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Antibacterial and Antibiofilm Activity Related to the Mechanism of Work of the Active Fraction of Moringa Leaves (Guilandina Moringa L.) to Staphylococcus Aureus

Armayanti Rindiarko Hasbrianti[1*], Opstaria[2], Wiwin Herdwiani[3]
Department of Pharmaceutical Science, Faculty of Pharmacy, Setia Budi University, Surakarta
Corresponding Author: Armayanti Rindiarko Hasbrianti
maya.Hasbrianti326@gmail.com

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ABSTRACT

Biofilm-associated S. aureus bacterial infections are difficult to treat because bacterial cells encased in biofilms can be highly resistant to antibiotics and the immune response of the host. Moringa leaf extract contains active compounds of tannins, saponins, alkaloids, and phenols that have activity as antibacterial and antibiofilm. This study aims to determine the antibacterial activity and of extracts, mechanism of action of the most active fraction of moringa leaf ethanol extract against S. aureus.Extraction of Moringa leaves using maceration method with 96% ethanol solvent, fractionated using n-hexane, ethyl acetate, and water solvents. Antibacterial test using disc diffusion method, followed by dilution test. Observations were made using SEM and AAS. Data from the diffusion test, inhibition of biofilm formation, and biofilm degradation were analyzed using One Way ANOVA. Based on the results of the study, the ethyl acetate fraction is the most active fraction in inhibiting the growth of S. aureus in the diffusion test with an inhibition zone diameter of 15,7 mm at a concentration of 100 mg/mL and a minimum kill concentration value of 25 mg/mL. The water fraction was the most active fraction in inhibiting the formation and degradation of biofilm against S. aureus with an IC50 value of 4,049 ± 0,063 mg/mL and an EC50 value of 4,246 ± 0,050 mg/mL

INTRODUCTION

Infections are caused by bacteria, viruses, fungi and parasites. One of the bacteria that causes infection is Staphylococcus aureus. Prevalence S. aureus remains difficult to predict and large geographic differences are a major factor contributing to differences in health care systems and infection control practices. In developed countries, the estimated incidence is 80-190 cases per 100,000 population per year.

Bacteria S. aureus, is a gram-positive type of bacteria which is estimated to be 20-75% found in the upper respiratory tract, face, hands, hair and vagina. S. aureus is one of the most common bacterial infections in humans and is the cause of several human infections, including bacteremia, infective endocarditis, skin and soft tissue infections (e.g. impetigo, folliculitis, boils, carbuncles, cellulitis, burns), osteomyelitis, septic arthritis, prosthetic infections, lung infections (e.g. pneumonia and empyema), gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections

Biofilm is a collection of microbial cells attached to a substrate, tissue, or material covered by polysaccharides or extracellular matrix produced from bacterial cell secretions. Biofilm formation greatly increases the risk of cross-contamination and leads to increasingly serious microorganism infections. S. aureus capable of adhering and forming biofilm which can largely be regulated by production polysaccharide intercellular adhesin (PIA). PIA plays a key role in cell interactions or quorum-sensing (QS). Bacterial infection S. aureus Biofilm-related infections are difficult to treat because the bacterial cells encased in the biofilm can be highly resistant to antibiotics and the immune response of the host.

The use of resistant antibacterial drugs is one of the reasons for switching to herbal medicines. One plant that has antibacterial and antibiotic properties is Moringa leaves. In research, Moringa leaf extract (Moringa oleifera L.) contains 8.22% tannin, 1.75% saponin, 0.42% alkaloid, and 0.19% phenol. Research conducted on Moringa leaf water extract (Moringa oleifera L.) contains active compounds of alkaloids, saponins, tannins, phenols, flavonoids, triterpenoids, steroids, and glycosides. In previous research, it was said that the chemical compounds contained such as flavonoids, alkaloids and phenols could inhibit bacterial activity

Research conducted by, antibacterial activity of Moringa leaf extract (Moringa oleifera L.) against Staphylococcus aureus with varying extract concentrations of 5%, 10%, 20%, 40%, and 80% respectively produced an inhibitory power of 12.16 mm, 13.66 mm, 16.00 mm, 18.66 mm, and 20, 50mm. Moringa leaf extract (Moringa oleifera L.) as an antibacterial against S. aureus with variations in extract concentration of 0.3%, 0.6%,

