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Effect of Ashitaba Leaf as Antibiofilm Against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

Infection is one of the serious health problems that will continue to grow in Indonesia. Indonesian people have used herbal medicines derived from plants such as ashitaba leaves which contain chemical compounds tannin, alkaloids, flavonoids and saponins to treat several diseases. This study aims to see the potential of extracts and fractions (n-hexane, water and ethyl acetate) to inhibit the formation, destroy biofilms against Pseudomonas aeruginosa and Staphylococcus aureus. The method used is antibacterial testing using the dilution method, observation of bacterial cell morphology using SEM. Results active ashitaba leaf fraction inhibits the formation and degrades Pseudomonas aeruginosa and *Staphylococcus aureus* biofilms is water. The location of the most active water fraction against *Staphylococcus aureus* bacteria is on the bacterial cell wall. The water fraction has antibacterial activity against *Staphylococcus aureus* with an MBC value of 12.5%, the location of the water fraction is on the bacterial cell wall.

Keywords: antibacterial, antibiofim, ashitaba, *pseudomonas aeruginosa*, *staphylococcus aureus*

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BACKGROUND

Infection is one of the most serious health problems that will always develop in Indonesia (Pratiwi, 2008). Treatment of infections originating from natural materials has been widely developed. Bacteria are one of the things that can cause infectious diseases. As a form of self-defense, a protective layer of bacteria will be formed from bacteria called biofilm which is a structural form of a collection of microorganisms that are protected by an extracellular matrix called Extracellular Polymeric Substance (EPS) which is a product obtained from the microorganisms themselves and can be protected from environmental influences that are not good (Prakash, 2003).

Pseudomonas aeruginosa is included in opportunistic pathogens, The biofilm protects bacteria from penetration by antibiotics, complement, antibodies, and phagocytic cells (Bakung 2014). These properties cause resistance to antibiotics (Mansouri, 2013)). *Staphylococcus aureus* is a type of Gram-positive bacteria (Diyantika 2017). That can cause several types of skin and body tissue infections and then spread through the blood vessels which can be life-threatening (Chessa, 2015).

Biofilm is one of the results of quorum sensing (QS) originating from each microorganism (Alhede, 2011). The process of biofilm formation begins when the microbe is attached to the appropriate surface, then attachment will occur and the Quorum Sensing signal will be released. The Quorum Sensing signal is released, the bacteria will release EPS (extracellular polymeric substance) which is a matrix that protects the bacteria that is strong and sturdy. Bacteria will form a microcolony and then develop to form a biofilm (Michael, 2003).

Plants that have high flavonoid content are ashitaba leaves (Aviantina, 2019) The active content in ashitaba shows that ashitaba leaves contain chemical compounds of the flavonoid, alkaloid saponin, and tannin groups (Sembiring, 2011). In this study, Ashitaba leaf extraction was carried out using the maceration method, the maceration method was chosen because the procedures and equipment used were simple and no heating was carried out, so that natural materials did not decompose. The extract obtained was then fractionated, the fractions used were the water fraction, the ethyl acetate fraction and the n-hexane fraction.

The selection of these fractions is based on the polarity of each solvent. Water is a polar solvent, ethyl acetate is a semi-polar solvent and n-hexane is a non-polar solvent, so the compounds contained in ashitaba leaves will dissolve in the solvent based on the polarity of each compound in ashitaba leaves (Putri, 2017). Inhibition and degradation of biofilms and antibacterials using extracts and fractions of ashitaba leaves which also contain quite high flavonoids, then the most active fraction obtained from the results of antibiofilm testing on *Staphylococcus aureus* bacteria and *Pseudomonas aeruginosa* bacteria which produced the lowest IC_{50} and EC_{50} values, the smaller the IC_{50} value means the stronger the biofilm inhibition power, the further test is dilution which aims to determine the value of the MIC (Minimum Inhibitory Concentration) and the value of the MBC (Minimum Killing Concentration). Previous research (Kusuma, 2017), conducted a test of the antibacterial activity of the ethyl acetate fraction from ashitaba leaves against *Staphylococcus aureus* with a MBC value of 10%. Ashitaba leaves have antibacterial effects because the leaves contain flavonoid compounds that play a role in damaging the permeability of bacterial cell (Mercy, 2013).

