NASKAH PUBLIKASI

EKSPLORASI BIOAKTIVITAS EKSTRAK DAUN GELINGGANG (*Cassia alata* L.) TERHADAP BIOFILM *Staphylococcus aureus* FASE PERTENGAHAN, PEMATANGAN DAN ERADIKASI SERTA POTENSI ANTIOKSIDANNYA

EXPLORATION OF GELINGGANG LEAF EXTRACT (Cassia alata L.) BIOACTIVITY AGAINST Staphylococcus aureus BIOFILMS IN THE MID PHASE, MATURATION AND ERADICATION AND ITS ANTIOXIDANT POTENTIAL

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Naskah Publikasi

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Exploration of Gelinggang leaf extract (*Cassia alata* L.) bioactivity against *Staphylococcus aureus* biofilms in the mid phase, maturation and eradication and its antioxidant potential

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Abstract: *Staphylococcus aureus* is one of the dangerous biofilm-forming bacteria with the highest resistance. According to World Health Organization (WHO) data, the highest mortality rate occurs when infections are caused by microorganisms that are resistant to various drugs. The people of Kalimantan island have used gelinggang leaf (*Cassia alata* L.) as a medicine for skin diseases. This study aims to determine the bioactivity and antibacterial and antibiofilm effectiveness of gelinggang leaf extract against *S. aureus* bacteria in the mid-phase, maturation, and eructation activity, and to see its antioxidant potential. Phytochemical analysis was performed using qualitative phytochemical screening-tube methods. Antioxidant assay was performed using the DPPH method. Antibacterial testing uses agar diffusion method and antibiofilm testing uses microdilution method. The results of this study indicate that gelinggang (*Cassia alata* L.) leaf extract contains phenolic compounds, flavonoids, saponins, and tannins, and has strong antioxidant activity with an IC_{50} value of 23,119 µg/ml. Gelinggang (*Cassia alata* L) leaf extract has antibacterial activity against *S. aureus* and antibiofilm in the middle and maturation phases. The results also showed that gelinggang leaf extract was able to eradicate *S. aureus* biofilms above 50%. Therefore, gelinggang leaf extract has the potential to be developed as a new antibiofilm against *S. aureus*.

Keywords: Gelinggang plant, Secondary metabolites, antioxidant, antibiofilm, MIC50.

1. Introduction

According to World Health Organization (WHO) data, the highest mortality rate occurs when infections are caused by multidrug-resistant microorganisms. Antibacterial-resistant infections are estimated to cause 700,000 deaths annually worldwide, a number that is expected to increase to 10 million annually by 2050. Overall, 65% of all microbial infections and 80% of all chronic infections are biofilm-related, including osteomyelitis, infective endocarditis, wound infections, and implant-related infections, such as catheters and joint prostheses. Biofilm refers to a complex sessile community of microorganisms attached to a biotic surface or tissue and incorporated into a self-produced protective matrix composed of extracellular polymeric substances (EPS)[1]. The biofilm matrix surrounds the bacterial cells and makes them resistant to adverse conditions, including antibacterial treatments. It is estimated that bacteria in biofilms become 1000 times more resistant to antibacterial agents than their planktonic counterparts[2]

Staphylococcus aureus is one of the most resistant biofilm-forming harmful bacteria. The bacteria can be found in water, soil, air, or skin. *Staphylococcus aureus* bacteria are the most common cause of hospital-acquired infections and have an extensive list of virulence factors. *Staphylococcus aureus* can express up to 24 cell wall-attachable proteins, which can promote biofilm formation, and impair the neutralization of the innate immune system[3].

The relationship between *Staphylococcus aureus* bacteria and biofilms is that *Staphylococcus aureus* is one of the biofilm-forming harmful bacteria with the highest resistance. The bacteria can be found in water, soil, air, or skin. *Staphylococcus aureus* bacteria are the most common cause of hospital-acquired infections and have an extensive list of virulence factors. *Staphylococcus aureus* can express up to 24 cell wall-anchored proteins, which can promote biofilm formation, and impair neutralization of the innate immune system[3].

