

Antibacterial Activity and Fractional Antibiofilm Black Mulberry Leaf (*Morus Nigra*. L) against Bacterial *Staphylococcus Aureus* ATCC 25923

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ABSTRACT

The purpose of this study is to determine the antibacterial activity of extracts and fractions against *S. aureus* bacteria, the most active fraction of black mulberry leaves has antibiofilm activity against *S. aureus* bacteria, the most active fraction is located in the cell membrane wall of *S. aureus* bacteria. The leakage of K^+ metal ions and Ca^{2+} was carried out using the AAS method and the morphology of *S. aureus* bacterial colonies was observed using the SEM method. The test result of the concentration inhibition % of 12.5 mg/ml had the highest average percentage, which was 40.01. The average IC_{50} value of inhibition is 24.57. The biofilm degradation test had with an average of 34.98. EC_{50} value 48.98.

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium that is the main cause of various clinical infections in humans. These bacteria are often found in crowded environments and hospitals, causing serious infections if they enter the internal tissues or bloodstream (Taylor et al., 2023). *S. aureus* can cause a variety of infections, including bacteremia, infective endocarditis, skin and soft tissue infections, osteomyelitis, septic arthritis, prosthetic device infections, lung infections, gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections (Taylor et al., 2023). One of the major problems in the treatment of *S. aureus* infection is the emergence of strains that are resistant to antibiotics, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA). A study by Jinghua in 2017 showed that *S. aureus* was the leading cause of pneumonia in 51 of the 119 samples found to be Gram-positive, with high resistance to some antibiotics. According to the Indonesian Ministry of Health in 2016, the high rate of antibiotic resistance caused about 700,000 deaths per year in 2014, and is expected to increase to 10 million over the next 30 years (Agustina et al., 2019).

One of the factors contributing to *S. aureus* resistance is its ability to form biofilms. Biofilms are extracellular polymeric substances (EPS) that help bacteria fight microbes and minimize the effects of antibacterial drugs. *S. aureus* biofilm consists of water (97%) and organic matter, including extracellular polymer substances (50%-90%) and microcolonies (10%-25%) (Idrees et al., 2021). Bacteria coated with biofilm can be 10,000 times more resistant to antibiotics compared to bacteria in planktonic form (Rabin et al., 2015). Given the complexity of biofilms and their role in antibiotic resistance, the development of antibiofilm strategies is essential to address *S. aureus* infection. Several studies have shown the potential of natural compounds in inhibiting the formation of biofilms. One of the plants that attracts attention is the black mulberry (*Morus nigra* L.), which is known to have a variety of bioactive compounds with antibacterial and antibiofilm activity.

Black mulberry leaves contain a variety of bioactive compounds, including alkaloids, anthocyanins, and flavonoids (Kurnia Hadi Saputra, 2020). The polyphenols in mulberries, such as tannins, terpenoids, phenolics, and steroids, are known to have antibacterial and antibiofilm activity. Tannins act as antiadherents that prevent bacterial adhesion to surfaces and damage EPS matrices at biofilm maturation (Jeffrey, Mieke H Satari, 2019). Phenolic acids decrease the motility of flagel-mediated bacteria, while steroids can cause leakage in liposomes. Terpenoids can damage bacterial cell membranes, modulate bacterial efflux pumps, suppress biofilm growth, and inhibit virulence factors such as enzymes and toxins (Wijaya et al., 2024). Several studies have shown the antibacterial activity of black mulberry leaf extract against *S. aureus*. Zainab & Munawarroh (2019) reported that the ethyl acetate fraction from 50% ethanol extract of black mulberry leaves has antibacterial activity with a Barrier Area Diameter (DDH) of 4.7 mm at a concentration of 40%. (Nasir, 2023) showed that ethanol extract of 95% black mulberry leaves had a Minimum Resistance Concentration (KHM) of 0.32 mm and a Minimum Kill Concentration (KBM) of 0.64 mm against *S. aureus*. (Kusumawardani et al.,

2022) reported that methanol extract of black mulberry leaves at a concentration of 10 mg/ml produced 18 mm DDH against *S. aureus*. Although previous studies have shown the potential of black mulberry leaves as an antibacterial agent, more research is still needed to optimize its activity. This study aims to evaluate the antibacterial activity and antibiofilm of black mulberry leaf fraction against *S. aureus* ATCC 25923 using 96% ethanol as a solvent. The use of 96% ethanol is expected to result in a higher Barrier Area Diameter (DDH) compared to previous research. The results of this study are expected to contribute to the development of alternative treatments to overcome antibiotic-resistant *S. aureus* infections.