Further test development was carried out, namely seeing the potential of extracts and fractions (n-hexane, water and ethyl acetate) from ashitaba leaves to inhibit biofilm formation, destroy biofilms and antibacterial against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and see the location of the most active fraction.

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METHODS

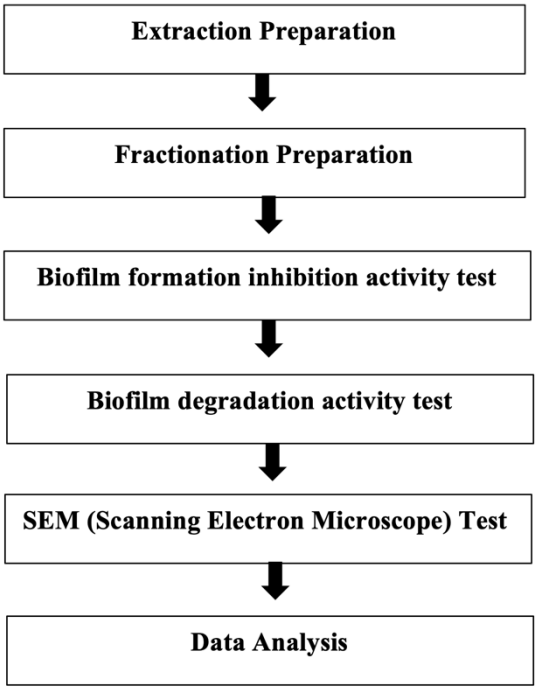
Tools and Materials

Tools

Autoclave, incubator, freezer, 96 well polystyrene flat-buttom microtitterplate, I Mark- Biorad Microplate Reader, separating funnel, analytical balance (AND GF-02), evaporator cup, ose needle, Bunsen, UV-Vis spectrophotometer (shimadzu), rotary evaporator, Sterling-Bidwell apparatus, moisture balance, UV 366, Laminar Air Flow, Scanning Electron Microscope (SEM).

Materials

Staphylococcus aureus and *Pseudomonas aeruginosa* obtained from the UESBE Laboratory of Setia Budi University, Vogel Johnson Agar (VJA), Potato Sucrose Agar (PSA), Mannitol Salt Agar (MSA), Crystal Violet 1% (brand), Brain Heart Infusion (BHI), safranin (brand), Mc.Farland solution and 10% DMSO, Amoxicillin.



Data Analysis

The results of the absorbance value reading or Optical Density (OD) which indicates the quantity of biofilm formation and biofilm degradation were then analyzed. The data used in this study used One Way Anova with a significance value of $p < 0.05$. As well as calculating the IC50 value from the average percentage of biofilm inhibition using the Linear Regression test to determine the potential of the ashitaba fraction in inhibiting *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms.

RESULTS

Extraction Preparation

The yield of the n-hexane ashitaba fraction obtained was 35.11%, the ethyl acetate ashitaba fraction obtained was 26.04%, and the water ashitaba fraction obtained was 36,02%. The yield of each solvent was not the same because the strength of each solvent in the process of extracting the compound content in the ethanol extract of ashitaba leaves was different.

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Test of inhibition activity of biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The reading of the beam in double light changes into data or interpretation of the test results called Optical Density (OD). The average results of inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms can be seen in the average inhibition table.

Table 1. Average results of *Pseudomonas aeruginosa* biofilm inhibition

No	Test sample	Average percentage of biofilm inhibition at concentration (%) ± SD			
		2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL
1.	Extract	42.05 ± 2.18	47.19 ± 0.88	55.04 ± 1.49	58.53 ± 1.65
2.	n-hexane fraction	34.79 ± 1.94	41.38 ± 1.17	44.19 ± 1.05	53.39 ± 1.02
3.	Ethyl acetate fraction	38.47 ± 1.09	45.06 ± 2.22	48.84 ± 1.05	55.5 ± 1.91
4.	Water fraction	42.64 ± 2.06	48.64 ± 1.43	57.91 ± 1.25	63.56 ± 1.30

Table 2. Average results of *Staphylococcus aureus* biofilm inhibition

No.	Test sample	Mean percent biofilm inhibition at concentration (%) ± SD			
		2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL
1.	Extract	57.44 ± 2.52	64.39 ± 0.68	73.86 ± 1.0	79.23 ± 0.13
2.	n-hexane fraction	51.45 ± 0.95	61.55 ± 2.37	67.61 ± 0.82	76.83 ± 0.61
3.	Ethyl acetate fraction	58.39 ± 5.27	71.27 ± 0.39	76.20 ± 0.85	80.05 ± 0.87
4.	Water fraction	61.93 ± 4.32	66.09 ± 1.55	80.99 ± 1.08	84.77 ± 1.99