The medicinal periwinkle plant (*Cassia alata* L.), native to tropical

America, will be the focus of this study. This plant can be found everywhere from sea level to 1,400m above sea level. The periwinkle plant, or *Cassia alata* L., has been used for centuries as a laxative, treatment for skin diseases, and even malaria prevention [4]

To date, research from the gelinggang plant (*Cassia alata* L) against S.aureus biofilm and its antioxidant potential has not been reported, therefore this study will explore the antibiofilm and antioxidant potential of gelinggang leaves (*Cassia alata* L) taken on the island of Kalimantan.

2. Materials and Methods

The tools used include rotary evaporator, analytical balance, filter, test tube, glass beaker, micropipette, volumetric flask, vortex, water bath and UV-VIS spectrophotometer, autoclave, buchner funnel, magnetic stirrer, microplate 96 well flat bottom, oven, blender, incubator, filter paper, destilator flask, destilator heater, microplate reader, ose needle, bunsen, analytical balance, blue and yellow tip, micropipette. The materials used were gelinggang plants obtained from the forests of East Kalimantan, DPPH, methanol PA, 96% ethanol solvent, 2N HCL, concentrated HCL, Dragendorf reagent, 1% and 10% FeCI3 reagent, Mg powder, warm water, and Liebermann-Burchard reagent. Burchard, *Staphylococcus aureus bacteria*, 96% ethanol, NA medium, 1% crystal violet, sterile distilled water, chloramphenicol.

Sample data collection using gelinggang (*Cassia alata* L) plants obtained from the seas of East Kalimantan Mariyam River area Anggana District and using *Staphylococcus aureus* bacteria. Determination was carried out in the laboratory of the Faculty of Forestry, Mulawarman University.

Simplisia was extracted using a maceration method at room temperature and 96% ethanol solvent. There are two to three macerations. The results of maceration were then concentrated using a rotary evaporator, the solvent was then evaporated in a water bath to produce a thick extract, the weight of the resulting extract was weighed, and finally the extract yield was calculated.

2.1 Secondary Metabolite Testing Alkaloid Test

Extracts and fractions (2 mL) were put into test tubes. Then added with HCl 2N as much as 1 ml and then dripped with 3 drops of Dragendorff reagent, a positive test is indicated by the formation of an orange precipitate.

Phenolic test

Extracts and fractions (1 mL) are put into a test tube, then 3 drops of 1% FeCl3 reagent are added, A positive test is indicated by the formation of a black color.

Flavonoid Test

Extracts and fractions (2 mL). Put into a test tube, then added 0.05 mg of Mg powder and 1 mL of concentrated HCl and shaken, Positive test is indicated by the formation of red, yellow or orange color.

Saponin Test

Extracts and fractions (2-3 mL) are put into a test tube, then added with 10 mL of warm water, and then shaken vigorously for 10 seconds. A positive test is indicated by the formation of a stable froth as high as 1-10 cm for 10 minutes.

Steroid and Terpenoid test

Extracts and fractions (2 mL) are put into a test tube, then 1-3 drops of Lieberman Burchard reagent are added and the solution is shaken gently, A positive test for steroids is indicated by the formation of a blue or green color, while terpenoids give a brownish red color.

Tannin Test

Extracts and fractions (1 ml), put into a test tube, then added 1-3 drops of 10% FeCl3 solution, Positive test is indicated by the formation of greenish-black color.

Anthocyanin Test

Extracts and fractions (2 ml) were put into a test tube, then added NaOH 2N drop by drop. If the red color turns blue-green and fades

slowly, it indicates the presence of anthocyanins.

