

Antibiofilm Test of Ethyl Asetate Extract of Jarak Tintir Stem Bark (*Jatropha multifida* L.) Against *Pseudomonas aeruginosa*

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Abstract Biofilm is defined as colony of microorganism in the extracellular polymeric substance that produced by bacteria. *Pseudomonas aeruginosa* is a pathogen bacteria that causing acute and chronic infections. Biofilm of *Pseudomonas aeruginosa* is difficult to be cured because of their resistance against antibiotics. Ethyl acetate extract of *Jatropha multifida* L. stem bark that contains alkaloids, flavonoids, and phenols are expected as an antibacterial agent. Previous study showed that the ethyl acetate extract of *Jatropha multifida* L. stem bark has antibiofilm activity against *Staphylococcus aureus* and *Methicillin-Resistant Staphylococcus aureus* (MRSA) with IC50 values of 300 µg / mL and 760 µg / mL, respectively. The aim of this study was to examine the inhibition and destruction of ethyl acetate extract of *Jatropha multifida* L. stem bark against biofilm of *Pseudomonas aeruginosa*. Antibiofilm activity test was performed using crystal violet microtiter plate assay method. The results showed that ethyl acetate extract of *Jatropha multifida* L. stem bark has biofilm inhibition percentage of 84.90% and biofilm destruction percentage of 75.86% against *Pseudomonas aeruginosa* at a concentration of 31.25 µg / mL. Ethyl acetate extract of jarak tintir stem bark (*Jatropha multifida* L.) has antibiofilm activity against *Pseudomonas aeruginosa*.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *Jatropha multifida* L. stem bark.

1. Introduction

Biofilm is a colony of bacteria attached to a surface that covered by extracellular matrix, which commonly called Extracellular Polymer Substance (EPS). EPS is a matrix which structured by several components including polysaccharides, DNA and proteins [1]. The National Institute of Health (NIH) reports that more than 80% of microbial infections caused by biofilm[2]. *Pseudomonas aeruginosa* is an opportunistic pathogenic bacteria which is commonly associated with Cystic Fibrosis infections and is one of the bacteria that has a strong ability to forming a biofilm. *P.aeruginosa* biofilms could make failure result of antibiotics

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treatment [1]. The difficulty to destructing *P.aeruginosa* biofilm can be prolonged the treatment of infection and required a high medical cost. Therefore, a compound that is not only able to inhibit but also can destruct *P.aeruginosa* biofilm is needed to overcome infection due to *P.aeruginosa* biofilm. The use of plant extracts which have an antibacterial and antibiofilm effect can be an alternative solution in therapeutical care[3]. Not only safe, but the use of traditional medicine has also been accepted by people since ancient times[4].

Jatropha multifida L. is a plant that is widely cultivated in almost all tropical regions. *Jatropha*

multifida L. have many benefit for treatment including as an antibacterial agent. Stems of *Jatropha multifida* L. has been used as a traditional medicine to treat infectious skin diseases[5]. The previous study about antibacterial activity test revealed that ethyl acetate extract of *Jatropha multifida* L. leaves exhibits the antibacterial activity against *Pseudomonas aeruginosa* at MIC values of 19.75 $\mu\text{g} / \text{mL}$ [6]. Another study reports that ethyl acetate extract of *Jatropha multifida* L. stem showed antibiofilm activity against *Staphylococcus aureus* and *Methicillin-Resistant Staphylococcus aureus* (MRSA) in IC_{50} values of 300 $\mu\text{g} / \text{mL}$ and 760 $\mu\text{g} / \text{mL}$ respectively[7]. Study on the antibiofilm test against *Pseudomonas aeruginosa* which includes inhibition and destruction test is still limited. Therefore, the researchers were interested to investigate the antibiofilm capability of ethyl acetate extract of *Jatropha multifida* L. stem bark against *Pseudomonas aeruginosa*.

2. Methodology

2.1 Ethyl Acetate Extract of *Jatropha multifida* L.

The amount of 20 gram powder of jarak tintir stem bark obtained from the previous study. Then extraction was conducted by soxhletation method with 200 mL ethyl acetate as a solvent. Extracts obtained in the form of a viscous extract 2.5 grams in weight.

