish green color appears, the tannin test shows a positive result.
d. ***Identification of saponins.*** Extract and fraction as much as0.1 gr plus 5 ml of distilled water then shaken vigorously. Positive saponin test results are indicated by the formation of stable foam for 15 minutes approximately 1 cm high.
e. ***Steroid Identification***.2 ml of test solution was evaporated in an evaporator cup. The residue was dissolved with 0.5 ml of chloroform, added 0.5 ml of anhydrous acetic acid and 2 ml of concentrated sulfuric acid through the tube wall. The formation of a bluish green ring indicates the presence of steroids.

Antibacterial Test

a. ***Diffusion method***. Activity test was conducted using disc diffusion method, first sterile petri dish and sterile glass bottle were prepared then 15 mL MHA media was poured into the glass bottle and 0.2 mL of test bacterial suspension was added, then the glass bottle was slowly rotated so that the suspension was mixed with the media. After that, it was put into the petri dish, leveled and left to solidify. Then in the media, a 6 mm disc paper (blank disk) was inserted which had been dipped into each test solution (extract, n-hexane fraction, ethyl acetate and water). Furthermore, positive control in the form of chloramphenicol antibiotic and 5% DMSO were also inserted as negative control then repeated 3 times and incubated for 24 hours at 370C.
b. ***Dilution method.***Determination of Minimum Inhibitory Concentration (MIC) and Maximum Bactericidal Concentration (MBC) of the most active fractionkatuk leaves were measured using the dilution method. Making the most active fraction stock solution using 5% DMSO solvent. This process was carried out using 1 row of tubes consisting of 10 tubes, with concentrations of 75; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78 mg/mL, control (+) and control (-). BHI media was added 0.5 mL in each tube except tubes 1, 2 and 10. Aseptically, add 1 mL of the stock solution to be tested in tube 1. Then 0.5 mL of stock solution was added to tube 2 and tube 3, then 0.5 mL was pipetted into tube 3 and inserted into tube 4 and so on until tube 11

then discarded. Add 0.5 mL of bacterial culture from tube 2 to tube 11, 1 mL was added to tube 12. All tubes were incubated at 370C for 24 hours, then the turbidity was observed. *Minimum Bactericidal Concentration* (MBC) was determined by inoculating clear media tubes by streaking on selective media incubated at 37^0C for 24-48 hours. Then observed whether or not there were colonies growing on the surface of the plate media.

Antibiofilm Test

a. Optimization of Biofilm Formation Time. The purpose of this optimization is to obtain the best incubation time to form biofilm. The differences in incubation periods used are 1, 2 and 3 days. After the incubation period, the microplate was washed using running water 3 times, and 200 µL of 1% crystal violet solution was added to each well and then incubated for 15 minutes at room temperature. The microplate was washed again with running water 3 times. A total of 200 µL of 96% ethanol solution was added to each well and incubated for 15 minutes at room temperature. Biofilm formation was read with a value (Absorbance OD 595) using the iMark-Biorad *Microplate Reader*. The highest absorbance value is considered as the optimal formation of *Staphylococcus aureus* biofilm.

b. Biofilm Formation Inhibitory Activity Test. Biofilm inhibition test was conducted to obtain the effectiveness of katuk leaves in inhibiting biofilm growth against *Staphylococcus aureus* bacteria. The biofilm formation inhibition activity test was conducted using a *96-well polystyrene* round bottom microplate with BHI media. A total of 70 µL of sample in the media was added to each well, then 70 µL of bacterial suspension in the media equivalent to 1,5 x 108 CFU/mL was added to the well containing the sample, incubation was carried out at a temperature of ± 370 C for 72 hours. Following incubation, the well's contents were disposed out, and the plate was cleaned under running water before being inverted and allowed to dry for fifteen minutes at room temperature.

