

# Antibiofilm Activity of *Aloe barbadensis* Miller Extract against *Staphylococcus aureus*

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**Keywords:** Biofilm, *Staphylococcus aureus*, *Aloe barbadensis*, microtiter plate, antibiofilm activity.


**Abstract:** Background: *Aloe barbadensis* Miller is a type of plant that can potentially inhibit the growth of bacteria causing infections. Improper use of antibiotics can result in the occurrence of bacterial resistance caused by the formation of biofilms on a living creature. The goal of this study was to investigate the antibiofilm activity of *Aloe barbadensis* Miller against the biofilm produced by *Staphylococcus aureus*. This study is classified as an experimental in vitro with the post-test only control group design. The *Aloe barbadensis* Miller rind macerated with methanol solvent. The extract was divided into concentrations of 20%, 10%, 5%, 2.5%, 1.25% and 0.63%, tested against biofilm formation by *Staphylococcus aureus* ATCC 25923. The antibiofilm activity was tested by microtiter plate biofilm assay method with 3 replications on a 96 well round-bottom microplate. Then observed by calculating the value of optical density using a microplate reader ( $\lambda = 620$  nm). Extract concentrations 20%, 10%, 5%, 2.5%, 1.25% and 0.63% showed a minimum percentage of inhibition of biofilm concentration were -30.3%; 10.9%; 10.5%; 45.9%; 19.3% and 16.3% respectively. Statistical analysis using One-Way ANOVA ( $p = 0.008$ ) followed by post-hoc LSD which showed a concentration of 2.5% had no significant difference ( $p > 0.05$ ) with positive control using ciprofloxacin. Although the methanol extract of *Aloe barbadensis* Miller rind showed inhibitory activity against *S. aureus* biofilms was equivalent to positive control but had a Minimum Biofilm Inhibition Concentration (MBIC50) percentage <50%.

## 1 INTRODUCTION

*Staphylococcus aureus* is one of the most common causes of bacterial infection in humans worldwide (Singh et al., 2017; Chen et al., 2014; Tong, et al., 2015). *S. aureus* can produce an extracellular polymer matrix consisting of polysaccharides, air, nucleic acids, proteins, and extracellular DNA (Archer et al., 2011). This matrix ensures the survival of the biofilm colony and protects it against the phagocytic activity of macrophages, the host immune system, temperature, and pH fluctuations (Leseigneur et al., 2020). Antibiotic therapy, in general, will only kill planktonic bacterial cells, while bacteria that are tightly arranged in biofilm will remain alive so that they can cause chronic infections that resistant to antibiotics (Tong et al., 2015; Zaman et al., 2017). The Indonesian Ministry of Health (2015) states that from the 2013 WHO data, the death rate due to

resistance is around 700 thousand people per year (Kemenkes, 2015). It's estimated, by 2050 the death rate could increase to 10 million per year. The existence of biofilms has the opportunity to develop drugs by utilizing the same bioactive plants (Abraham et al., 2012). Also, Indonesia is mega biodiversity which is rich in medicinal plants that have the potential to be developed (Kusmana et al., 2015). *Aloe vera* (*Aloe barbadensis* Miller), is currently processed as food, drink, and medicine. However, only part of the gel is used, while the skin of the leaves becomes waste (Aryanti et al., 2013). The active ingredients that have been identified in the extract of the bark of the leaves of *Aloe barbadensis* Miller include saponins, sterols, acemannan, and anthraquinones which are toxic to bacterial cells (Marimuthu et al., 2012; Benzidia et al., 2018; Devaraj et al., 2011). Through this research, hopefully that the incidence of *S. aureus* resistance

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can be overcome with antibiofilm as an alternative therapy that can reduce the prevalence of infection. The purpose of this study was to determine the antibiofilm activity of the methanol extract of the Aloe barbadensis Miller leaf bark against the biofilm of Staphylococcus aureus ATCC 25923.

## 2 MATERIALS AND METHODS

### 2.1 Research Design

This type of research is experimental in vitro study with a post-test only control group design. The effect of Aloe barbadensis Miller in inhibiting biofilm of S. aureus ATCC 25923 using the Microtiter Plate Biofilm Assay method (14). S. aureus ATCC 25923 was bred from the Yogyakarta Health Center.