Based on the background that has been described, the formulation of the problem in this study is as follows: What is the antibacterial activity and antibiofilm of black mulberry leaf fraction (*Morus nigra* L.) against *Staphylococcus aureus* ATCC 25923 bacteria? Can the use of 96% ethanol as a solvent result in a higher Barrier Area Diameter (DDH) compared to previous studies? How effective is the fraction of black mulberry leaves in inhibiting the formation of *S. aureus* biofilm? The study will also investigate the optimal concentration of black mulberry leaf fractions needed to achieve significant antibacterial and antibiofilm effects.

This study aims to evaluate the antibacterial activity and antibiofilm of black mulberry leaf fraction (*Morus nigra* L.) against *Staphylococcus aureus* ATCC 25923. Specifically, this study will analyze the effectiveness of black mulberry leaf fractions extracted using 96% ethanol in inhibiting bacterial growth and biofilm formation. Another objective was to determine the Minimum Inhibition Concentration (KHM) and Minimum Kill Concentration (KBM) of the black mulberry leaf fraction against *S. aureus*, as well as identify the optimal concentration required to achieve maximum antibacterial and antibiofilm effects. In addition, this study also aims to compare the results obtained with previous research, especially in terms of the Diameter of the Obstacle Area (DDH) produced.

This research is expected to provide benefits both theoretically and practically. Theoretically, the results of this study will enrich scientific knowledge about the antibacterial and antibiofilm potential of the black mulberry leaf fraction, specifically against *S. aureus*. This could contribute to the development of new strategies in addressing infections caused by antibiotic-resistant bacteria. Practically, the findings of this study could be the basis for the development of a natural product based on black mulberry leaves as an alternative or companion to conventional treatment for *S. aureus* infection. In addition, the results of this study can also be the basis for further research in optimizing the use of natural materials as antibacterial agents and antibiofilms, as well as encouraging further exploration of the potential of traditional medicinal plants in overcoming the problem of antibiotic resistance.

LITERATURE REVIEW

Antibacterial agents are substances used to kill bacteria, especially bacteria that are harmful to the human body. Drugs designed to eradicate bacteria that

cause infections in humans must show selective toxicity. Antimicrobials are chemicals with antibacterial properties produced by microorganisms (Worthington et al, 2012). Based on the type of selective toxicity, antibacterial agents can be classified into two types, namely bactericidal and bacteriostatic. Bactericidal agents kill bacteria while bacteriostatic agents have the ability to inhibit bacterial growth but do not kill bacteria. The ideal antimicrobial has selective, relative, not absolute toxicity (Costerton, 2001).

Amoxicillin is an antibiotic that is often chosen in antibacterial and antibiofilm tests against *S. aureus* for the following reasons. Amoxicillin is a penicillin-type antibiotic with a fairly broad spectrum of activity, covering many types of Gram-positive bacteria, including *S. aureus*. Although some strains of *S. aureus* (such as Methicillin-resistant *S. aureus* or MRSA) can be resistant to amoxicillin, this antibiotic is still relevant for sensitive strains.

Antibacterial testing of black mulberry leaf extract can be carried out using two main methods, namely the dilution method and the diffusion method.

Dilution Method (Weakening Test)

Minimum inhibitory concentration (MIC) and minimum killing concentration (MBC) can be determined using this antibacterial test procedure. Minimum inhibitory concentration, or MIC, is the lowest concentration of an antibiotic that can stop the growth of a microbe or completely kill it. The lowest concentration of antibacterial that can kill germs is MBC. The basic idea of the dilution method involves a test tube containing a liquid medium and a predetermined number of target microorganism cells, The tube rack is incubated at 37°C for 24 hours while measuring the turbidity in each tube. The lowest concentration in the tube that produces clearly visible culture results is called MIC (Minimum Inhibitory Concentration) while MBC (Minimum Killing Concentration) is the lowest concentration that does not produce the formation of microbial colonies (Radji et al., 2002).