A total of 200 µL of 1% crystal violet solution was added to each well with a staining time of 15 minutes. The contents of the wells were discarded and the wells were rinsed again with running water. The microplate was dried by inverting it at room temperature for one hour. Then 200 µL of 96% ethanol solution was added to each well on the plate and the optical density was read at λ 595 nm optimization. Each test was replicated 3 times. The control in the test used was a negative control (bacteria + media) which is a control where bacterial growth is not disturbed to see the biofilm formed. The test control (bacteria + media + fractions) is a control for the activity of katuk leaves in inhibiting biofilm growth. Positive control using Chloramphenicol.

$$\% \text{ inhibition} = \frac{Do\ Negative\ control - Do\ sample}{Do\ Negative\ control} \text{x } 100\%$$

*Note: DO (Optical Density)

After obtaining the average percentage of inhibition of biofilm formation from each concentration of fractions, the IC50 value was determined using a linear regression line equation between the percentage of inhibition of biofilm

formation and the concentration of fractions to see the relationship between concentration and the percentage of biofilm inhibition in inhibiting 50% of biofilm.

c. ***Biofilm Degradation Activity Test.*** Biofilm degradation activity test was carried outusing a 96-well polystyrene round bottom microplate with BHI media. Biofilms were formed by adding 70 µL of media to each well and then adding 70 µL of bacterial suspension in BHI equivalent to 1,5x108 CFU/mL. The microplate was then incubated at a temperature of ± 37^0C for 72 hours. The contents of the wells were then discarded and the wells were washed using running water. Each well was filled with 200 µL of sample in the media and then the microplate was incubated again at a temperature of ± 370C for 24 hours. For the determination of biofilms, the same method as in the biofilm formation inhibition activity test can be applied. The control in this test is a negative control (bacteria + media) which is a biofilm formation control where bacterial growth is not disturbed, Sample control (bacteria + media + fraction) is a control for the activity test of katuk leaves in degrading biofilms.

$$\% \text{ Destruction} = \frac{Do\ Negative\ control - Do\ sample}{Do\ Negative\ control} \text{x } 100\%$$

*Note: DO (Optical Density)

The concentration of the test substance that kills the biofilm by 50%, or the EC50 (Effective Concentration) value, indicates the Staphylococcus aureus biofilm destruction activity test. The EC50 value is determined from the linear regression equation between the sample concentration and the percentage of biofilm damage. Linear regression equation, r table value with a confidence level of 0.95. The EC50 value is different from the biofilm destruction activity, the higher the EC50 value, the smaller the *Staphylococcus aureus* biofilm destruction activity, which means that the concentration needed to destroy the biofilm by 50% is greater.

Data Analysis

Data analysis in this study used the IBM SPSS Statistic analysis application. The initial steps that can be taken are the normality test and homogeneity test. If a value of > 0.05 is found from sig. or significance or probability value, then it can be said that the data distribution is normal. After that, a homogeneity test can be carried out where if a value of > 0.05 is obtained, it can be said that the data variance is not significantly different or homogeneous variance (Nuryadi *et al*., 2017).

Next, a parametric test is carried out in the form of One Way ANOVA. If the data is not normally distributed and not homogeneous, a non-parametric test can be carried out in the form of Kruskall Wallis. If the results of the One Way ANOVA test show Sig. <0.05 then there is a significant difference, whereas if the data shows the results of Sig. >0.05 then there is no significant difference. Furthermore, the data is tested for Post Hoc Test to determine the significant differences in values in the treatment group.

RESEARCH RESULT

Katuk Leaf Extract Results

Table 1. Thick Extract Yield Results

Weight of Simple Ingredients (g)	Extract Weight (g)	Yield (%)
1000 g	136 g	13,6%

The results of the thick extract of katuk leaves obtained in this study were 136 g with a yield value of 13,6%, where these results indicate that the yield obtained meets the requirements of the Indonesian Herbal Pharmacopoeia II Edition literature, namely not less than 7,6% (Ministry of Health of the Republic of Indonesia, 2017). In the research Surya *et al*., (2022) maceration of 1500 g of katuk leaf powder using 96% ethanol solvent obtained an extract yield of 13,07%.