### 2.2 Time and Place of Research

The process of determining aloe vera plants is carried out at the Plant Systematics Laboratory of the Faculty of Biology, Gadjah Mada University. The plant extraction process is carried out at the Integrated Laboratory of the Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia, in the division of Pharmaceutical Biology. Testing the potential of Aloe barbadensis Miller leaf bark extract against Staphylococcus aureus ATCC 25923 biofilm was conducted at the Microbiology Laboratory of the Faculty of Medicine, Islamic University of Indonesia. The research was conducted in June-July 2020.

### 2.3 Research Tools and Materials

The tools used in this study include a round-bottom microplate, microplate reader, analytical scale, refrigerator, oven, autoclave, incubator, Laminar Air Flow (LAF), bunsen, loop wire, beaker glass, Erlenmeyer, petri dish, test tube, test tube rack, cotton, micropipette, micro tip, dropper pipette, mortar, pestle, vial, microscope, Staphylococcus aureus ATCC 25923 bacteria, sterile distilled water, physiological NaCl, Gram stain, McFarland solution, nutrient agar (NA) media, nutrient broth (NB) media, catalase, and coagulase reagents.

### 2.4 Time and Place of Research

The process of determining aloe vera plants is carried out at the Plant Systematics Laboratory of the Faculty of Biology, Gadjah Mada University. The plant extraction process is carried out at the Integrated

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### 2.5 Preparation of Aloe Barbadensis Miller Leaf Bark Extract and Secondary Metabolites Test.

Aloe vera leaves are washed with tap water and then peeled to separate the gel and the skin of the leaves. The extraction process starts from chopping the sample, drying the sample in a dry cabinet, pollinating the alar miller, macerated dry Simplicia with methanol solvent, filtering, evaporation, and packaging then testing phytochemicals.

### 2.6 Purification and Characterization of Test Bacteria

Purification and characterization of tested bacteria by inoculating bacteria on NA media with streak plate techniques, gram staining, catalase test, and catalase test on glass objects (Kaiser et al., 2016).

### 2.7 Determination of the Minimum Inhibitory Level (MIL) and Minimum Killing Level (MKL).

The dilution series of extract concentration tested were 20%, 10%, 5%, 2.5%, 1.25% and 0.63% compared to positive control (Ciprofloxacin 0.00125 mg/ml), negative control (bacterial suspension), and media control which was carried out 3 times replication (Bazargani et al., 2017)

### 2.8 The Biofilm Formation Test of Staphylococcus aureus ATCC 25923

Two bacteria were used in the biofilm formation test, S. aureus ATCC 25923 and S. epidermidis non-biofilm as a negative control, tested 3 times. The microplate was washed 3 times with sterile phosphate buffer saline (PBS) (Teapasian et al., 2016). Then stained with 0.1% crystal violet and then rinsed 3-4 times with sterile distilled water (Lestari et al., 2017). Then add 275  $\mu$ L of 33% acetic acid to each well to dissolve the crystal violet and incubate for 10-15

minutes. Then OD (Optical Density) was measured with a microplate reader with a wavelength of 620 nm. OD cut off is calculated by the formula:

$$\text{OD cut off} = \text{O average negative control} + (3 \times \text{standard deviation OD negative control})$$

(Singh et al., 2017)

## 2.9 The Antibiofilm Performance Test

Antibiofilm testing was done using the microdilution method with a series of dilutions using a 96 well round-bottom microplate. The group used was the same as the test group in the antibacterial test but incubated for 2 days. Washing was done using the same as in the biofilm formation test. Then entered into the formula MBIC50 [Inhibitory% =  $\{((\text{OD-negative control test group}) / (\text{OD negative control})) \times 100\}$ ] (Singh et al., 2017).

## 2.10 Data Analysis Method

The data obtained were analyzed using SPSS. The inhibition of *S. aureus* ATCC 25923 biofilm formation was obtained from the Optical Density (OD) value measured using a microplate reader. The data were analyzed using Shapiro Wilk and Levene's test to determine the distribution and homogeneity of the data. Data that is normally distributed ( $p > 0.05$ ) is followed by the One-Way ANOVA test with testing criteria based on probability, namely  $H_0$  is accepted test for bacteria each showed positive results indicated by the presence of gas bubbles ( $O_2$ ) or foam and the formation of clots / deposits Figure 2(19)(20).

if  $p \text{ value} > 0.05$  and  $H_0$  is rejected if  $p\text{-value} < 0.05$ . Then the post hoc test of Least Significant Difference (LSD) was carried out to determine the MBIC50 (Minimum Biofilm Inhibitory Concentration 50%).