IC_{50} value of extract, n-hexane fraction, ethyl acetate fraction and water with linear regression. The IC_{50} value produces a value that is inversely proportional to the biofilm inhibition activity. The higher the IC_{50} value, the lower the biofilm inhibition activity, meaning that the concentration required to produce 50% biofilm inhibition activity will be higher (10).

Table 3. IC_{50} results of *Pseudomonas aeruginosa* biofilm inhibition

No	Test sample	Linear Regression	IC_{50} results of *Pseudomonas aeruginosa* biofilm (µg/ml)
1.	Extract	Y= 0.3638 + 0.02864x	1.73 µg/ml
2.	n-hexane fraction	Y= 0.3246 + 0.02378x	1.69 µg/ml
3.	Ethyl acetate fraction	Y= 0.3325 + 0.02743x	1.81 µg/ml
4.	Water fraction	Y= 0.3605 + 0.03518x	1.38 µg/ml

Table 4. IC_{50} results of *Staphylococcus aureus* biofilm inhibition

No.	Test sample	Linear Regression	IC_{50} results of *Staphylococcus aureus* biofilm (µg/ml)
1.	Extract	Y= 0.5002 + 0.03742x	1.32 µg/ml
2.	n-hexane fraction	Y= 0.4381 + 0.04110x	1.20 µg/ml
3.	Ethyl acetate fraction	Y= 0.5400 + 0.03495x	1.41 µg/ml
4.	Water fraction	Y= 0.5259 + 0.04171x	1.19 µg/ml

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Biofilm degradation activity test of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The results of the biofilm degradation test in this study showed that the extract and fraction of ashitaba leaves have biofilm degradation activity of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Table 5. Results of *Pseudomonas aeruginosa* biofilm degradation test

No.	Test sample	Average percentage of biofilm degradation at concentration (%) ± SD			
		2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL
1.	Extract	50.48 ± 0.88	55.23 ± 1.26	57.27 ± 2.38	62.88 ± 1.21
2.	n-hexane fraction	52.8 ± 0.67	56.1 ± 0.87	59.92 ± 0.7	62.21 ± 1.16
3.	Ethyl acetate fraction	47.67 ± 1.74	55.39 ± 1.04	51.16 ± 1.43	60.56 ± 2.87
4.	Water fraction	55.71 ± 0.93	58.43 ± 0.77	62.69 ± 0.60	72.87 ± 0.73

Table 6. *Staphylococcus aureus* biofilm degradation test results

No.	Test sample	Average percentage of biofilm degradation at concentration (%) ± SD			
		2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL
1.	Extract	58.27 ± 0.77	63.26 ± 0.75	71.15 ± 0.86	75.06 ± 0.77
2.	n-hexane fraction	53.91 ± 0.82	60.67 ± 1.23	63.26 ± 0.75	72.98 ± 0.95
3.	Ethyl acetate fraction	57.83 ± 0.89	64.27 ± 0.57	68.62 ± 0.39	76.52 ± 0.56
4.	Water fraction	60.54 ± 1.45	78.79 ± 1.58	81.31 ± 0.19	84.22 ± 0.58

The results of the percentage of biofilm degradation determined the EC_{50} value of the extract, water fraction, ethyl acetate fraction, and n-hexane with linear regression, the linear regression table used to determine the EC_{50} value. The EC_{50} results of *Pseudomonas aeruginosa* and *Staphylococcus aureus* can be seen in tables 7 and 8.

Table 7. EC_{50} results of *Pseudomonas aeruginosa biofilm degradation.*

No.	Test sample	Linear Regression	EC50 results of *Pseudomonas aeruginosa* biofilm (µg/ml)
1.	Extract	Y= 0.4665 + 0.01962x	2.53 µg/ml
2.	N-hexane fraction	Y= 0.4974 + 0.01602x	3.09 µg/ml
3.	Ethyl acetate fraction	Y= 0.4297 + 0.02145x	2.31µg/ml
4.	Water fraction	Y= 0.4849 + 0.02787x	1.78 µg/ml

Table 8. EC_{50} results of *Staphylococcus aureus* biofilm degradation.