1.25% did not show any inhibitory power, while concentrations of 2.5%, 5%, and 10% showed an inhibitory power of 8 mm, 12 mm, and 14 mm. successively. KBM test results (Minimum Inhibitory Concentration) of Moringa leaf ethanol extract (Moringa oleifera L.) against S. aureus at an extract concentration of 5% it gave an average inhibition zone of 8.85 mm and the results of the KBM (Minimum Kill Concentration) test did not show any bacterial growth. Antibacterial strength criteria are divided into 4, namely very strong with an inhibition zone of >20 mm, strong criteria with an inhibition zone of 10-20 mm, medium criteria with an inhibition zone of 5-10 mm, and weak criteria with an inhibition zone of <5 mm. Moringa leaf extract (Moringa oleifera L.) which was tested as an antibiofilm against S. aureus Varying extract concentrations of 20 mg/mL, 40 mg/mL, and 60 mg/mL, resulted in biofilm inhibition of 4.96%, 13.97% and 28.57%. The active compounds contained in Moringa leaves such as flavonoids, saponins, tannins and alkaloids which have antibacterial and anti-biofilm effects are believed to cause cell leakage which can lead to death. S. aureus. Ion leaks are caused by cell wall damage.

METHODS

A. Antibacterial Activity Test

1. Diffusion Method Antibacterial Activity Test

Testing antibacterial activity using the paper disk diffusion method, first prepared a sterile petri dish and sterile glass bottle, then poured 15 mL of MHA media into the glass bottle and added 0.2 mL of the test bacterial suspension then slowly rotated the glass bottle so that the suspension mixed with the medium. . After that, put it in a petri dish, flatten it and let it sit until it solidifies. Then, in the media, insert a paper disc (blank disk) measuring 6 mm with an absorption capacity of 50 µL per disc dipped into each test solution (extract, fraction n-hexane, ethyl acetate fraction and water), streptomycin as a positive control and DMSO 5% as a negative control, carried out 3 times, then incubated for 24 hours at 37°C.

2. Dilution Method Antibacterial Activity Test

Dilution method antibacterial activity test, MIC determination was carried out using the liquid dilution method broth dilution test (Serial dilution). The test media used is Brain Heart Infusion (BHI). This method uses 1 row of test tubes consisting of 12 sterile tubes containing active fractions with concentrations. MIC testing was carried out by making the concentration of the Moringa leaf fraction, namely 100 mg/mL; 75 mg/mL; 50 mg/mL; 25mg/ml; 12.5 mg/mL; 6.25 mg/mL; 3.12 mg/mL; 1.56 mg/mL; 0.78 mg/mL; 0.19 mg/mL, positive control (+), and negative control (-). Tubes 2-11 contain 0.5 mL of each concentration with graded dilutions then 0.5 mL of bacterial

suspension is added aseptically. Tube 12 as a positive control only contains bacterial suspension, then incubated at 37ºC for 24 hours. The smallest concentration in the tube that looks clear without any growth of test bacteria is determined as the MIC value. The MIC value is expressed as the lowest concentration of the active fraction of Moringa leaves that can still inhibit the test bacteria. Minimum Kill Concentration (KBM) is determined by inoculating a clear media tube by scratching onto selective media and incubating at a temperature of 37ºC for 24-48 hours. Observe whether or not colonies grow on the surface of the plate media.

B. Optimization of Biofilm Formation Time

Research uses microtitier plate flat-bottom polystyrene 96 wells, carried out by adding 200 µL of bacterial suspension into each well and then optimizing the incubation time. Aims to obtain optimal incubation time to form biofilm. Variations in incubation time used were 1, 2, 3, and 4 days. After the incubation period, microplate washed using running water 3 times, then added 200 µL of 1% crystal violet solution to each well and incubated at room temperature for 15 minutes. Microplate washed again using running water 3 times. 200 µL of 96% ethanol solution was added to each well and incubated at room temperature for 15 minutes. Next, the biofilm growth (Absorbance OD595) was read using the iMark-Biorad Microplate Reader. Testing is carried out aseptically inside Laminar Air Flow (LAF) which has previously been cleaned with alcohol, then sterilized with UV turned on for approximately 2 hours before use. The result of the largest absorbance value is expressed as biofilm formation S. aureus the optimal one.