2.2 Preparation of bacterial subcultures

Standard biofilm-forming *Staphylococcus aureus* was cultured in Brain-heart infusion broth (BHI) medium and incubated at 37ºC for 72 h. The optical densities (OD600) of microbial cultures were adjusted to 0.1 (equal to the 0.5 Mc Farland standard ~1.5 x 108 CFU/ml), and subsequently diluted in fresh medium to OD600 0.01 for each microbial species.

2.3 Antibacterial activity test

The antibacterial activity test was carried out by diffusion method at extract concentrations of 0.125%, 0.25%, 0.5%, and 1% to determine bacterial activity by measuring the inhibition zone around the paper disk. Testing for antibacterial activity using the agar diffusion method. This diffusion method uses a 6 mm paper disk that has an absorbency of 50 µl. Observations can be made after 24 hours by observing the clear zone formed around the paper disk. The clear zone formed around the paper disk indicates that the gelinggang and kelubut plant extracts have antibacterial activity. The diameter of the inhibition zone was calculated using a caliper with millimeter units.

2.4 Biofilm Inhibition Activity Test of *Staphylococcus aureus* bacteria

To assess the effect of the test isolates on S. aureus mono-species biofilm formation, polystyrene flat bottom 96-well microtiter plates were used. (Pierce dkk., 2010). A total of 100 µL of S. aureus suspension (107 CFU/mL) was added to each well of the microtiter plate and incubated at ±37°C for 90 minutes for the biofilm attachment phase. After the incubation period, the plate was washed using 150 µL of sterile distilled water three times to remove non-adherent cells. A total of 100 µL of media containing pure isolates with concentration series (1%, 0.5%, 0.25%, 0.125% b/v), was added to each washed well. As media control, media without microbial growth was used, and microbial suspension was used as

a negative control. As a positive control, a microbial suspension treated with 1% b/v chloramphenicol was used. The plate was then incubated at 37°C for 24 hours for mid-phase biofilm formation and 48 hours for maturation phase biofilm formation. Next, the plate was washed using distilled water three times and dried at room temperature for 5 minutes to remove the remaining water. A total of 125 µL of 1% crystal violet solution was added to each well to color the biofilm that had formed, both dead cells and living cells which are also constituent components of the biofilm, then incubated at room temperature. After incubation at room temperature, the microplate was washed under running water three times to remove the remaining crystal violet, and 200 µL of 96% ethanol was added to each well to dissolve the biofilm formed. Optical Density (OD) readings were taken with a microplate reader at a wavelength of 620 nm. Data obtained from biofilm inhibition analysis in the form of OD values of each concentration of test compounds and controls without test compounds (growth control) obtained from reading with a microplate reader.

2.5 Antioxidant Test

Prepared 6 10 ml volumetric flasks that have been wrapped in aluminum foil and have been given each concentration label, namely 5, 10, 20, 40, 100, and 200, then put 3 ml of DPPH solution into each volumetric flask and pipetted the sample stock solution according to their respective concentrations, namely 5 µl (concentration 5), 10 µl (concentration 10), 20 µl (concentration 20), 40 µl (concentration 40), 100 µl (concentration 100), and 200 µl (concentration 200), then add methanol PA until the limit mark, after that shake until all solutions are evenly mixed and let stand for 30 minutes in a dark place, then read the absorbance on a spectrophotometric device at a wavelength of 517 nm twice, for the blank used methanol PA.

2.6 Data analysis technique

Secondary metabolite testing is done directly by testing alkaloid, phenolic, flavonoid, saponin, steroid and terpenoid compounds, tannins, and also anthocyanins.

In this antioxidant test using the DPPH method with absorbance measurements using UV-VIS spectrophotometry, to calculate the IC_{50} value using the formula, namely:

$$\% \text{ Inhibisi} = \frac{Absorbansi\ kontrol - absorbansi\ sampel}{absorbansi\ kontrol}\ x\ 100\%$$

Perhitungan nilai OD hasil analisis menggunakan rumus yaitu:

$$\%\text{penghambatan} = \frac{(ODrenata\ kontrol\ negatif - ODrenata\ sampel\ uji)}{OD\ rerata\ kontrol\ negatif}\ x\ 100$$

The sample level that can inhibit 50% of biofilm formation is considered the Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$).