No.	Test sample	Linear Regression	EC50 results of *Staphylococcus aureus* biofilm (µg/ml)
1.	Extract	Y= 0.5237 + 0.02913x	1.69 µg/ml

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2.	n-hexane fraction	Y= 0.4775 + 0.02990x	1.99 µg/ml
3.	Ethyl acetate fraction	Y= 0.5170 + 0.03021x	1.63 µg/ml
4.	Water fraction	Y= 0.5782 + 0.03678x	1.34 µg/ml

Antibacterial activity test of the most active fraction of ashitaba leaves against *Staphylococcus aureus* bacteria

The antibiofilm test of the three fractions between the water fraction, n-hexane fraction and ethyl acetate fraction of the ethanol extract of ashitaba leaves produced data on the most active fraction that inhibited the growth of *Staphylococcus aureus* bacteria with the smallest IC_{50} value being the water fraction, with IC_{50} values of concentrations of 2, 4, 6, 8 mg/mL being 1.19 mg/mL and EC_{50} being 1,34 mg/mL, then further testing was continued by conducting further testing, namely the dilution test by conducting multilevel dilutions using a concentration series of 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781%, 0.391%, 0.195%, 0.098%. The results of the antibacterial activity test of the ethyl acetate fraction using the dilution test method can be observed in table 9.

Table 9. Results of antibacterial activity test of ashitaba leaf water fraction against *Staphylococcus aureus*

Concentration (% b/v)	Water fraction		
	Replication I	Replication II	Replication III
Control (-) Water	-	-	-
50	-	-	-
25	-	-	-
12.5	-	-	-
6.25	+	+	+
3.125	+	+	+
1.562	+	+	+
0.781	+	+	+
0.390	+	+	+
0.195	+	+	+
0.097	+	+	+
Control (+) Bacterial Suspension	+	+	+

Information :
(-) : There is no bacterial growth
(+) : There is bacterial growth Control

SEM (Scanning Electron Microscope) Test

The use of Scanning Electron Microscope (SEM) aims to see the surface characterization of biomaterial structures, changes in bacterial morphology and measurement of cell attachment. The advantage of SEM testing is the ability to see results with high magnification, with microscopic structural details (20).

The results of the observation test of morphological changes in *Staphylococcus aureus* due to exposure to the ashitaba leaf water fraction with a concentration of 6,25% are changes in the shape of the cell wall which is no longer intact. The cell wall experiences slight thickening and the surface of the cell wall becomes uneven. Shrinkage is seen forming towards the inside of the *Staphylococcus aureus* bacterial cells, which can be compared to

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Figure A showing a normal picture, namely the size and shape are almost the same, the cell wall looks normal, thick and arranged to form a collection (21).

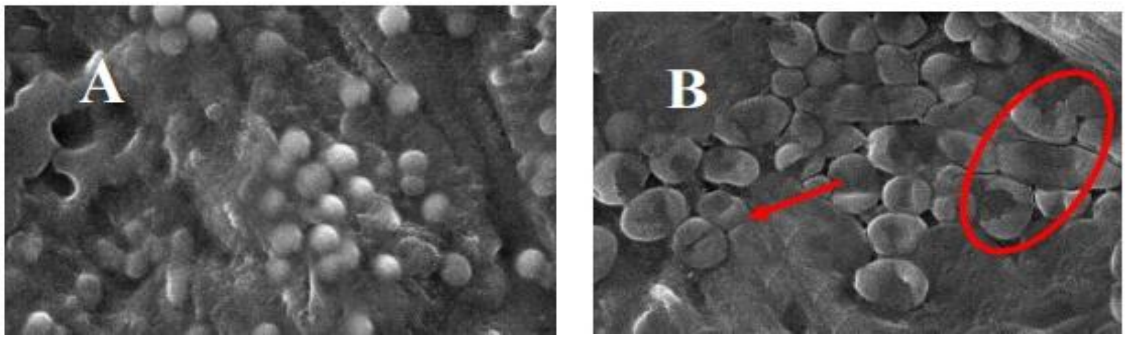

Fig 1. Morphological changes of *Staphylococcus aureus*.