C. Biofilm Formation Inhibitory Activity Assay

Biofilm formation inhibitory activity tests were carried out using microplate round bottom polystyrene 96 wells 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 was equivalent to 1.5x108 CFU/mL is added to the well containing the sample, incubation is carried out on temperature ± 37°C for 72 hours. After incubation, the contents of the wells were discarded and the plate was washed with running water then dried for 15 minutes by turning it upside down microplate at room temperature.

A total of 200 µL of 1% crystal violet solution was added to each well for a staining time of 15 minutes. The contents of the well are thrown away and the well is rinsed again with running water. Microplate dried by turning over at room temperature for one hour. Then 200 µL of 96% ethanol solution was given to each well on the plate optical density read at optimized λ 595 nm. Each test was replicated 3 times. The control in the test used is a negative control (bacteria + media) which is a control where bacterial growth is not disturbed to see the biofilm that forms. The test control (bacteria+media+fractions) is a test control for the activity of Moringa leaves in inhibiting biofilm growth. The positive control used streptomycin.

$$\% \text{ inhibition} = \frac{Negative\ control\ OD - Treatment\ OD}{OD\ Negative\ Control} \text{ x } 100\%$$

After obtaining the average percent inhibition of biofilm formation from each fraction concentration, proceed with determining the IC value50 by using the linear regression line equation between the percent inhibition of biofilm formation and the concentration of the fractions to see the relationship between the concentration and the percent inhibition of the biofilm in inhibiting 50% of the biofilm.

D. Biofilm Degradation Activity Assay

Biofilm degradation activity tests were carried out using microplates roundbottom polystyrene 96 wells 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 which was equivalent to 1.5x108 CFU/mL. Microplate then incubated at a temperature of ±37°C for 72 hours. The contents of the well are then discarded and the well is washed using running water. Each well was then filled with 200 µL of sample in media microplate Again incubated at a temperature of ±37°C for 24 hours. To determine biofilm, the same method as the biofilm formation inhibitory activity test can be applied.

The control in this test is a negative control (bacteria + media) which is a control for biofilm formation where bacterial growth is not disturbed. The sample control (bacteria + media + fraction) is a control test for the activity of Moringa leaves in degrading biofilms.

$$\% \text{ degradation} = \frac{Negative\ control\ OD - Treatment\ OD}{OD\ Negative\ Control} \text{ x } 100\%$$

Biofilm destruction activity assay S. aureus expressed by EC parameters50 (Effective Concentration) namely the concentration of the test compound that destroys the biofilm by 50%. EC value50 determined 46 from the linear regression equation between sample concentration and the percentage of biofilm destruction. Linear regression equation, r table value with a confidence level of 0.95. EC Price50 Inversely proportional to the biofilm destruction activity, the greater the EC value50 hence the biofilm destruction activity S. aureus The smaller it is, the greater the concentration required to destroy the biofilm by 50%.

E. Analysis Atomik Absorption Spectrophotometer AAS

AAS was used to analyze cell leakage in this study. Ion leak analysis was carried out on tubes which was determined by calculating one KBM and 2 times the KBM in the dilution method antibacterial activity test. AAS is used to detect leaks of ions such as K+ and Ca2+. The results from 1 KBM and 2 times KBM were then taken in 1 mL and then diluted in a 100 mL measuring flask. Results were measured by wet digestion using HNO3 (Park, 1996). Leakage is expressed by measuring metal ions present in the test bacteria after contact with the active fraction at concentrations 0 (control), 1 and 2. The supernatant fluid is analyzed using AAS Thermo Elemental Solar MS type. The cell solution resulting from contact with the fraction is taken to measure its ion content.