3. Results and Discussion

3.1 Extraction

In the extraction process carried out by the maceration method using 96% ethanol solvent, from the wet weight of the gelinggang plant, namely 500 grams, 150 grams of simplisia powder was also obtained, the obtained simplisia powder was then extracted and obtained a thick extract of 24.91 grams with a percent extract yield of 16.60%, the results of the yield calculation can be seen in table 1.

Simplified weight	Weight of condensed extract	Yield (%)
150 gr	24,91 gr	16,60%

Table 1. Extract Yield Calculation Results

The preparation of gelinggang plant simplisia begins with separating impurities from the leaves and stems, then weighing the wet weight of 500 grams after which the leaves are washed

thoroughly with the aim of separating foreign particles. After obtaining simplisia from the gelinggang plant, the gelinggang simplisia was blended until smooth and then weighed using an analytical balance of 150 grams, the powdered simplisia that had been mashed and weighed was soaked in water to dissolve the metabolite compounds contained in the gelinggang plant. The results of the soak were filtered with a white cloth and then filtered again with filter paper, the pulp obtained was macerated three times. The next stage is the concentration of the remaining solvent with a rotary evaporator at a temperature of 60° C so that the content of gelinggang plant compounds is not damaged, after which the extract that has been rotary put into a heat-resistant glass bowl, then concentrated again on a water bath until the desired thick extract is obtained, the percentage yield of the weight of the thick extract can be calculated with the equation below.

$$rendemen = \frac{\text{bobot ekstrak}}{\text{bobot simplisia}}\ x\ 100\%$$

The maceration process was chosen to extract Gelinggang leaves because it is suitable for leaf materials that cannot sustain high temperatures and also destroys oil if heated too much, and because the equipment used is very simple and the procedure is not difficult [11].

The best solubility of maceration extraction requires organic solvents.Ethanol solvent of 96% purity was used.Since Yuswi's study (2017) found that the best treatment test results were obtained with 96% ethanol solvent type treatment, this solvent was used for this extraction [11].

3.2 Secondary Metabolite Test

In the extraction process carried out by the maceration method using 96% ethanol solvent, from the wet weight of the gelinggang plant, which is 500 grams, 150 grams of simplisia powder is also

obtained, the obtained simplisia powder is then extracted and obtained a thick extract of 24.91 grams with a percent extract yield of 16.60%, the results of the yield calculation can be seen in table 1.

Compound	Solvent	Indicator	Results
Alkaloids	Dragendorff Pre Action	Orange discoloration	(-)
Phenolic	FeCl 5%	Turns black in color	(+)
Flavonoids	HCL and magnesium powder	Turns red in color	(+)
Saponins	Warm distilled water	Stable foam	(+)
Steroids	Lieberman burchard	Turns blue-green in color.	(-)
Terpenoids	Lieberman burchard	Turns brownish-red in color	(-)
Tannins	FeCl 1%	Turns blackish green in color.	(+)
Anthocyanins	NaOh 2N	It turns blue-green in color and does not fade slowly.	(-)

Table 2. Secondary Metabolite Assay Results ((+) Indicates the presence of the compound. (-) Indicates the absence of the compound.)