Diffusion Method (Diffusion Test)

The diffusion method is a technique to determine how well antibacterial substances suppress microorganisms. The circular diffusion method (Kirby-Bauer) is an antibacterial test that measures the inhibition zone. Disc method (Kirby Bauer method). This method is a method used for antibacterial tests with disc paper that has been dripped with extract samples, fractions as antimicrobials, then placed on agar media that has hardened and already contains test bacteria, incubated at optimal temperature. The results of the observation show the presence or absence of an inhibition zone, then the inhibition zone formed is measured (mm) (Radji, 2002).

Biofilm

Biofilm is a collection of microorganisms that adhere to each other on a living or non-living surface in an Extracellular Polymeric Substance (EPS) matrix or extracellular substances produced by the microorganisms themselves. EPS can cover 50% to 90% of the total organic carbon of the biofilm and can be considered the primary matrix material of the biofilm (Purbowati, 2016).

(Stoodley, 2019) Determine the criteria or specific characteristics that can be used to describe biofilms in general. Includes a thin layer underneath ranging from a single layer of attached cells to a thick multilayer layer with water channels. Studies have shown that biofilm thickening can be caused by a number of bacterial components. The structure of the biofilm can also be influenced by the interaction of non-microbial particulate components, both host and environment, such as red blood cells and fibrin that accumulate in the biofilm. The difference between biofilm and infectious colony is the presence of a collection of microcolonies of cells attached to a surface. The formation of biofilm as a defense mechanism has significant consequences for the host, because microorganisms that grow in aggregates surrounded by the matrix are more resistant to antibiotics and host defense mechanisms. Some examples of bacteria that can form biofilms are *P. Aeruginosa*, *S. aureus*, *Klebsiella pneumoniae*, *flavobacterium spp*, *S. epidermis* and *streptococcus sp* (Homenta H, 2016).

Scanning Electron Microscope (SEM)

SEM is a microscope tool used to observe the surface of solid objects with a magnification of 10 to 3 million times, a depth of field of 4 to 0.4 mm, and a resolution of 1 to 10 nm. Combining high magnification, deep depth of field, extraordinary resolution, and the ability to obtain compositional and crystallographic information. SEM is a tool used for research and industry. Producing image scanning with electrons, the interaction of the electron beam with the sample produces secondary electrons (SE) and backscattered electrons (BSE), which are received by the SE/BSE detector and converted into signals. The signal data is amplified by a video amplifier and synchronized with the scanning circuit to produce images on a cathode ray tube (Farikhin, 2016).

Atomic Absorbance Spectrophotometer (AAS)

AAS or atomic absorption spectrophotometry has a basic principle is the occurrence of action related to electromagnetic radiation and the sample being tested. SSA has a very accurate analysis of samples with low concentrations and is a method for elemental analysis. This technology is based on the emission and absorption of atomic vapor. This SSA system is to obtain the results of atomic vapor released from the sample.

METHODOLOGY

This study is an experimental study that aims to evaluate the antibacterial activity and antibiofilm of black mulberry leaf fraction (*Morus nigra* L.) against *Staphylococcus aureus* ATCC 25923. Black mulberry leaf samples were obtained from Tawangmangu District, Karanganyar Regency, Central Java. The leaves used are dark green leaves that are still fresh and taken randomly. The research was carried out from March to June 2024 at the Laboratory of Microbiology and Pharmaceutical Technology, Setia Budi University, Surakarta. Sample preparation begins by washing the leaves using running water, followed by the drying process using an oven at a temperature of 45-50°C for 24 hours. The dried simplisia is then mashed into powder and sifted using a mesh

sieve number 40/20. Extraction was carried out by maceration method using 96% ethanol solvent with a ratio of 1:10 for 24 hours, followed by remasuration. The extract obtained is concentrated using a rotary evaporator at a temperature of 50°C until a thick extract is obtained.

The fractionation of ethanol extract of black mulberry leaves was carried out using the liquid-liquid partition method with n-hexane, ethyl acetate, and water solvents. A thick extract of 30 grams is dissolved in hot water, then partitioned successively with n-hexane and ethyl acetate. Each fraction obtained is concentrated using a rotary evaporator at a temperature of 50°C.