2.2 Antibacterial Activity Test of Ethyl Acetate Extract of *Jatropha multifida* L. against *Pseudomonas aeruginosa*

Ethyl acetate extract of *Jatropha multifida* L. made in 8 concentration series (4000 μg - 31.25 μg). The extract was dissolved with 10% DMSO. The bacteria culture have been grown on Mueller Hinton Broth media. The bacteria were adjusted the turbidity with McFarland standard by suspended with NaCl to obtain 10^8 CFU/mL. After this procedure, bacterial dilution was carried out by taking 100 μL then added with 10 mL NaCl to obtain 10^6 CFU/mL. Microplate 96 wells was prepared by pouring the amount of 160 μL of Mueller Hinton media Broth (MHB), 20 μL of bacterial suspension 10^6 CFU/mL and extract of 20 μL for each concentration into the well. Media control contained 200 μL of MHB media, solvent control contained 160 μL of MHB media, 20 μL of bacteria, and 20 μL of DMSO and bacterial control

contained 180 μL of MHB media and 20 μL of bacteria. Then microplate 96 wells were incubated at 37°C for 24 hours. After 24 hours, subculture as a confirmation test was done by preparing the MHA media then transferred an aliquot from wells to plates that would be streaked by ose. The plates were incubated at 37°C for 24 hours. The lowest concentration which inhibits visible growth of bacteria on media agar considered as MIC value.

2.3 Antibiofilm Activity Test

The antibiofilm activity test used the three variations of extract and antibiotic concentrations ($\frac{1}{2}$ MIC, $\frac{1}{4}$ MIC, and $\frac{1}{8}$ MIC). The MIC (Minimum Inhibitory Concentration) value was obtained from the antibacterial activity test of ethyl acetate extract against *Pseudomonas aeruginosa*. The MIC value of extract was 62.5 $\mu\text{g} / \text{mL}$.

2.3.1 Biofilm Inhibition Test

The ethyl acetate extract stock solution was prepared with various concentration (31.25 $\mu\text{g} / 100\mu\text{L}$, 15.62 $\mu\text{g} / 100\mu\text{L}$, and 7.81 $\mu\text{g} / 100\mu\text{L}$). The prepared bacteria that have been grown on TSB + 1% glucose was suspended with NaCl and adjusted the turbidity with McFarland standard to obtained 10^8 CFU/mL bacteria. The suspensions of bacteria dilution was carried out by taking 100 μL add 10mL NaCl to obtain 10^6 CFU/mL bacteria. Microplate 96 wells was prepared then put 160 μL of TSB + 1% glucose, 20 μL of 10^6 bacterial suspension and 20 μL of extract solution into each well. Each concentration of extract is made in 6 wells. The media control contained 200 μL of TSB + 1% glucose, a solvent control contained 160 μL of TSB + 1% glucose, 20 μL of 10^6 bacterial suspensions and 20 μL of 10% DMSO and a bacterial control containing 180 μL of TSB + 1% glucose and 20 μL of 10^6 bacterial suspensions. The microplate was incubated at 37°C for 48 hours. The bacteria suspension from each well of the microplate, was discarded using a micropipette. Each wells was washed three times with 300 μL sterile NaCl 0.9% then fixed with 150 μL of methanol and allowed to stand for 20 minutes. After 20 minutes, 150 μL of 0.1% crystal violet was added and incubated at room temperature for 15 minutes. Then, the crystal violet was removed and the microplate washed with aquadest. Next, 200 μL of 96% ethanol was added and allowed to stand for 30 minutes. The absorbance of bacterial biofilm was measured at a wavelength of 570 nm using a microplate reader. The percentage of

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biofilm inhibition can be calculated by the following formula:

% Inhibition=

$$\frac{(OD \text{ bacterial control} - OD \text{ media control}) - (OD \text{ sample} - OD \text{ media control})}{(OD \text{ bacterial control} - OD \text{ media control})} \times 100\% \quad (1)$$

2.3.2 Biofilm Destruction Test

Biofilm destruction test was carried out to determine the ability of ethyl acetate extract to penetrate into the *Pseudomonas aeruginosa* biofilm so that it could destroy the formed biofilm. This method as same as in the biofilm inhibition test. However, the biofilm destruction test for ethyl acetate extract was added after biofilm formed. First, 160 μL of TSB + 1% glucose and 20 μL of 10^6 CFU/mL bacteria were added in each well on microplate then incubated at 37°C for 48 hours. After biofilm formation occurred the supernatant in the microplate was taken out and put 20 μL of ethyl acetate extract with variations in concentrations of $\frac{1}{2}$ MIC, $\frac{1}{4}$ MIC, and $\frac{1}{8}$ MIC and 160 μL of TSB media. The microplate was re-incubated at 37°C for 48 hours. Furthermore, it was treated as in the biofilm inhibition test. The percentage of biofilm destruction can be calculated by the following formula:

% Destruction=

$$\frac{(OD \text{ bacterial control} - OD \text{ media control}) - (OD \text{ sample} - OD \text{ media control})}{(OD \text{ bacterial control} - OD \text{ media control})} \times 100\% \quad (2)$$