RESULTS AND DISCUSSION

A. Plant Determination

Determination Moringa leaf plant (Guilandina moringa L.) carried out in

B2P2TOOT (Research and Development Center for Medicinal Plants and Traditional Medicine) in Tawamangu, Central Java. The purpose of plant determination is to ensure that the samples used are accurate, prevent errors in the material collection process, and eliminate the potential for combining materials with other plants. The results of the determination can be seen in Appendix 1. The results show that the samples come from the Moringaceae family and species. Moringa oleifera Lam, with synonyms Guilandina moringa L.

Table 1. Moringa Leaf Powder Extract Yield

Simplicity weight (grams)	Extract weight (grams)	Yield % (w/w)
1.000	176	17,6

Objective The process of filtering is to withdraw or separate compounds from simplicia or mixtures. Research conducted by Arum Sari et al., (2023) The yield of 96% ethanol extract of Moringa leaves was 21.54%. The yield of Moringa leaf extract in this study was 17.6%, this value is in accordance with the requirements stated in the Indonesian Herbal Pharmacopoeia (FHI), namely not less than 9.2%. Calculation of extract yield

B. Phytochemical Screening

Phytochemical screening is one way that can be used to identify the content of secondary metabolite compounds in a natural substance. Screening phytochemicals is a preliminary stage that can provide an overview of the content of certain compounds in the natural materials to be studied. Phytochemical screening can be carried out either qualitatively, semi-quantitatively or quantitatively according to the desired objectives. Qualitative phytochemical screening methods can be carried out through color reactions using certain reagents. Important things that influence the phytochemical screening process are the choice of solvent and extraction method. An unsuitable solvent means that the desired active compound cannot be attracted properly and completely (Kristanti et al., 2008).

Table 2. Phytochemical Screening of Moringa Leaf Powder, Extract and Fractions

Chemical compounds	Library	Results			
	Powder	ExtraK	hexane	ethyl acetate	Air

Flavonoid	Positive results if a yellow-orange to red layer of amyl alcohol is formed	Yellow color on amyl alcohol layer (+)	Dark red orange color on the amyl alcohol layer (+)	No amyl alcohol coating (-)	Yellow color on amyl alcohol layer (+)	Dark red orange color on the amyl alcohol layer (+)
Alkaloid	Mayer: Reaction with Mayer's reagent will form a white precipitate	No white precipitate (+)	No white precipitate (+)	No white precipitate (+)	No white precipitate (+)	No white precipitate (+)
	Dragendorff: with Dragendorff's reagent a red-orange precipitate is formed	There is an orange-red precipitate (+)	There is an orange-red precipitate (+)	There is an orange-red precipitate (+)	There is an orange-red precipitate (+)	There is an orange-red precipitate (+)
Saponin	A positive reaction if foam forms in less than one minute with a height of 1-2	Foam formed 1-10 cm (+)	Formed bubbles 1-10 cm (+)	Formed bubbles 1-10 cm (+)	No bubbles formed 1-10 cm (-)	Foam formed 1-10 cm (+)
Tannin	The formation of a greenish brown or blackish blue color	Blackish blue (+)	Blackish blue (+)	Blackish blue (+)	Blackish blue (+)	Blackish blue (+)

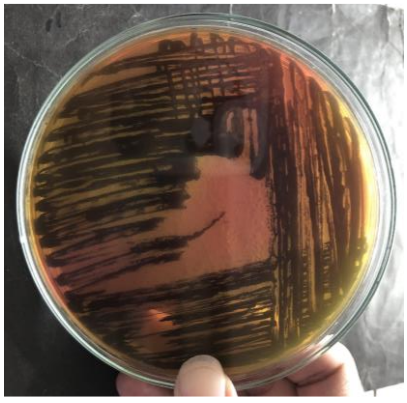
	indicates that the sample is positive for containing tannin	

The results of the phytochemical screening in table 12 show that the powder, extract and water fraction are positive for containing flavonoids, alkaloids, saponins and tannins. Faction nPositive hexane contains alkaloids, saponins and tannins. The positive ethyl acetate fraction contained flavonoids, alkaloids and tannins.