Phytochemical tests were carried out on extracts and fractions to identify the content of secondary metabolite compounds including flavonoids, alkaloids, saponins, tannins, terpenoids, and steroids. The characterization of the extract includes organoleptic test, determination of drying shrinkage, total ash content, acid insoluble ash content, water soluble juice content, and ethanol soluble juice content. The antibacterial activity test was carried out using disc diffusion and dilution methods. The test bacteria *S. aureus* ATCC 25923 was identified through Gram staining, catalase test, coagulase test, and culture on Vogel Johnson Agar (VJA) media. The concentration of the extracted and fraction tested was 12.5%, with amoxicillin 10% as a positive control and DMSO 10% as a negative control. The determination of the Minimum Inhibition Concentration (KHM) and the Minimum Kill Concentration (KBM) was carried out by the dilution method using the radiant concentration.

The antibiofilm activity test began with the optimization of the formation time of *S. aureus* biofilm using a 96-well microplate with variations in incubation time of 1, 2, and 3 days. The activity of inhibiting biofilm formation and biofilm degradation was tested using the most active fractions at concentrations of 20%, 40%, and 80%. Measurements were made using a microplate reader at a wavelength of 595 nm. The IC₅₀ values for inhibition of biofilm formation and EC₅₀ for biofilm degradation were calculated using a linear regression equation. The analysis of the antibacterial mechanism of action was carried out through observation of K⁺ and Ca²⁺ ion leakage using the Atomic Absorption Spectrophotometer (AAS) at concentrations of 1 KBM and 2 KBM. The morphology of bacterial cells was observed using a Scanning Electron Microscope (SEM) to evaluate changes in cell structure due to exposure to the most active fractions. Data analysis was carried out using SPSS 29 software. The normality test was carried out with Kolmogorov-Smirnov and Shapiro-Wilk. Differences in effectiveness between treatments were analyzed using the one-way ANOVA or Kruskal-Wallis non-parametric test, followed by an appropriate post-hoc test. Linear regression analysis was used to determine IC₅₀ and EC₅₀ values in antibiofilm assays.

RESEARCH RESULT

Plant Determination

The initial stage of this research is the determination of black mulberry leaf plants (*Morus nigra** L.) (Umar et al., 2016). The determination was made in B2P2TOOT Kalisoro, Tawangmangu District, Central Java Regency. The determination results (Appendix 1) confirm that the species used is from the

Moraceae Family, Species **Morus nigra** L. This process is important to ensure accuracy and precision in the collection of research materials.

Ingredient Intake Levels

Black mulberry leaves (**Morus nigra** L.) were obtained from farmers in Tawangmangu, Karanganyar Regency, Central Java. Leaf samples were taken randomly and sorted. After drying, the leaves are smoothed and sifted using a 40 mesh.

Table 1. Results of Simplisia Yield of Black Mulberry Leaves

Wet Weight (g)	Dry Weight (g)	Yield (%)
12000	1300	10.83

The simplicia yield obtained was 10.8%. This value shows good results, because according to the Ministry of Health of the Republic of Indonesia (2017), a good yield is more than 10%. A high yield indicates a high concentration of compounds that are interested in the feedstock. Some of the factors that affect the simplicia yield include quality of harvested raw materials, drying method and moisture content in plants. The lower yield compared to previous studies (Kristina E, 2018) which reached 60%, may be due to differences in these factors.

Manufacture of Thick Extracts

The viscous extract is obtained through the process of maceration and evaporation.

Table 2. Results of Yield of Thick Extract

Simplisia Weight (g)	Weight of Thick Extract (g)	Yield (%)
1200	133	11.08

The yield of the viscous extract obtained was 11.0%. According to the Indonesia Herbal Pharmacopoeia (2017), the yield of mulberry leaf thick extract that meets the requirements is not less than 8.3%. Thus, the results of this study meet these standards. The yield of thick extract in this study (11.0%) was lower than that of Kristina Erni Sanul (2018) which reached 45.9%. This difference may be caused by differences in solvent concentrations, different soaking and reccassation times, other factors such as extraction method and sample condition.