3. Result and Discussion

3.1 Antibacterial Activity of Ethyl Acetate Extract of *Jatropha multifida* L. against *Pseudomonas aeruginosa*

Antibacterial activity test of ethyl acetate extract *Jatropha multifida* L. stem bark was carried out to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The control used in this test include media control for aseptic sterility parameters, bacterial control that serves as a comparison of bacterial growth, and solvent control to ensure that the solvent was used does not have antibacterial activity. Minimum Inhibitory Concentration (MIC) is the lowest concentration of extract which can inhibit bacterial growth. Minimum Bactericidal Concentration (MBC) is the lowest concentration of extract which does not produce bacterial colonies on solid media.

Table 1. MIC and MBC of Ethyl Acetate Extract of *Jatropha multifida* L.

Concentration ($\mu\text{g/ml}$)	Bacterial Growth	
4000	-	
2000	-	
1000	-	
500	-	
250	-	
125	-	MBC
62,5	+	MIC
31,25	++	

(-) : no bacterial growth, (+) : little bacterial growth, (++) : many bacterial growth

Based on Table 1, it can be seen that at concentration of 62.5 $\mu\text{g/mL}$ the extract produces less bacterial growth than bacterial control so it can be concluded that concentration of 62.5 $\mu\text{g/mL}$ is the Minimum Inhibitory Concentration (MIC) and at concentration 125 $\mu\text{g/mL}$ there was no bacterial growth, so the concentration of 125 $\mu\text{g/mL}$ could be expressed as Minimum Bactericidal Concentration (MBC). Study on the antibacterial activity test ethyl acetate extract of *Jatropha multifida* L stem bark against *Pseudomonas aeruginosa* has never

been reported before. Previous studies tested the antibacterial activity of ethyl acetate extract from *Jatropha curcas* L. stem against *Pseudomonas aeruginosa* and MIC values at a concentration of 100 $\mu\text{g/mL}$ [8]. Compared with the previous study, the ethyl acetate extract of the *Jatropha multifida* L. stem has a lower MIC value of 62.5 $\mu\text{g/mL}$.

3.3 Antibiofilm Activity

3.3.1 Biofilm Inhibition Activity

The results showed that the ethyl acetate extract of *Jatropha multifida* L stem bark had inhibition activity against *Pseudomonas aeruginosa* biofilm. The absorbance value is directly showed the biomass of biofilm formation. The crystal violet microtiter plate assay method was first described by Christensen et al. (1985) which until now has been modified to calculate the amount of biofilm biomass in wells[10]. The comparison chart is used

to compare the biomass of bacterial control biofilms and extract. Comparison chart of *Pseudomonas aeruginosa* biomass biofilm on the inhibition test can be seen in Figure 1. The increased of absorbance will showed the increased of the biofilm formation. The absorbance of biofilm of bacterial control is higher than the absorbance of extract. It caused the bacterial control only contains bacteria so the biofilm formed is denser than the extract. The decreasing absorbance of the bacterial biofilm treated with extract indicated that extract had inhibitory activity against the biofilm formation.

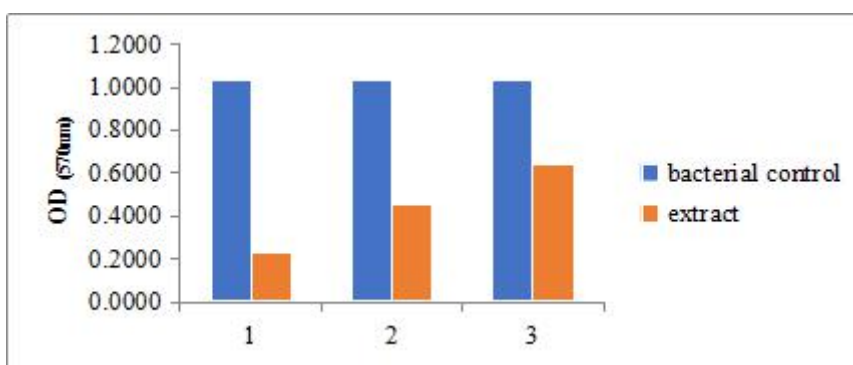


Fig 1. Comparison *Pseudomonas aeruginosa* biomass biofilm on the inhibition test