## 2.11 Research Ethics

This research received permission from the Ethics Committee for Medical and Health Research, Faculty of Medicine, Islamic University of Indonesia with number 4 / Ka.Kom.Et / 70 / KE / XII / 2019.

## 3 RESULTS

Based on the results of plant determination, it was proven that the plant used in this study was Aloe vera (L.) Burm.f. with the synonym Aloe barbadensis Miller. Then from 900 grams of fresh material, 6.1223 grams of extract were obtained. The extract was tested for secondary metabolites and it was found that it contains active compounds of flavonoids, alkaloids, saponins, and polyphenols. The results of purification and characterization of *S. aureus* ATCC 25923 bacteria on nutrient media to obtain the results of bacterial colonies that grow yellowish white. Gram staining obtained the test bacteria showing a purple colour, cocci-shaped, single or clustered irregularly like grapes and without spores Figure 1. The catalase and coagulase

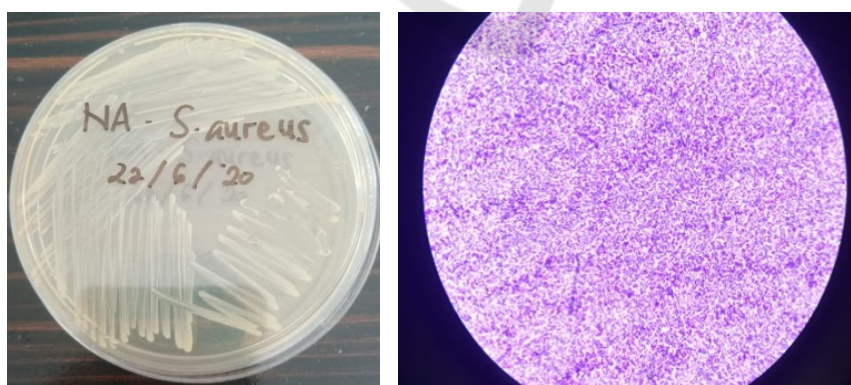


Figure 1. Purification and characterization of *S. aureus* bacteria on NA media (left) and microscopic appearance at 100x magnification (right).

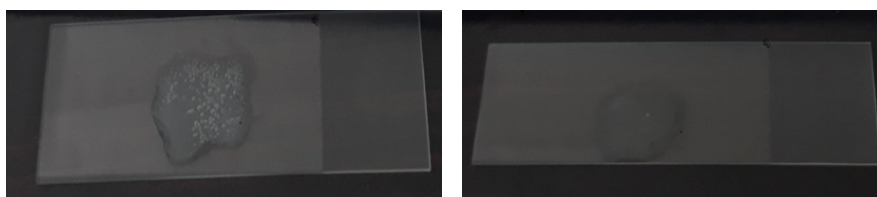


Figure 2. Positive catalase test results, there are air bubbles (left); The coagulase test results are positive, there is clumping (right).

The results of the antibacterial test on NA media with Petri dish 1 to 4 were clear and no visible colonies were growing. Meanwhile, in Petri dish 5 and 6, the growth of *S. aureus* was shown Figure 3.

The results of the *S. aureus* biofilm formation test were formed purple biofilm rings on the good walls. The absorbance / OD values of *S. aureus* and *S. epidermidis* non-biofilm formation tests were obtained in Table 1 and the antibiofilm test in Table 2.

The absorbance value was analyzed using statistical tests. The results of the distribution normality test using the Shapiro-Wilk test, obtained a p value > 0.05 in each test solution group, meaning that the data were normally distributed. Levene's test obtained a p-value < 0.05 in each test solution group, meaning that the data did not have the same variance.

Obtained normal data distribution and variance which are not the same. One-Way ANOVA test obtained a p-value of 0.008 which indicates that there is a significant difference in the test solution group. In the post-hoc LSD test (Table 3) it was found that some groups showed significant differences with p-value < 0.05 (significant result) at a concentration of 20% with a concentration of 2.5%, a concentration of 20% with 1.25%, a concentration of 20% with the positive control, 20% concentration with media control, 10% concentration with media control, 5% concentration with media control, 1.25% concentration with media control, 0.63% concentration with media control, negative control with the positive control, control media with a concentration of 5% and control media with the negative control.