Black Mulberry Leaf Extract Fraction

Fractionation is performed to separate compounds based on their polarity into three fractions: the water fraction (polar), the ethyl acetate fraction (semi-polar), and the n-hexane fraction (non-polar).

Table 3. Results of Black Mulberry Leaf Fraction

Solvent	Sample Weight (g)	Weight of Thick Extract (g)	Dry Fraction Weight (g)
Water fraction	30	6	5
Ethyl acetate	30	5	4
n-hexane	30	16	15
Entire	90	27	24

- a. The n-hexane fraction had the highest yield (53.33%), indicating that non-polar compounds were more dominant in the extract.
- b. The ethyl acetate fraction has the lowest yield (16.67%), indicating less semi-polar compound content.
- c. The water fraction has a medium yield (20%), indicating the presence of a moderate amount of polar compounds.

Test Specific Parameters

- a. Organoleptis Test

The results of the organoleptis test of black mulberry leaf powder and extract are presented in Table 4.

Table 4. Results of Observation of Organoleptis Powder and Mulberry Leaf Extract

Dosage	Aroma	Color	Taste
Powder	Typical Mulberry Leaves	Dark Green	Bitter
Thick Extracts	Typical Mulberry Leaves	Brownish Black	Bitter

The results of the organoleptis test showed that black mulberry leaf powder and extract had characteristics that were in accordance with the standard, including the distinctive aroma of mulberry leaves, dark green color for powder and brownish-black color for thick extract, as well as bitter taste. These results are in accordance with the desired quality requirements based on organoleptic test standards with a simple method (Ma'ruf et al., 2023).

b. Phytochemical Screening Test

The results of phytochemical screening of thick extracts and fractions of black mulberry leaves are presented in Table 5.

Table 5. Results of Chemical Content Testing of Condensed Extracts by Phytochemical Screening

Metabolite Seconds	Crude Extract	Water Faction	Ethyl Acetate Faction	n-Hexane fraction
Flavonoids	+	+	+	+
Alkaloids	±	±	±	±
Saponins	+	+	+	+
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Steroids	-	-	-	-

Description: (+): contains test compounds, (-): does not contain test compounds

The results of phytochemical screening showed that the extract and fraction of black mulberry leaf contained a variety of secondary metabolites, including flavonoids, alkaloids (positive on the Wagner test), saponins, tannins, and terpenoids. No steroids were found in the tested samples. The chemical compound content in black mulberry can be affected by various environmental factors, including the conditions of the place where it grows, the type of soil, nutrients, the altitude of the place, and the intensity of light that the plant receives.

Non-Specific Parameter Test

The results of determining the drying shrinkage of black mulberry leaf extract are presented in Table 6.

Table 6. Shrinkage of Drying of Mulberry Leaf Extract

Replication	Starting Weight (g)	Final Weight (g)	Drying Shrinkage (g)
1	2	1,82	0,18
2	2	1,83	0,17
3	2	1,84	0,16
Average	2	1.83 ± 0.01	0.17 ± 0.01

The results of the determination of drying shrinkage showed an average of 8.5% ± 0.005, which met the requirements of the 2017 Herbal Pharmacopoeia Indonesia (FHI), which was less than 10%. This indicates that the extract has good quality in terms of water content and volatile compounds.

a. Determination of Total Ash and Acid Insoluble Ash Levels

The results of the determination of total ash and acid insoluble ash levels are presented in Table 7.

Table 7. Results of Determination of Total Ash and Acid Insoluble Ash

Parameters	Test Result (%)	FHI Standard 2017 (%)
Up to Total Ash	11,19	< 13.0
Acid Insoluble Ash	3,26	< 4.5

The results of determining the total ash content (11.19%) and acid insoluble ash (3.26%) met the requirements of FHI 2017. The relatively low total ash content indicates a low content of inorganic compounds and heavy metal contaminants in the extract.

b. Determination of Water Soluble Juice and Ethanol Levels

The results of the determination of water and ethanol soluble juice levels are presented in Tables 8 and 9.