The inhibition of biofilm biomass is different in every concentration of extracts. It means that each concentration of extract has a different inhibitory activity against *Pseudomonas aeruginosa* biofilm. Ethyl acetate extract at a concentration of 31.25 µg/mL showed the highest *Pseudomonas aeruginosa* biofilm inhibition activity with an inhibition percentage of 84.90%. The lowest percentage of ethyl acetate extract at a concentration of 7.81 µg/mL was 36.38%. It can be concluded that ethyl acetate extract have abilities in inhibiting *Pseudomonas aeruginosa* biofilm. Study on the test of antibiofilm activity of ethyl acetate extract of *Jatropha multifida* L. stem bark against *Pseudomonas aeruginosa* has not been reported. The previous studies tested the antibiofilm activity of ethyl acetate extract of *Mangifera indica* L. against *Pseudomonas aeruginosa*. In this study, the percentage of biofilm inhibition was 48% at a concentration of 200 mg/mL[11]. Compared with the study, the ethyl acetate extract of *Jatropha multifida* L. stem bark had a higher inhibitory percentage. Ethyl acetate extract of *Jatropha*

multifida L. stem bark with a concentration of 31.25 µg/mL had the percentage of biofilm inhibition of 84.90%.

3.3.1 Biofilm Destruction Activity

The results showed that ethyl acetate extract had biofilm destruction activity against *Pseudomonas aeruginosa*. The absorbance of biofilm formation which added with extract showed a lower value than the bacterial control which indicated that the extract had the destruction activity of *Pseudomonas aeruginosa* biofilm. Most biofilm biomass calculation are carried out conventionally. However, over the past few years, the method of calculating the biofilm biomass has been replaced with a microtiter plate assay[12]. The microtiter plate assay method with crystal violet staining or commonly called crystal violet microtiter plate assay could be used to quantify biomass biofilm[10]. Comparison of biofilm biomass in bacterial control and ethyl acetate extract can be seen in Figure 2.

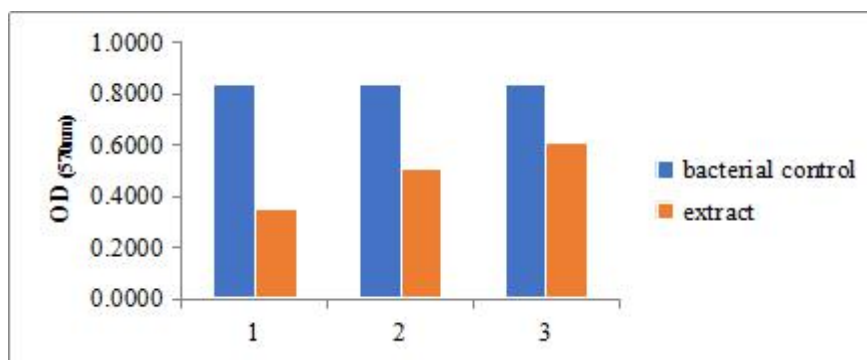


Fig 2. Comparison *Pseudomonas aeruginosa* biomass biofilm on the destruction test

Based on Figure 2. it can be seen that there is a quite high difference in biomass of bacterial control biofilm compared with biofilm biomass which added with extract. This indicates that the extract have the activity to destructs the *Pseudomonas aeruginosa* biofilm. Based on the biofilm biomass data can be calculated the percentage of *Pseudomonas aeruginosa* biofilm destruction by extracts. The results of the calculation showed that ethyl acetate extract of *Jatropha multifida* L. stem bark had biofilm destruction activity produced by *Pseudomonas aeruginosa*. The highest average percentage of *Pseudomonas aeruginosa* biofilm destruction (75.86%) was indicated by the concentration of ethyl acetate extract at 31.25 $\mu\text{g/mL}$. Extract concentration of 7.81 $\mu\text{g/mL}$ has the lowest ability of biofilm destruction (35.53%). Research on *Pseudomonas aeruginosa* dari biofilm destruction test of ethyl acetate extract of *Jatropha multifida* L. stem has never been carried out. Research by Pratiwi et al., (2015) concerning *Pseudomonas aeruginosa* biofilm destruction test of methanol extract of *Cinnamomum burmannii* Nees ex Bl. stem resulted in 49.54% destruction percentage at a concentration of 120 $\mu\text{g/mL}$ [13]. The results of the study of ethyl acetate extract of *Jatropha multifida* L. stem bark had a higher percentage of biofilm destruction which was 79.42% at a lower concentration of 31.25 $\mu\text{g/mL}$.

4. Conclusion

These results showed that ethyl acetate extract of *Jatropha multifida* L. stem bark has antibiofilm activity against *Pseudomonas aeruginosa*. Ethyl acetate extract with a concentration of 31.25 $\mu\text{g/mL}$ was able to inhibit *Pseudomonas aeruginosa* biofilm with an inhibition percentage of 84.90% and capable of destroying *Pseudomonas aeruginosa* biofilm with a percentage of 75.86% damage.

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