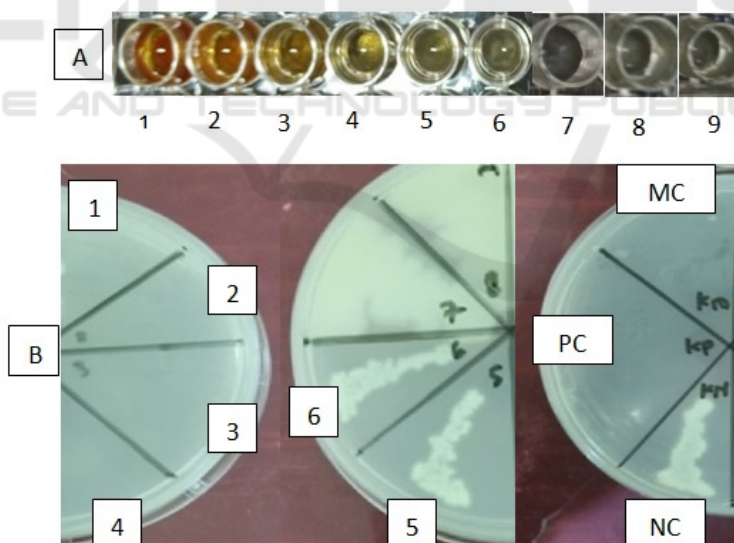


Figure 3. Antibacterial test result in A) microplate and B) nutrient agar.

Information

- |                      |  |
|----------------------|--|
| 1. 20% Concentrate   | 6. 0,625% Concentrate                      |
| 2. 10% Concentrate   | 7. Positive Control (Ciprofloxacin)        |
| 3. 5% Concentrate    | 8. Negative Control (bacterial suspension) |
| 4. 2,5% Concentrate  | 9. Media Control                           |
| 5. 1,25% Concentrate |  |

Table 1. Biofilm Inhibition Optical Density Staphylococcus aureus ATCC 25923

Treatment	Absorbance			Average	% Inhibition
	Replication 1	Replication 2	Replication 3		
Extract 20%	0.5495	0.6840	1.6010	0.9448	-30.35
Extract 10%	0.6053	0.5537	0.7781	0.6457	10.92
Extract 5%	0.7637	0.6089	0.5734	0.6487	10.51
Extract 2.5%	0.4006	0.3621	0.4122	0.3916	45.97
Extract 1.25%	0.4969	0.5847	0.6720	0.5845	19.36
Extract 0.625%	0.5231	0.7644	0.5318	0.6064	16.33
Positive Control	0.2443	0.2894	0.3663	0.30	58.61
Negative Control	0.6326	0.8336	0.7083	0.7248	
Media Control	0.1214	0.1721	0.1502	0.1479	

Table 2. Post-hoc analysis result LSD type

	E20%	E10%	E5%	E2.5%	E1.25%	E0.625%	KP	KN	KM
E20%	-	0.097	0.100	<b>0.005</b>	<b>0.049</b>	0.063	0.001	0.214	<b>0.000</b>
E10%	0.097	-	0.986	0.154	0.724	0.821	0.058	0.648	<b>0.009</b>
E5%	0.100	0.198	-	0.149	0.712	0.807	0.056	0.661	<b>0.009</b>
E2.5%	<b>0.005</b>	0.154	0.149	-	0.273	0.224	0.598	0.067	0.170
E1.25%	<b>0.049</b>	0.724	0.712	0.273	-	0.899	0.113	0.422	<b>0.020</b>
E0.625%	0.063	0.821	0.807	0.224	0.899	-	0.089	0.497	<b>0.015</b>
KP	<b>0.001</b>	0.058	0.056	0.598	0.113	0.089	-	<b>0.023</b>	0.385
KN	0.214	0.648	0.661	0.067	0.422	0.497	0.023	-	<b>0.003</b>
KM	<b>0.000</b>	<b>0.009</b>	<b>0.009</b>	0.170	<b>0.020</b>	<b>0.015</b>	0.385	<b>0.003</b>	-

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Information: E = extract concentration; KP = Positive Control; KN = Negative Control; KM = Media Control.