Table 8. Determination of Water Soluble Juice Rate

Replication	Sample Weight (g)	Test Result (%)	FHI Maximum Limit 2017 (%)
1	5	16,78	>14.0
2	5	19,34	>14.0
3	5	30,96	>14.0
	5	22.36	>14.0
Average		± 7.56	

Table 9. Determination of Ethanol Soluble Sari Content

Replication	Test Result (%)	FHI Maximum Limit 2017 (%)
1	11,16	>10.0
2	22,92	>10.0
3	10,04	>10.0
Average	14.71 ± 7.13	>10.0

The results of the determination of water-soluble juice content ($22.36\% \pm 7.557$) and ethanol soluble juice content ($14.71\% \pm 7.135$) met the requirements of FHI 2017. The higher water soluble content indicates that black mulberry leaf powder is more soluble in water solvents compared to ethanol.

Identification of *Staphylococcus aureus* Bacteria

The results of the identification of *S. aureus* bacteria in Vogel Johnson Agar (VJA) media showed a black spherical colony, with smooth edges, convex

surfaces, and areas around the colony were yellowish. These results correspond to the morphological characteristics of *S. aureus* described by (Ibrahim, Jumriani., Irnawaty, 2017). The results of the Gram staining showed spherical bacteria (coccus) clustered like grapes, purple in color, which confirmed that the bacteria were Gram-positive. This is in accordance with the characteristics of *S. aureus* as described by (Putu Risky Vidika Apriyanthi et al., 2022). Biochemical tests include coagulase and catalase tests. The results of the coagulase test showed positive results with the formation of clots, while the catalase test showed positive results with the formation of gas bubbles after the addition of H₂O₂. Both of these results confirm the identification of *S. aureus*.

S. aureus Antibacterial Diffusion Test

The results of the antibacterial diffusion test against *S. aureus* are presented in Table 10.

Table 10. Test Results of *S. aureus* Bacteria Diffusion Method with a Concentration of 12.5%

Sample	Replication 1 (mm)	Replicati on 2 (mm)	Replication 3 (mm)	Average SD ± (mm)
Extract	12	17,1	23	17.37 ± 5.50
Water fraction	17	17,2	17,1	17.10 ± 0.10
Ethyl acetate fraction	17	15,3	12,6	14.97 ± 2.22
N-hexane fraction	25	21,4	23	23.13 ± 1.80
(+) Control	32	34	34,3	33.43 ± 1.25
Control (-)	0	0	0	0 ± 0

Description: Control (+) = amoxicillin antibiotic disc, Control (-) = no treatment

The results of the diffusion test showed that the n-hexane fraction had the highest antibacterial activity with an average inhibition zone of 23.13 ± 1.80 mm, followed by extract (17.37 ± 5.50 mm), water fraction (17.1 ± 0.10 mm), and ethyl acetate fraction (14.97 ± 2.22 mm). One-way statistical analysis of ANOVA and Tukey's post hoc test showed significant differences between the samples (p<0.05), with the n-hexane fraction having the activity closest to positive control (amoxicillin). The high antibacterial activity of the n-hexane fraction can be caused by the content of non-polar compounds that are effective in inhibiting

bacterial growth. These compounds, such as terpenoids and alkaloids, can damage bacterial cell membranes or inhibit essential functions within bacterial cells (Anwar et al., 2023). Amoxicillin, as a positive control, showed the largest inhibitory zone (33.43 ± 1.25 mm), which is categorized as very strong antibacterial activity. The mechanism of action of amoxicillin involves inhibiting the synthesis of bacterial cell walls, leading to lysis of bacterial cells (Pratiwi et al., 2019).

S. aureus Antibacterial Dilution Test

The antibacterial dilution test of *S. aureus* was carried out using the n-hexane fraction which previously showed the largest inhibitory zone in the diffusion test. The results of the dilution test showed:

- The Minimum Inhibition Concentration (KHM) cannot be determined visually due to the high turbidity of the tubes after 24 hours of incubation.
- The Minimum Kill Concentration (KBM) was determined by inoculation on VJA (Vogel Johnson Agar) media.