C. Antibacterial Activity Test

Macroscopic Test Results of the Scratch Method

The results of macroscopic identification on VJA media showed the presence of colonies colored black and the medium around the colonies is yellow. This is due to S. aureus can ferment mannitol to acid and the presence of indicators phenol red causes the color of the medium around the colony to change from red to yellow, while the black color of the colony is caused S. aureus reduces potassium tellurite to metallic tellurium and the presence of lithium chloride. Macroscopic test results S. aureus can be seen in the picture.



Picture 1. Results of Macroscopic Identification S. Aureus

Method Gram stain

Coloring Gram aims to observe the morphology of staphylococcus cells and determine the purity of bacterial cells. S. aureus is a Gram positif bacterium and is in the form of a cocci which produces a purple color on Gram staining. The purple color is caused by bacteria retaining the first color, namely crystal violet. Differences in Gram properties are influenced by the content of the cell wall, namely

Gram positif bacteria contain thicker peptidoglycan compared to Gram negative bacteria. Microscopic test results S. auerus can be seen in the picture.

Biochemical Methods

The function of the catalase test on cocci-shaped bacteria is to differentiate between staphylococcus and streptococcus, where the staphylococcus group is catalase positive. Catalase is an enzyme that catalyzes the breakdown of hydrogen peroxide into H2O to O2. Hydrogen peroxide is toxic to cells because this ingredient inactivates enzymes in cells. Hydrogen peroxide is formed during aerobic metabolism, so microorganisms that grow in an aerobic environment must decompose this material. all staphylococcus strains were catalase positive. Catalase test results S. aureus can be seen in picture 16.

Picture 2. Catalase test Results S. Aureus

Test coagulase The aim is to determine the ability of bacteria to produce coagulase enzymes. The coagulase enzyme is an extracellular protein produced by S. aureus which can coagulate plasma (Dewi, 2013). Plasma clotting occurs because there are proteins that resemble enzymes which, when added to oxalate or citrate, can cause clotting. Serum factors react with coagulase to form esterase, clotting activity, and activate prothrombin to thrombin. Thrombin will form fibrin which influences plasma clotting. The ability to coagulate plasma is an important virulence factor in pathogenesis S. aureus. Coagulase test results S. aureus can be seen in the picture.

Picture 3. Coagulase Test Results S. Aureus

Dilution Method

The dilution method is divided into 2, namely liquid and solid dilution. Liquid dilution method used to measure MIC (minimum inhibitory content) while the solid dilution method is used to determine KBM (minimum bactericidal content). The method used in the liquid dilution method is to make a series of dilutions of the antibacterial agent in a liquid medium to which the test bacteria are added.

Table 3. MIC Test Results for the Ethyl Acetate Fraction

Concentration mg/mL	Ethyl acetate fraction		
	Replication I	Replication II	Replication III
100	-	-	-
75	-	-	-
50	-	-	-
25	-	-	-
12,5	+	+	+
6,25	+	+	+
3,12	+	+	+
1,56	+	+	+
0,78	+	+	+
0,39	+	+	+
(K+) Streptomycin	-	-	-

(K-) Kontrol media BHI	+	+	+

Information:
(-) : No bacterial growth
(+) : There is bacterial growth

The MIC test results showed turbidity which indicated that there was still bacterial growth at a concentration of 12.5 mg/mL; 6.26 mg/mL; 3.12 mg/mL; 1.56 mg/mL; 0.78 mg/mL; and 0.39 mg/mL, which means that these concentrations cannot inhibit bacterial growth. Further test by inoculating a concentration of 100 mg/mL; 75 mg/mL; 50 mg/mL; and 25 mg/mL in VJA media.

Table 4. KBM Test Results for the Ethyl Acetate Fraction

Concentration mg/mL	Ethyl acetate fraction		
	Replication I	Replication II	Replication III
100	-	-	-
75	-	-	-
50	-	-	-
25	+	+	+

Information:
(-) : No bacterial growth
(+) : There is bacterial growth

Results test KBM with the scratch method on VJA media showed that the ethyl acetate fraction with a concentration of 25 mg/mL contained bacterial growth. The positive control used was the antibiotic streptomycin. Streptomycin is used as a comparison in antibacterial activity tests because its mechanism of action is to bind to ribosomal subunits, thus interfering with protein synthesis. If protein synthesis is disrupted, it will prevent the formation of new bacterial cells and cause death of the cells.