Katuk Leaf Fractionation Results

Fractionation is a method to separate organic compounds according to the solubility of the compounds using two or more solvents that do not mix with each other. This study used n-hexane, ethyl acetate, and water as solvents. These three solvents have a polarity level from non-polar to polar. The following are the results of the fractionation yield percentage obtained:

Table 2. Fractionation Yield

Extract weight (g)	Faction	Fraction weight (g)	Yield (b/b) (%)
60	*n*-hexane	12	20
	ethyl acetate	8	13,3
	water	14	23,3

The total fraction obtained in this process was 34 g, which means the weight of the fraction is less than the weight of the extract used, which is 60 gr. The reduction in the weight of this fraction is caused by the residue left in the separating funnel in the fractionation process. The results of the fractionation yield percentage obtained in table 8 show that the water fraction (23,3%) has a higher yield percentage compared to the n-hexane fraction (20,0%) and ethyl acetate (13,3%). Fractionation process shows that the compounds contained in the katuk leaf extract can be identified based on their polarity. More polar compounds will be more contained in the water and ethyl acetate fractions, while more nonpolar compounds will be more contained in the n-hexane fraction. The difference in yield results obtained is because each solvent has a different ability to attract compounds contained in the katuk leaf extract (Khaira Rusdi *et al*., 2018). N-hexane solvent is a type of organic solvent that is non-polar. This is because n-hexane solvent is insoluble in water and is more effective for extracting non-polar compounds such as fats and oils (Utomo, 2016). Non-polar compounds in katuk leaves will be attracted by the n-hexane solvent

Phytochemical Screening

Phytochemical screening aims to determine the content of active metabolite compounds in plants to be tested such as alkaloids, flavonoids, tannins and saponins. The results of phytochemical screening on 96% ethanol extract of katuk leaves are shown as follows:

Table 3. Phytochemical Screening of Katuk Leaves

Test Sample	Alkaloid	Flavonoid	Tannin	Saponins	Steroid
Extract	+	+	+	+	+
n-hexane fraction	-	-	-	+	-
Ethyl Acetate Fraction	+	+	+	+	+
Water fraction	-	+	+	+	+

Note: (+) contains active compounds
(-) does not contain active compounds

Antibacterial Activity Test Results

Diffusion test

Table 4. Diffusion Test Inhibition Zone Results

Sample	Concentration	Replication			Average±SD
		1	2	3	
Extract	75 mg/mL	11,5mm	12,5 mm	10 mm	11,3 ± 1,02
N-hexane fraction	75 mg/mL	0	0	0	0
Ethyl Acetate Fraction	75 mg/mL	18,5mm	21mm	15mm	18,1 ± 2,46
Water Fraction	75 mg/mL	0	0	0	0
Positive control	30µ/mL	38,5 mm	37,5 mm	32mm	36 ± 2,85
Negative control	0	0	0	0	0

Information:
K+= Chloramphenicol 30µ/mL
K-= DMSO 5%

Dilution Test

Table 5. Results of the Dilution Test of Katuk Leaves

Concentration (mg/mL)	Replication I	Replication II	Replication III
75	-	-	-
50	-	-	-
25	-	-	-**
12.5	+	+	+ *
6.25	+	+	+
3.12	+	+	+
1.56	+	+	+
0.78	+	+	+
Chloramphenicol	-	-	-
BHI	+	+	+

Information:

(-): there is bacterial growth
(+): no bacterial growth
(*): MIC
(**): MBC

Optimization of Biofilm Formation Time

Table 8. Optimization of Biofilm Formation

No	Absorbance		
	24 hours	**48 Hours**	**72 Hours**
1	0.127	0.347	0.578
2	0.132	0.353	0.587
3	0.138	0.368	0.555
4	0.143	0.347	0.586
5	0.123	0.382	0.565
6	0.155	0.369	0.568
7	0.147	0.374	0.572
8	0.115	0.365	0.584
9	0.152	0.391	0.579
10	0.141	0.386	0.577
Average	0.137 ±0.012	0.368 ± 0.015	0.578 ± 0.009