## 4 DISCUSSION

Table 1 shows the ODcut value calculated from the negative control (*S. epidermidis* non-biofilm) of 0.1341. The average *S. aureus* biofilm with a value of 0.3991 is a value that is between 2xODcut and 4xODcut. So it can be concluded that the *S. aureus* bacteria used in this study were moderate biofilm-former (Singh et al., 2020).

Based on the microplate reader result in Table 2. Higher OD score indicates the increasing survived biofilm microorganism amount (Locke et al., 2012). From the percentage inhibition formula, it was found that the higher the absorbance value / OD of the test solution, the lower the percentage value of inhibition (inversely proportional). The absorbance/OD value of Aloe vera leaf extract which was the best in inhibiting the growth of *S. aureus* biofilm was produced at a concentration of 2.5%, namely 0.3918 and had an inhibition percentage of 45.9% (Teaupaisan et al., 2017). When compared with a positive control containing Ciprofloxacin and bacterial suspension, it had an absorbance / OD value of 0.3000 and an inhibition percentage of 58.6%. This shows that the

percentage of inhibition in the positive control is higher than the series test solution for the dilution of the extract concentration. The percentage value of inhibition by the series dilution test solution with the concentration of 2.5% extract did not reach the MBIC50 requirement, called the minimum inhibitory concentration of biofilm of 50% (Pirbalouti et al, 2010; Pratiwi et al., 2015).

The results of statistical analysis, the post hoc LSD test showed that the extract concentration of 2.5% had the best value based on the percentage of MBIC50 compared to the concentration of other extracts. The extract concentration of 2.5% had a significance value of 0.113 against the positive control group, the antibiotic Ciprofloxacin. This indicates a meaningless relationship. Thus, the extract concentration of 2.5% had the same ability as the positive control in inhibiting *S. aureus* biofilm. So, it can be concluded that although the methanol extract of aloe vera leaf bark (*Aloe barbadensis* Miller) has statistically shown inhibitory activity against *S. aureus* biofilms is equivalent to that of positive controls, it has a Minimum Biofilm Inhibition Concentration (MBIC50) percentage <50%.

Several factors can cause the methanol extract of the aloe leaf bark (Aloe barbadensis Miller) to have MBIC<sub>50</sub> <50%. First, the methanol solvent was used in the extraction of the test plants. The methanol has a polar group that is stronger than its nonpolar group, this can be seen from the chemical structure of methanol which contains a hydroxyl group (polar) and a carbon group (nonpolar). The methanol can extract a greater amount of phytochemical compounds so that it can extract more bioactive components that have higher polarity properties (Seidel et al., 2012).

According to Seidel (2012), the high polarity index in methanol solvents can extract secondary metabolites that have polar properties such as flavonoids glycosides, tannins, and some alkaloids. This solvent is also effective for extracting phenolic compounds with low molecular weight and moderate polarity levels (Lin et al., 2009) flavonoid aglycones (Dehkarghanian et al., 2010) anthocyanins, terpenoids, saponins, flavones, and polyphenol compounds. Non-polar solvents such as n-hexane which has a zero polarity index are effective in dissolving lipophilic compounds, such as alkanes, waxes, colour pigments, sterols, some terpenoids, and alkaloids (Romadanu et al., 2014).

Lawrence et al, shows that the ethanol extract of Aloe vera gel has a wider diameter of inhibition zone against S. aureus bacteria than the methanol extract and acetone extract of Aloe vera gel. It is suspected that the methanol extract of Aloe barbadensis Miller leaf bark used in this study has not been able to dissolve other secondary compounds/metabolites that are lipophilic (Lawrance et al., 2009).

Also, considering the effect produced by the methanol extract of the Aloe barbadensis Miller is still the result of the combined work of various secondary compounds/metabolites that can affect the mechanism of action of one compound with another. So for further development, it is necessary to isolate pure compounds for antibiofilm activity tests.

## 5 CONCLUSION

The methanol extract of aloe vera leaf bark (Aloe barbadensis Miller) has an inhibitory activity against S. aureus biofilms equivalent to positive controls but has a Minimum Biofilm Inhibition Concentration (MBIC<sub>50</sub>) <50%.

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