The MIC and KBM test results show that the ethyl acetate fraction of Moringa leaves is capable hinder bacterial growth S. aureus. In phytochemical screening, the ethyl acetate fraction of Moringa leaves contained flavonoid, alkaloid and tannin chemical compounds. The mechanism of flavonoids as antibacterials is to form complex compounds with extracellular and soluble proteins so that they can damage bacterial cell membranes, followed by the release of intracellular compounds. Alkaloids as antibacterials work by disrupting the peptidoglycan components in bacterial cells so that the cell wall layer does not form

completely and causes cell death. The antibacterial ability of tannins is thought to be because tannins can shrink cell walls, thereby disrupting the permeability of the cells themselves and causing cell wall damage..

D. Biofilm Activity test results

Optimization of Biofilm Formation Time

Optimization Biofilm formation time aims to determine the optimum time for bacteria S. aureus ATCC 25923 in forming the best biofilm. Optimization time variants used are 24 hours, 48 hours, 72 hours. Before optimizing the time for biofilm formation, the turbidity of the bacterial suspension was adjusted to the turbidity McFarland 0.5 (1.5 x 108 CFU/mL). The results of the time for biofilm formation can be seen in table 17.

Table 5. Results of Biofilm Formation Time

No	Absorbance (λ 595 nm)		
	24 hours	48 hours	72 hours
1	0,134	0,377	0,546
2	0,126	0,345	0,558
3	0,137	0,357	0,578
4	0,124	0,363	0,574
5	0,153	0,356	0,569
6	0,155	0,379	0,587
7	0,121	0,358	0,564
8	0,135	0,396	0,576
9	0,146	0,367	0,578
10	0,127	0,398	0,587
Rate-rate	0,135±0,011	0,369±0,016	0,571±0,012

Formation Biofilms begin when bacteria attach to a surface through organic molecules. The attachment of microbes to the surface of objects is influenced by the characteristics of the liquid media used, for example pH, temperature, nutrient levels and ionic strength. Temperature control in this study was carried out during the incubation process, namely at a temperature of 37°C. Hydrodynamic properties can also influence the level and extent of bacterial attachment, for example the fluid velocity characteristics. The level of bacterial attachment is influenced by the condition of the bacterial cell surface, for example the production of extracellular polymers (EPS), cell surface hydrophobicity, and the presence of fimbriae and flagella (Mahami & Adu-Gyamfi, 2011). The process of biofilm formation has five

stages. In the first stage, bacterial cells stick together on the substrate surface due to the influence of Van der Waals forces. This stage is

a process of cell attachment which is still temporary, but in the second stage, the bacterial cells are attached permanently due to the formation of exopolymer material which is a stronger adhesive compound. The third stage is marked by the formation of microcolonies and biofilms begin to form. In the fourth stage, more and more biofilms are formed and form a three-dimensional structure containing cells covered in several groups that are connected to each other. The final stage, the development of the biofilm structure, results in cell dispersion so that the cells are released from the biofilm, attach to a new substrate and form a new biofilm.

Biofilm Formation Inhibition Test

The biofilm formation inhibition test aims to obtain the activity of leaf Moringa in inhibiting biofilm formation S. aureus. Biofilm formation in this study was measured quantitatively using the crystal violet assay method. Biofilm on S. aureus will form a microcolony surrounded by a polysaccharide matrix. The matrix is composed of several components such as extracellular DNA, polysaccharides and several proteins. The percentage of inhibition of biofilm formation can be seen in the table.