Table 10. Results of Inoculation n-hexane Fraction Dilution Test on VJA Media

It	Concentrati on (% B/V)	Replic ation 1 (mm)	Replicat ion 2 (mm)	Replicat ion 3 (mm)	Average (mm)
1	(+) Control	-	-	-	-
2	50	-	-	-	-
3	25	-	-	-	-
4	12.5	-	-	-	-
5	6.25	+	+	+	+
6	3.12	+	+	+	+
7	1.56	+	+	+	+
8	Control (-)	+	+	+	+

Description: (-) : no bacterial growth, (+) : no bacterial growth

The results showed that the KBM of the n-hexane fraction against *S. aureus* was 12.5%. The absence of bacterial growth at high concentrations (50%, 25%, 12.5%) indicates strong antibacterial activity in the n-hexane fraction of black mulberry leaves.

Optimization and Time of Biofilm Formation of S. aureus Bacteria

Optimization of biofilm formation time was carried out by incubation for 24, 48, and 72 hours. The results showed:

Table 11. Results of Bacterial Optimization Test with Absorbance of 595 nm

Incubation Time (hours)	Average Inhibition Zone Diameter \pm SD (mm)
24	0.351 \pm 0.044
48	0.761 \pm 0.215
72	0.800 \pm 0.134

The optimal time of biofilm formation is 72 hours with the highest average absorbance (0.800 \pm 0.134). ANOVA's one-way statistical analysis showed a significant difference between incubation times ($p < 0.05$). The formation of biofilms is influenced by various factors such as surface properties, film conditions, hydrodynamics, media characteristics, and the surface state of bacterial cells. The use of plastic microplates supports bacterial attachment due to its hydrophobic properties.

Biofilm Formation Inhibition Test

The test of inhibition of biofilm formation was carried out with n-hexane fraction at concentrations of 12.5, 6.25, and 3.12 mg/ml. Inhibition percentage results:

Table 12. Results of Percentage Inhibition of Biofilm Formation of n-hexane Fraction

Concentration (mg/mL)	Average Percentage of Inhibition \pm Elementary School (%)
12,5	40.01 \pm 0.68
6,25	34.15 \pm 0.96
3,12	32.09 \pm 1.34
Positive Control (Amoxicillin)	89.61 \pm 0.95

The highest percentage of inhibition was found at a concentration of 12.5 mg/ml. Statistical analysis showed a significant difference between concentrations ($p < 0.05$). IC50 value of biofilm formation inhibition: mean IC50 = 24.57 \pm 2.72 mg/ml compounds in the n-hexane fraction can inhibit microbial attachment and damage the extracellular matrix (EPS) of biofilms, disrupting cell and nutrient communication between microbes.

Biofilm Degradation Test

Biofilm degradation tests were carried out on biofilms that had been formed, with the following results:

Table 13. Results of Percentage Degradation of Biofilm N-hexane Fraction

Concentration (mg/mL)	Installment (%)
12,5	34.98 ± 1.99
6,25	33.47 ± 2.65
3,12	30.82 ± 2.32
(+) Control	90.88 ± 1.37

The highest percentage of degradation was found at a concentration of 12.5 mg/ml. EC50 value of biofilm degradation: mean EC50 = 48.97 ± 6.77 mg/ml. Compounds in the n-hexane fraction can degrade biofilms by removing EPS from existing biofilms.

Morphological Observation of S. aureus Bacteria SEM Method

SEM observations showed morphological differences between the control sample and the sample treated with the n-hexane fraction:

- Control sample: Biofilm is clearly visible around the surface of the bacteria, cell size 11.5 µm.
- Sample treatment: The shape of the cell changed from round to oval, the size shrank to 10.9 µm, the surface of the biofilm was reduced.

These morphological changes indicate damage to cell walls and membranes due to the activity of compounds in the n-hexane fraction, such as saponins, tannins, flavonoids, and terpenoids.

Observation of Ca²⁺ and K⁺ Metals by AAS Method

Observation Results of K⁺ Metal Ion Leakage Test

The results of the ion leakage test of *S. aureus* bacteria that had been treated with the N-Hexane fraction from black mulberry leaves with a concentration of 12.5% stated that there were K⁺ and Ca²⁺ ions that were read on the Atomic Absorption Spectrophotometer (AAS), K⁺ ions were read in the cell wall and Ca²⁺ ions were found in the cell membrane indicating that the bacterial cell wall was leaking and damaged.