Biofilm Formation Inhibition Test Results

Table 9. Percentage of Inhibition of Biofilm Formation

Mean inhibition of biofilm formation (%) ± SD					
Sample	**Concentration**				**Chloramphenicol**
	6,25 mg/mL	12,5 mg/mL	25 mg/mL	50 mg/mL	
Extract	37.263 ± 1.616	56.872 ± 0.799	70,320 ± 1,046	77.113 ± 0.477	83.235 ± 0.466
N-hexane fraction	34.538 ± 0.604	54,384 ± 1,454	68.187 ± 0.768	71.979 ± 0.654	
Ethyl acetate fraction	42.239 ± 1.473	60.012 ± 0.952	71.090 ± 0.966	78.495 ± 0.906	
Water Fraction	34,360 ± 1,814	53,614 ± 2,304	69.254 ± 0.580	73.637 ± 0.887	

Note:
K+= Chloramphenicol 0.076 mg/mL

Table 10. IC50 value of biofilm formation inhibition

Sample	**Replication**			**Mean±SD**
	I	**II**	**III**	
Extract	10,046	10,814	9,885	10.248±0.405
N-hexane	11,534	11,561	12,302	11.799±0.355
Ethyl Acetate	7,603	8,356	9,036	8.332±0.585
Water	10,592	11,857	11,668	11.372±0.557

Biofilm Degradation Test Results

Table 11. Percentage Value of Biofilm Degradation

Mean inhibition of biofilm formation (%) ± SD					
Sample	**Concentration**				**Chloramphenicol**
	6.25 mg/mL	12.5 mg/mL	25 mg/mL	50 mg/mL	
Extract	38,667±1,208	59.188±0.729	71,536±1,288	74.494±0.574	83.235±0.466
N-hexane fraction	37.507±0.640	55.942±1.208	69.159±2.040	72,754±1,007	
Ethyl acetate fraction	43.130±0.931	60,812±1,288	72,638±1,208	75.188±0.715	
Water Fraction	42,609±2,149	58,029±2,386	69.913±0.710	73.449±0.807	

Note:
K+= Chloramphenicol 0.076 mg/mL

Table 12. EC50 value of biofilm degradation

Sample	**Replication**			**Mean±SD**
	I	**II**	**III**	
Extract	8,590	9.418	10,020	9,343±0.586
N-hexane	10,000	10,423	11,168	10,530±0.483
Ethyl Acetate	7,095	7,691	8.298	7,695±0.491
Water	6,934	9.204	9,332	8,490±0.954

DISCUSSION

Phytochemical Screening

Ethyl acetate solvent is a semi-polar solvent that can dissolve polar and non-polar bioactive compounds. Semi-polar solvents such as ethyl acetate are able to dissolve components from the alkaloid, aglycone and glycoside groups (Houghton *et al*., 2016).The fraction of ethyl acetate also attracts more flavonoid compounds which have antibacterial properties, where the compounds This can disrupt the integrity of the cell membrane components in bacteria. In addition, flavonoids found in katuk leaves also work by forming complex compounds in extracellular proteins in the bacterial cell membrane, the presence of these bonds causes an imbalance in the membrane components until the bacterial cell membrane lysis occurs (Suhaillah *et al*., 2017).