Table 6. Results of Percentage Inhibition of Biofilm Formation

Sample	**Mean Inhibition of Biofilm Formation (%) ± SD**			
	Concentration			**Streptomycin**
	12.5 mg/mL	25 mg/mL	50 mg/mL	
Extract	71,25	78,258	80,87	
n-hexane	68,75	73,61	76,00	87,75
ethyl acetate	70,06	74,60	77,95	
Air	75,52	80,30	82,33	

The results of the percentage inhibition of biofilm formation from each test sample can be seen that the greater the concentration of Moringa leaf extract or fraction, the higher the percentage results of inhibition of bacterial biofilm formation. S. aureus. The greater the test concentration, the greater the secondary metabolite content contained in the extract or fraction, so the greater the potential to inhibit biofilm formation.

Control positive used in this study was the antibiotic streptomycin. Streptomycin is an antibiotic belonging to the aminoglycoside group and is an option for biofilm therapy. Based on a docking study

conducted by Khan et al., (2020), it is concluded that aminoglycosides can interact with the QS receptor (quorum-sensing). Based on molecular docking studies, this aminoglycoside can be used as a potential anti-biofilm and virus weakening drug. The use of streptomycin can inhibit biofilm formation. The principle of QS is that when a single bacterial cell releases an autoinducer into the environment, the concentration is too low to be detected because it is diluted in the environment. The autoinducer concentration will increase when the bacterial population is large enough and can reach a threshold concentration at which bacterial cells can recognize the bacteria again and then simultaneously activate the expression of target genes.

Data percent The inhibition of biofilm formation obtained was followed by calculating the IC value50 by using a linear equation between the percent inhibition of biofilm formation and the fraction concentration. IC value50 can be seen in the table.

Table 7. Values of IC_{50} Inhibition of Biofilm Formation

Sample	**Nilai IC_{50} inhibition of biofilm formation (mg/mL)**			**IC $rate_{50}$**
	Replication I	**Replication II**	**Replication III**	
Extract	4,315	4,178	4,149	4,14
hexane	4,864	4,742	4,666	4,75
ethyl acetate	4,666	4,518	4,549	4,57
Water fraction	3,962	4,111	4,073	4,04

The inhibition value of biofilm formation shows that the IC value50 smallest shown by the water fraction, namely 4.049 ± 0.063 mg/mL, the

smaller the IC value50 the greater the inhibitory power of biofilm formation. Inhibition of biofilm formation with IC value50 smallest to largest is indicated by the water fraction, extract, ethyl acetate fraction, and fraction n-hexane.

Based on the results of the anti-biofilm test, the extract and fractions of Moringa leaves were proven own activity in inhibiting biofilm formation in S. aureus ATCC 25923. Analysis using methods Oneway ANOVA to see whether or not there is a significant difference between the extract and fractions of Moringa leaves.

The normality test results obtained a significance value of $p > 0.05$. These results indicate that H0 accepted which states that the data is normally distributed. One of the requirements to proceed to the next testing stage, namely the ANOVA test, is that the data is normally distributed. Test

results Oneway ANOVA on S. aureus ATCC 25923 showed a significant difference in the inhibition of biofilm formation, with a significance result of $p < 0.05$ indicating that H0 rejected.

Tukey test results show a sign (*) in the column mean difference which shows that there is a real difference between the results of inhibiting biofilm formation from the extract and fraction n-hexane, ethyl acetate fraction, and water fraction from Moringa leaves and reinforced in the probability column (Sig) whose value is ($p < 0.05$). If there is no sign (*), the inhibition of biofilm formation is not significant, indicating no difference. Tukey test results can be seen in Appendix 23.

Leaf Moringa contains active compounds, namely flavonoids, alkaloids, saponins and tannins. Flavonoids, alkaloids, saponins and tannins are secondary metabolites that dissolve in polar solvents (Sa'adah & Nurhasnawati, 2015). Water is a polar solvent so it will attract secondary metabolites that dissolve in polar solvents. The water fraction shows the IC value50 The lowest possibility is that in the ethyl acetate fraction there are secondary metabolites of flavonoids, alkaloids, saponins and tannins which have activity in inhibiting bacterial biofilm formation.