According to the results obtained, the gelinggang plant positively contains phenolic compounds, flavonoids, saponins, and tannins.

a. Based on the research of Mawaddah et al., (2020) stated that gelinggang plants contain alkaloid compounds, but in this study it is not in line because there is no change in color to orange.
b. In the phenolic test, the color changes to black so that it can be said to be in line with the research of Oktavia et al., (2021) which states that phenolic compounds can be seen when there is a change in color to black.
c. This flavonoid test is in line with research by Oktavia et al., (2021), which shows that gelinggang includes flavonoid compounds when identified there is a color change to orange.
d. In this saponin compound test research shows that gelinggang contains saponin compounds, this statement is in accordance with research conducted by Fajri et al., in 2018 which states that gelinggang contains saponins which are characterized by the

presence of stable froth as high as 1-10 cm for 10 minutes.

e. In this study, the test of steroid and terpenoid compounds showed that gelinggang did not contain steroid and terpenoid compounds, this statement is not in accordance with research conducted by Ulfasari, S. in 2021 which states that gelinggang plants contain steroid and terpenoid compounds.

f. In this tannin compound test research states that gelinggang plants contain tannin compounds, this statement is in accordance with research conducted by Noviyanty, Y. in 2020, which is marked by a change in color to greenish black.

g. In this anthocyanin compound test research states that gelinggang plants do not contain anthocyanin compounds, this statement is not in accordance with research conducted by Noviyanty, Y. in 2020, which is characterized by no change in color from red to blue green and fades slowly.

3.3 Antioxidant Test

Antioxidant testing of gelinggang plants using the DPPH method with Uv-Vis spectrophotometric devices with a wavelength of 517 nm, the results of antioxidant testing can be seen based on the linear regression equation in Figure 1 below.

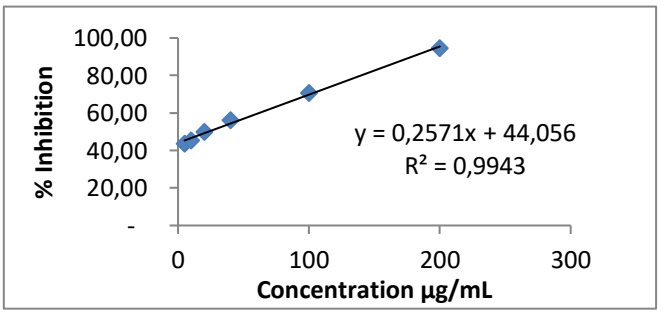

Figure 1.Concentration relationship curve of algae sample against % inhibition

In this study, antioxidant testing of gelinggang plants was carried out using the DPPH method with 6 concentrations, namely 5 µl, 10 µl, 20 µl, 40 µl, 100 µl, and 200 µl, the difference in concentration is with the intention of comparing how much antioxidant activity in each sample.

The DPPH method is used because it is easy, fast, and sensitive, and requires only a few samples (the decay of the purple hue of DPPH to yellow is assessed at a wavelength of 517 nm). The DPPH method is also effective because antioxidant chemicals can provide reactions through a hydrogen atom donation mechanism, which causes the purple DPPH color to degrade and turn yellow [34].

The IC_{50} value of the gelinggang plant was calculated using a linear regression equation Curve relationship of gelinggang sample concentration to % inhibition with the equation formula y=ax + b, gelinggang sample concentration marked as x-axis and % inhibition marked as Y-axis.

Based on research conducted by Safitri & Rohama, in 2020, which states that gelinggang plants have weak antioxidant compounds with an IC_{50} of 185.037 µg/mL, so that specifically a compound is said to be a very strong antioxidant if the IC_{50} value is less than 50 ppm (IC_{50}< 50 ppm), strong (with an IC_{50} value of 50 ppm-100 ppm), moderate (IC_{50} value of 100 ppm-150 ppm), weak (150 ppm < IC_{50}< 200 ppm), and very weak (IC_{50} value> 200 ppm). However, this study is not in line because it obtained the % inhibition result with an IC_{50} value of 23,119 µg/mL in the linear regression equation of the gelinggang sample concentration relationship curve, which means that there is a very strong antioxidant content in the gelinggang plant[27].

The mechanism of action of flavonoid compounds is to provide hydrogen atoms quickly to free radical compounds so that the effects of free radicals can be stabilized[29].