Antibacterial Activity Test Results

Diffusion test

The test results above indicate the presence of inhibition against *Staphylococcus aureus* bacteria. The inhibition zone produced from the extract and the three fractions shows that the extract (11,3 mm) and ethyl acetate fraction (18,1 mm) have the best inhibition zone. While the n-hexane and water fractions show the presence of an inhibition zone that is formed but is very small, so it is unlikely that the inhibition zone can be measured.This is because n-hexane solvents tend to extract compounds that are non-polar or lipophilic (fat-soluble). The active antimicrobial compounds in katuk leaves may not be dissolved in n-hexane, so if the desired antimicrobial compounds are polar,

they will not be dissolved or detected in the n-hexane fraction and will not show antibacterial activity. While the water fraction tends to contain polar compounds, such as sugars, salts, and other hydrophilic compounds, which may not have strong antibacterial activity. The active compounds responsible for antibacterial activity may be more abundant in organic fractions such as ethanol or methanol.

Ethyl acetate solvent is a semi-polar solvent that can dissolve polar and non-polar bioactive compounds. Semi-polar solvents such as ethyl acetate are able to dissolve components from the alkaloid, aglycone and glycoside groups (Houghton *et al*., 2016).The fraction of ethyl acetate also attracts more flavonoid compounds which have antibacterial properties, where the compounds This can disrupt the integrity of the cell membrane components in bacteria. In addition, flavonoids found in katuk leaves also work by forming complex compounds in extracellular proteins in the bacterial cell membrane, the presence of these bonds causes an imbalance in the membrane components until the bacterial cell membrane lysis occurs (Suhaillah *et al*., 2017).

Tannin is also a compound that has antibacterial properties. Antibacterial effects include damage to bacterial cell membranes, this will inhibit bacterial growth and bacteria will die. Tannin can also damage cell walls and inhibit bacterial growth as an antibacterial mechanism.

In addition, saponin compounds have a working mechanism as antibacterial by inhibiting the function of the cell membrane so that it damages the permeability of the membrane which causes the cell wall to be damaged or destroyed. Saponin can damage the peptidoglycan structure in the cell wall of Staphylococcus aureus, forming porins which cause the permeability of the cell membrane to be disrupted (Ramadheni *et al*., 2017).

The positive control used was the antibiotic chloramphenicol. Based on table 10, it can be seen that the positive control chloramphenicol has the largest inhibition zone. This shows that chloramphenicol has greater antibacterial activity compared to the sample solution tested. In addition, chloramphenicol is an antibiotic that has been commonly used to inhibit bacterial growth. According toKatzhung *et al*, (2004) Chloramphenicol works by inhibiting protein synthesis in bacterial cells. By diffusing into the bacterial cell wall and reversibly binding to the bacterial 50S ribosomal subunit. This binding interferes with peptidyl transferase activity, thereby preventing the transfer of amino acids to the peptide chain, resulting in bacterial protein synthesis being inhibited and proliferation not occurring.

The negative control in this study used 5% *Dimethyl Sulfoxide* (DMSO) which showed results with no inhibition zone diameter. This is because DMSO <10% does not have antibacterial activity so it does not affect bacterial growth. In accordance with research conducted by(Septiani and Reni, 2017), stated that DMSO can inhibit bacterial growth at concentrations above 10%.

After obtaining the data on the diameter of the inhibition zone of the extract and fraction of katuk leaves, statistical data analysis of the normality test was carried out. The results of the normality test obtained had a value of >0.05, so it can be said that the data is normally distributed. Furthermore, a

homogeneity test was carried out where the value obtained was 0.008 (p>0.05), so it can be said that the data is not significantly different or homogeneous. Then the data was tested using the *One Way Annova* parameter. The results of the significance value of the One Way Annova test obtained were 0.000 (p<0.05), so it can be concluded that the values obtained from each treatment were significantly different. The data was then tested for *Post Hoc* to determine the difference in significant values in the treatment groups. The data obtained showed that the n-hexane and water fractions did not show any difference in the diameter of the inhibition zone with the negative control. While the extract and ethyl acetate fraction showed a difference in the diameter of the inhibition zone with the negative control. However, the inhibition zone activity of the extract and ethyl acetate fraction of katuk leaves could not match the inhibition zone activity of the positive control of the antibiotic chloramphenicol

Dilution Test

The results of the observations showed that there was turbidity and clarity in different tubes. Tubes with different concentrations12.5; 6.25; 3.12; 1.56; 0.78; and 0.39 mg/mL showed turbidity, while at 75-25mg/mL did not show any turbidity. Furthermore, at a concentration of 75-25mg/mL from the dilution results, inoculation was carried out using the scratch method on VJA selective media to determine the MBC value. The positive control in this test used the antibiotic chloramphenicol. Chloramphenicol works by disrupting the process of bacterial protein synthesis, thereby inhibiting bacterial growth and reproduction.