The results of the analysis can be seen the absorbance value without treatment (negative control) and with N-Hexane fraction treatment, the absorbance value without treatment obtained a value of 21.00 mg/L while the absorbance results with N-Hexane treatment at a concentration of 12.5% obtained a value of 1.725 mg/L it can be concluded that the results of the ion leakage test or cell contents out in the N-Hexane fraction treatment there is a leakage of K⁺ metal ions of 1.725 mg/L. The addition of the N-Hexane Fraction can cause the release of metals from the bacteria, especially K⁺ and Ca²⁺ metals. K⁺ metal functions as a component of the cell wall of Gram-positive bacteria and for the function of ribosome unity.

Ca²⁺ Metal Ion Test Results

The results of the Ca²⁺ metal ion test using AAS showed that the bacterial suspension without treatment (negative control) obtained a Ca²⁺ ion leakage value of 2.13 mg/L, while the bacterial suspension with N-Hexane fraction treatment obtained a Ca²⁺ ion leakage value of 5.456 mg/L. The results of this study stated that there was an increase in the level of K⁺ metal ions outside the cell indicating damage to the permeability of the *S. aureus* bacterial cell membrane, while the increase in Ca²⁺ indicated damage to the bacterial cell wall (Harsini et al., 2018). The release of Ca²⁺ ions from the cell caused by interference from compounds that can disrupt the stability of the cell wall and cause bacteria to die (Syarifuddin et al., 2018). The results of this study stated that there was an increase in the level of K⁺ ions outside the cell indicating damage to the membrane permeability, while the increase in Ca²⁺ metal ions indicated damage to the cell wall (Sudarmi et al., 2017).

DISCUSSION

This study shows that the black mulberry leaf fraction (*Morus nigra* L.) has significant potential as an antibacterial agent and antibiofilm against *Staphylococcus aureus* ATCC 25923. The n-hexane fraction showed the highest antibacterial activity with an average inhibitory zone of 23.13 ± 1.80 mm at a concentration of 12.5%, and a Minimum Kill Concentration (KBM) of 12.5%. In the antibiofilm test, the n-hexane fraction inhibits the formation of biofilms with an IC₅₀ of 24.57 ± 2.72 mg/ml and degrades the biofilms that have been formed with EC₅₀ of 48.97 ± 6.77 mg/ml. SEM analysis confirmed the morphological changes of bacterial cells and the reduction of biofilms after treatment with n-hexane fraction. AAS observations showed increased leakage of K⁺ and Ca²⁺ ions, indicating damage to bacterial membranes and cell walls. These results show that the n-hexane fraction of black mulberry leaves has the potential as a natural alternative in overcoming *S. aureus* infection and biofilm-related antibiotic resistance.

CONCLUSIONS AND RECOMMENDATIONS

This study shows that the black mulberry leaf fraction (*Morus nigra* L.) has significant potential as an antibacterial agent and antibiofilm against *Staphylococcus aureus* ATCC 25923. The n-hexane fraction showed the highest antibacterial activity with an average inhibitory zone of 23.13 ± 1.80 mm at a concentration of 12.5%, and a Minimum Kill Concentration (KBM) of 12.5%. In the antibiofilm test, the n-hexane fraction inhibits the formation of biofilms with an IC₅₀ of 24.57 ± 2.72 mg/ml and degrades the biofilms that have been formed with EC₅₀ of 48.97 ± 6.77 mg/ml. SEM analysis confirmed the morphological changes of bacterial cells and the reduction of biofilms after treatment with n-hexane fraction. AAS observations showed increased leakage of K⁺ and Ca²⁺ ions, indicating damage to bacterial membranes and cell walls. These results show that the n-hexane fraction of black mulberry leaves has the potential as a natural alternative in overcoming *S. aureus* infection and biofilm-related antibiotic resistance.

ADVANCED RESEARCH

Further research development is recommended for more in-depth development regarding the analysis of the effect of black mulberry leaf effectiveness on biofilm formation and biofilm degradation by making black mulberry leaf samples with the subfraction method or analyzing the difference in fractionation with different concentration variants. Analyzing black mulberry leaves with different bacteria than those tested by researchers, the bacteria tested except for *S. aureus* ATCC 25923 bacteria with other bacteria, the aim of which is to determine whether there is a difference if done with different bacteria between mulberry leaf samples will be affected by different concentration variants and bacteria.

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