Some factors that can cause errors in antioxidant testing are DPPH which is a compound that is very sensitive to light, so that if exposed to light it will damage the DPPH compound, DPPH is also very sensitive to temperature so that to store DPPH should be at cold temperatures, because hot temperatures will damage the

DPPH compound [16].

3.4 Bacterial Activity Test

In this antibacterial test using a positive control serves as a control of the test substance. The positive control uses the broad-spectrum drug chloramphenicol. Then the negative control uses aquadest which serves as a comparison.

Concentration	**Inhibition zone diameter (mm)**	**Bacterial response**
K (-)	0	None
K (+)	19,5 mm	Strong
1 %	11,23 mm	Strong
0,5 %	9,5 mm	Medium
0,25 %	4,25 mm	Weak
0,125 %	4 mm	Weak

Table 3. Observation of the inhibition of Gelinggang extract on *S. aureus* bacteria. (K (-) = Aquadest,K (+) = Chloramphenicol)

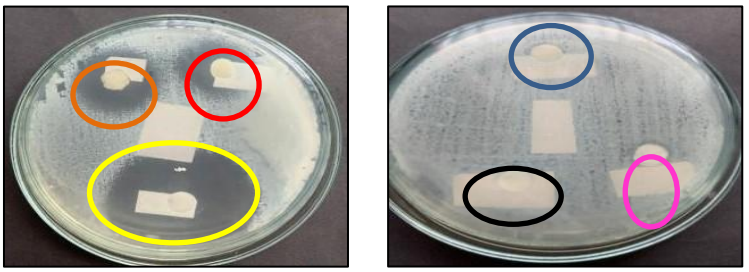

Figure 2. Observation result of inhibition of gelinggang extract on *S. aureus* bacteria

= Drug Control

= Concentration 1 %

= Concentration 0,5 %

= Concentration 0,25 %

= Concentration 0,125 %

= Aquadest Control

3.5 Mid-Phase Antibiofilm Test Results (24 Hours)

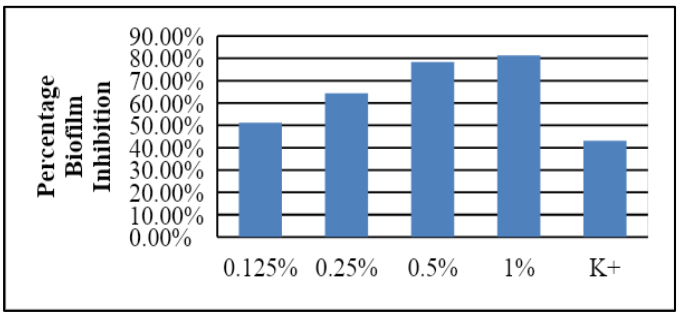

Figure 3. Percentage of mid-Phase Biofilm Inhibition (K+ = Positive Control: Chloramphenicol Antibiotics)

3.6 Maturation Phase Antibiofilm Test Results (48 Hours)

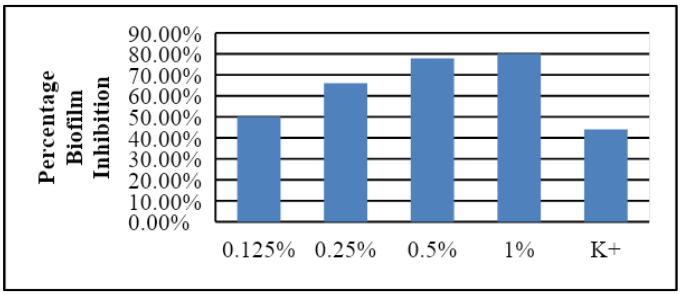

Figure 4. Percentage of Biofilm Inhibition maturation Phase

Staphylococcus aureus is a Gram-positive bacterium, which is the most common cause of human infection (Hamzah Hasyrul., 2021). Gram-positive bacteria called *Streptococcus pyogene* are commonly found in the respiratory tract and skin, although they don't usually result in any kind of illness.