The results of the dilution test showed that the ethyl acetate fraction had a *Minimum Bactericidal Concentration* (MBC) at a concentration of 25 mg/mL while the *Minimum Inhibitory Concentration* (MIC) was at a concentration of 12,5 mg/mL. This is because ethyl acetate solvent is a semi-polar solvent that can dissolve bioactive compounds in kauk leaves which have the potential to be antibacterial. Katuk leaves have the ability to inhibit bacterial growth due to the presence of saponin, flavonoid and tannin compounds (Wahyu *et al*., 2017). The ability of katuk leaves to inhibit growth *Staphylococcus aureus* It is suspected that katuk leaves contain flavonoid compounds that play a role in disrupting the integrity of bacterial cell membrane components. In addition, flavonoids found in katuk leaves work by forming complex compounds in extracellular proteins in bacterial cell membranes, the presence of these bonds causes an imbalance in membrane components until bacterial cell membrane iysis occurs (Suhaillah e*t al*., 2017).

Tannin functions as an antibacterial that will interfere with peptidoglycan synthesis so that the formation of cell walls becomes imperfect. Bacterial cells lose their shape and lysis occurs due to instability in the cell wall and cytoplasmic membrane. This instability causes selective permeability, active transport and protein composition control to be disrupted (Naim, 2014).

Optimization of Biofilm Formation Time

Optimization of bacterial biofilm formation is a process that aims to increase the ability of bacteria to form biofilms, which are organized

aggregations of microorganism cells in an extracellular matrix. Environmental conditions, including nutrient supplementation, greatly affect biofilm formation. The duration of bacterial biofilm formation varies depending on the type of bacteria and environmental conditions. Based on several studies conducted on bacteria *Staphylococcus aureus,* significant biofilm formation was observed after 12 hours of incubation, with a continued increase up to 48 hours. The measurement results showed that the *Optical Density* (OD) increased with increasing incubation time, indicating an increase in the amount of biofilm formed. In general, the biofilm formation process goes through several stages, including attachment, maturation, and spreading. In the initial stage, bacteria adhere to the surface, and after that, they begin to form colonies and produce exopolymer materials that make up the biofilm matrix.

Optimization of Biofilm Formation Time

The optimization value obtained shows that, the 24 hour incubation treatment OD is significantly different from the 36-hour incubation, and the 36-hour OD is significantly different from the 72 hour incubation. The lowest OD was obtained with a 24 hour incubation period and the highest OD was obtained with a 72 hour incubation period. Based on the table above, the best and optimal average values in forming biofilms*Staphylococcus aureus*obtained at 72 hours of incubation. In general, significant biofilm formation by Staphylococcus aureus bacteria is usually seen within a few days, but full maturation of the biofilm may take longer, depending on factors such as surface type, nutritional conditions, and species involved (Agrijanti *et al*., 2023).

Biofilm formation can occur on a variety of surfaces and in a variety of environmental conditions. Bacteria are more likely to adhere to hydrophobic and nonpolar surfaces such as plastic than to hydrophilic surfaces such as metal or glass (Jamal *et al*., 2018). The process of bacterial attachment is also influenced by the condition of the film. The surface submerged by the liquid media will be covered by the polymer of the media so that it can affect the growth and expansion of the attachment of microorganisms. Thus, the time required for biofilm formation can range from several hours to several days, depending on the species of bacteria and the conditions present (Agrijanti *et al*., 2023).