Flavonoid and tannins have activity in inhibiting biofilm formation by interfering with the expression of the icaA and icaD genes (Loresta et al., 2014). The icaA and icaD genes are regulatory genes in biofilm formation. Expression of the icaA and icaD genes can result Polysaccharide Intercellular Adhesin (PIA), which is the main ingredient that influences the adhesion process of bacteria to the host surface, states that PIA plays an important role in cell aggregation and formation Ekstracelullar Polymeris Substances (EPS) on biofilm formation. Inhibition of ica gene expression causes flavonoid and tannin compounds to disrupt bacterial cell adhesion which plays an important role in biofilm formation and disrupts the process of attachment of bacteria to substrate surfaces or between bacteria. The biofilm inhibition ability of a compound is related to the ability of the compound to penetrate into the biofilm that is formed. that is, it is able to penetrate the layer Extracelluler Polymeric Substance (EPS) or the mucus layer that surrounds bacteria.

Biofilm Degradation Test

Test degradation Biofilms start with bacteria S. aureus made to produce a biofilm first. Internal solution plate discarded and washed, then treated with Moringa leaf fractions with various concentrations. The absorbance value of biofilm degradation can be seen in the attachment. The absorbance value obtained then calculated the percentage of biofilm degradation can be seen in table 20.

Table 8. Biofilm Degradation Percentage Results

Sample	Mean Biofilm Degradation (%) ± SD			
	Concentration			Streptomycin
	12.5 mg/mL	25 mg/mL	50 mg/mL	
Extract	68,81	57,10	77,76	
hexane	66,04	70,86	73,21	85,44
ethyl acetate	67,93	71,73	75,07	
Water fraction	72,32	77,35	80,86	

The percent of biofilm degradation results are used to determine the EC value50 degradation biofilm from ethanol extract of Moringa leaves, fraction n-hexane, ethyl acetate fraction and water fraction. The positive control on biofilm degradation showed an average percentage of 85.430%. The water fraction is the highest percent value of biofilm degradation with a value of 80.866%, followed by the extract with a value of 77.765%, the ethyl acetate fraction with a value of 75.073% and the n-hexane with a value of 73.201%. Calculation of percent biofilm degradation using a linear equation between the percent concentration of the extract or fraction and the percentage value of biofilm degradation.

Table 9. EC Value50 Biofilm Degradation

Sample	EC value$_{50}$ inhibition of biofilm formation (mg/mL)			EC installment-installment$_{50}$
	Replication I	Replication II	Replication III	
Extract	4,68	4,54	4,49	4,57
n-hexane	5,24	5,14	5,09	5,17
ethyl acetate	5,03	4,89	4,93	4,95
Water fraction	4,17	4,295	4,26	4,24

EC value results50 shows that the ethyl acetate fraction has the lowest value, followed by the water fraction, fraction n-hexane and EC value50 The highest is the extract fraction. The smaller the EC value50 obtained, the greater the activity of this fraction in degrading S. bacterial biofilms. aureus.

The normality test results obtained a significance value of $p > 0.05$. These results indicate that H0 accepted which states that the data is normally distributed. One of the requirements to proceed to the next testing stage, namely the ANOVA test, is that the data is normally distributed. Test results Oneway ANOVA on S. aureus ATCC 25923 showed a significant difference in degrading biofilms, with a significance result of $p < 0.05$ indicating that H0 rejected.

Tukey test results show a sign (*) in the column mean difference which shows that there is a real difference between the results of biofilm degradation from extracts and fractions n-hexane, ethyl acetate fraction, and water fraction from Moringa leaves and reinforced in the probability column (Sig) whose value is ($p < 0.05$). If there is no sign (*), biofilm degradation is not significant, indicating no difference. Tukey test results can be seen in Appendix 25.

Conclusion

Faction n-hexane, ethyl acetate, and Moringa leaf water have antibacterial activity against Staphylococcus aureus, The mechanism of action of the antibacterial activity of the ethyl acetate fraction from the ethanol extract of Moringa leaves against Staphylococcus aureus is that it can cause cell leakage and cell wall damage. The most active fraction of Moringa leaf extract which has anti-biofilm activity against Staphylococcus aureus is the water fraction with the IC value50 of 4.049 ± 0.063 mg/mL and EC value50 amounting to 4.246 ± 0.050 mg/m

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