In the clear zone diameter, there is an increase from low concentration to high concentration, this is because the higher the concentration used, the higher the content or amount of extract used.

Table 3 shows that at all doses tested, gelinggang extract has an inhibitory effect on *Staphylococcus aureus*. In both the extract and bacteria, a concentration of 1% produced the highest antibacterial action.The higher the concentration of the extract, the stronger its inhibitory ability against germs, as stated by Lovista (2010).

When compared to the chloramphenicol-treated positive control, the zone of inhibition containing *Staphylococcus aureus* bacteria was still smaller.

According to the results of this study, it states that this research is in accordance with what was done by Oktavia, in 2021 which states that the bacteria *S.aureus* with gelinggang plants have a strong inhibitory power because they have an inhibitory diameter that is included in the strong activity range, namely 10-20 mm.

3.7 Antibiofilm Test

From the results of gelinggang extract research with *Staphylococcus aureus* bacteria, it was found that 1% concentration had a lower OD value than the positive control. The 0.125% concentration had the highest OD value, indicating the greatest biofilm formation. Because kelubut extract contains more active chemicals at lower concentrations.

The results of the biofilm inhibition test conducted with *Staphylococcus aureus* showed that increasing the concentration of gelinggang extract reduced biofilm formation. The OD values in the experimental group showed this trend as the concentration of the extract increased. The highest inhibitory activity of gelinggang extract with Staphylococcus aureus bacteria in the 24-hour phase was at a concentration of 1% with a percentage of 81.391% then the lowest inhibitory activity was at a concentration of 0.125% with a percentage of inhibition of 51.16%. The highest inhibitory activity of gelinggang extract with Staphylococcus aureus bacteria in the 48-hour phase was at a concentration of 1% with a percentage of 80.162% and the lowest inhibitory activity was at a concentration of 0.125% with a percentage inhibition of 50.096%. In the results of the biofilm inhibition test on *Staphylococcus aureus* bacteria in gelinggang extract, $MBIC_{50}$ is found at a concentration of 0.125%, which is 51.160% in the middle phase and 50.096% in the maturation phase.*Staphylococcus aureus* bacteria are the most

common cause of hospital-acquired infections and have an extensive list of virulence factors, infections sometimes occur on warm, moist skin or when skin is exposed due to eczema, surgical wounds, and intravenous devices. Infections with Staphylococcus aureus originate from contamination of wounds such as post-surgery and may result in endocarditis, acute hematogenous osteomyelitis, meningitis, or pulmonary infections[6].

Our results show that gelinggang leaf extract can inhibit biofilm growth, this can be seen from the inhibitory activity given in the maturation phase by 70 percent. The process of biofilm inhibition by gelinggang extract is due to the presence of flavonoid compounds where these flavonoids work to destroy the biofilm eps matrix, resulting in cell leakage until lysis occurs, this statement is reinforced by Manner et al, year (2013) stating that flavonoid compounds can inhibit the growth of biofilm bacteria, namely *Staphylococcus aureus*.

4. Conclusions

Gelinggang leaf extract contains phenolic compounds, flavonoids, saponins, and tannins and has antioxidant activity with a very strong category. The results also show that gelinggang leaf extract has antibacterial and antibiofilm inhibitory activities against *S. aureus*. The results also provide evidence that gelinggang leaf extract is able to eradicate *S. aureus* biofilm. Therefore, gelinggang leaf extract has the potential to be developed as a new antibiofilm against *S. aureus*.

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LAMPIRAN

Hasil Uji Turnitin

NP 1 : Exploration of Gelinggang leaf extract (Cassia alata L.) bioactivity against Staphylococcus aureus biofilms in the mid phase, maturation and eradication and its antioxidant potential

by Oktavia Triwanti

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