Biofilm Formation Inhibition Test Results

As shown by the percentage of inhibition of biofilm formation, from each test sample where the concentration of 50 mg/mL has the highest percentage in inhibiting biofilm formation. The higher the concentration of katuk leaf extract or fraction, the greater the percentage of inhibition of biofilm formation in *Staphylococcus aureus* bacteria and the higher the content of secondary metabolites in the fraction, so that the potential for inhibition of biofilm formation increases.

The IC50 value was obtained by calculating the percentage data of inhibition of biofilm formation and the concentration of extracts and fractions using a linear regression equation. The IC50 value of inhibition of biofilm formation is shown in the following table:

Table 10 shows that the results of the inhibition value of biofilm formation with the smallest to largest IC50 values were obtained from the ethyl acetate fraction, then the ethanol extract of katuk leaves, the n-hexane fraction and the water fraction. The results of the smallest IC50 value obtained by the ethyl acetate fraction were 8.332 ± 0.585mg/ml. This shows that the smaller the IC50 value, the greater the inhibition of biofilm formation.

After obtaining the IC50 value data, statistical data analysis was carried out for the normality test. Using the SPSS (*Statistical Package for the Social Sciences*) program.In the Shapiro Wilk normality test, the value obtained was 0.265 ($p>0.05$), indicating that the data was normally distributed, while the results of the homogeneity test obtained a value of 0.813 ($p>0.05$), so that the data was said to be homogeneous. Furthermore, the data was tested using the One-Way ANOVA test. In this test, the value obtained was 0.000 ($p>0.05$),so that the results are considered significant. WhileTukey test results showed that there was a significant difference between the results of inhibition of biofilm formation from the ethyl acetate fraction with the extract, n-hexane fraction, and katuk leaf water fraction.

The positive control used in this study was the antibiotic Chloramphenicol. In the context of biofilms, the antibiotic chloramphenicol can weaken the virulence activity of bacteria by inhibiting biofilm formation. In addition, chloramphenicol works by inhibiting bacterial protein synthesis which is an important process in biofilm formation. By interfering with protein production, chloramphenicol can reduce the ability of bacteria to form complex biofilm structures and protect themselves from the external environment.

The process of inhibiting bacterial biofilms by active compounds in plants involves several complex mechanisms. Biofilms are microbial communities that are bound to surfaces and protected by an extracellular matrix, which makes them difficult to remove. Active compounds from plants, such as flavonoids, tannins, and saponins, have the potential to inhibit biofilm formation and growth.

Katuk leaves are plants that contain many chemical compounds. Chemical compounds such as flavonoids, saponins, and tannins in katuk leaves have the ability to inhibit bacterial growth (Wahyu *et al*., 2017). Ethyl acetate is a polar solvent and will attract secondary metabolites that are soluble in polar solvents. The results of the ethyl acetate fraction of katuk leaves obtained the lowest IC50 value, this shows that in the water fraction there are secondary metabolites such as flavonoids, saponins and tannins which have activity in inhibiting the formation of bacterial biofilms.

The process of active compounds in plants in inhibiting biofilm formation is by inhibiting microbial attachment to the surface so that biofilm development will be disrupted. Disrupted biofilm development will affect the structure of the biofilm to increase defense against antimicrobials. In addition to inhibiting microbial attachment, compounds can damage the *extracellular matrix* (EPS) of the biofilm, this will cause the communication pathways of cells and nutrients between microbes to be cut off so that the microbes that will form

biofilms will become lysed or die, due to the loss of nutrients as components of the biofilm (Hamzah *et a*l., 2021).

Biofilm Degradation Test Results

Determination of the EC50 value of biofilm degradation from the extract and fraction of katuk leaves, was carried out by calculating the percentage value of biofilm degradation using a linear regression equation between the percentage of biofilm degradation and the concentration of the extract and fraction. Table 9 shows that the ethyl acetate fraction has the highest percentage value for biofilm degradation, with an average value of 75.188 ±0.715 at a concentration of 50mg/ml, then continued with ethanol extract of katuk leaves, n-hexane fraction and water fraction.

In the table above, the results of the biofilm degradation values show that the smallest EC50 value is shown by the ethyl acetate fraction, namely 7,695 ± 0,491 mg/ml and the highest value was shown by the n-hexane fraction, namely 10.530±0.483 mg/ml. This shows that, the smaller the EC50 value obtained, the greater the activity of the fraction in degrading biofilms against *Staphylococcus aureus* bacteria.

After obtaining the EC50 value data, statistical data analysis was carried out to test normality. Usingn the SPSS (*Statistical Package for the Social Sciences*) program.The results of the normality test obtained were 0.091 ($p>0.05$), so it can be said that the data is normally distributed, while the results of the homogeneity test obtained a value of 0.232 ($p>0.05$), so the data showed homogeneous results. Furthermore, the data was tested using the *One Way Annova* parameter test. The results of the significance value of the *One Way Annova* test results obtained were 0.021 ($p<0.05$), so it can be concluded that the value obtained is significant. The results of the Tukey test showed that there was a significant difference between the results of inhibition of biofilm formation from the ethyl acetate fraction and the n-hexane fraction, while the ethyl acetate fraction with the extract and water fraction did not show a significant difference.

Katuk leaves are plants that contain many chemical compounds. Chemical compounds such as tannins, flavonoids and saponins in katuk leaves have the ability to inhibit bacterial growth (Wahyu *et al*., 2017). Ethyl acetate is a polar solvent and will attract secondary metabolites that are soluble in polar solvents. The results of the ethyl acetate fraction of katuk leaves showed the highest degradation value, this shows that in the water fraction there are secondary metabolites such as flavonoids, saponins and tannins which have the activity to degrade bacterial biofilms.

Flavonoid compounds have activity in destroying biofilms. The mechanism of flavonoids in destroying biofilms is through the flavonoid structure, namely the hydroxyl group binds to the protein contained in the biofilm and forms a complex compound, causing the biofilm to denature (Winarsih *et al*., 2019). Tannin compounds damage bacterial biofilms by binding iron ions needed by bacteria to maintain the biofilm matrix, resulting in a decrease in bacterial viscosity and reduced biofilm matrix bonds. Tannins can

also affect EPS, by reducing the amount of EPS in bacterial biofilms (Aini *et al*., 2016). Saponin compounds work to destroy biofilms by changing the extracellular polymer matrix in the bacterial biofilm matrix. This can reduce polymer substances and change the integrity of the bacterial cell membrane, causing the bacterial cell wall to become unstable (Andrade *et al*., 2019).

CONCLUSIONS AND RECOMMENDATIONS

Extracts and ethyl acetate fractions of katuk leaves (Sauropus androgynus (L.) Merr.) have antibacterial activity against *Staphylococcus aureus* with MIC value at a concentration of 12,5 mg/mL and MBC value at a concentration of 25 mg/mL shown in the most active fraction of ethyl acetate. Extracts and fractions of katuk leaves (Sauropus androgynus (L.) Merr.) have inhibitory and degradation activity against *Staphylococcus aureus* biofilm with the lowest IC50 (8.332 ± 0.585) and EC50 (7.695 ± 0.491 mg/ml) values shown in the ethyl acetate fraction.

ADVANCED RESEARCH

The results of this study still have shortcomings, so further research is needed to determine the antibiofilm activity of n-hexane, ethyl acetate, and katuk leaf water fractions against bacteria. *Staphylococcus aureus.* In addition, further research is needed on the more specific chemical content in the extract and fraction of katuk leaves and other solvent variations quantitatively using certain analysis methods. Further research is also needed isolation of active antibacterial and antibiofilm compounds contained in the ethyl acetate fraction of katuk leaves.

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