A, *Staphylococcus aureus* without treatment,

B, *Staphylococcus aureus* given ashitaba leaf water fraction at a concentration of 6.25 mg/mL with a magnification of 100x

The changes that occur are due to flavonoids having a mechanism, namely inhibiting the function of cell membranes by forming complex compounds with extracellular proteins so that they are dissolved and then damage can occur to the cell membrane of bacteria followed by the release of intracellular compounds which cause shrinkage in bacteria.Shrinkage in the bacterial cell wall can be observed in Figure B which is marked with an arrow indicating a change in the shape of the bacteria.

DISCUSSION

Test of inhibition activity of biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The results of the percentage of biofilm inhibition were observed based on the smallest IC_{50} value, the smaller the IC_{50} value means the stronger the biofilm inhibition power. In *Pseudomonas aeruginosa*, the smallest IC_{50} value was given in the water fraction, which was 1.38 µg/mL, while in *Staphylococcus aureus* the smallest IC_{50} value was also obtained by the water fraction, which was 1.19 µg/mL. The IC_{50} value is used for inhibition tests on the biofilm formation process. The smaller the IC_{50} value, the more effective the sample is in inhibiting biofilm formation.[10] In the water fraction, it was concluded that it was the most effective in inhibiting biofilm growth, because water has an index of 10.2. The highest water polarity index value so that the level of polarity increases, the yield obtained increases (19).

The accumulation of solid surface biofilm occurs in two stages. The first stage is cell growth and the process of forming Extracellular Polymeric Substances, so that biofilm cells form accumulations. The second stage is that there can be release or reattachment. Bacteria use various extracellular organelles and proteins to be able to attach to the surface, including fimbriae, flagella pili and outer membrane proteins. Therefore, what makes it necessary to handle the prevention of biofilm growth is to inhibit or kill cells so that they do not continue to grow, and prevent the formation of EPS. At the stage of biofilm development, if inhibition is not carried out, what will happen is that the biofilm that is formed will increase and form a three-dimensional structure containing enveloped cells in several groups that will be connected to each other (10).

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Flavonoid compounds have the ability to suppress regulation originating from intercellular adhesion genes icaA and icaD which causes the production process of intercellular polysaccharides to decrease. With the decreasing production of Polysaccharide Intercellular Adhesin (PIA), the intercellular aggregation process in the maturation stage of the biofilm will be disrupted. If the biofilm does not mature, the subsequent process in the biofilm cycle can be disrupted. Based on the results of statistical tests, data were obtained for the inhibition of *Pseudomonas aeruginosa* biofilm for each test sample ($p<0.05$) from the negative control, this shows that there is a significant difference between the ashitaba leaf fractions and the negative control.

Biofilm degradation activity test of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The EC_{50} value is inversely proportional to the % biofilm degradation. The greater the EC50 value, the lower the biofilm degradation activity, meaning that the concentration required to produce 50% biofilm degradation activity is higher.[10] The table above shows that the water fraction has a lower EC_{50} value than the extract, ethyl acetate and n-hexane fractions. In *Pseudomonas aeruginosa* the EC_{50} value of the water fraction is 1.78 µg/mL, while in *Staphylococcus aureus* the EC_{50} value of the water fraction is 1.34 µg/mL. Statistical analysis of biofilm degradation begins with a normality test, the results show that the OD data for biofilm degradation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* are normally distributed. The homogeneity test shows that the OD data for biofilm degradation are homogeneous with a value ($P>0.05$). The data was then tested using one-way ANOVA test, the results showed a significant difference with a significance value of 0.00 ($p <0.005$).

The significance value between treatments showed that there was a significant difference in the OD of the negative control with the OD of the extract and fractions in the degradation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms. Based on the biofilm testing carried out, it can be concluded that the water fraction has the most effective biofilm inhibition and degradation activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria compared to the extract, ethyl acetate fraction and n-hexane fraction. Further tests were antibacterial activity tests using water fractions and using *Staphylococcus aureus* bacteria where the IC_{50} and EC_{50} values of the water fraction against *Staphylococcus aureus* bacteria were smaller than *Pseudomonas aeruginosa* bacteria, thus the ability of the ashitaba leaf water fraction to inhibit and degrade biofilms against *Staphylococcus aureus* bacteria was more active.

Antibacterial activity test of the most active fraction of ashitaba leaves against *Staphylococcus aureus* bacteria

From the scratch test conducted, the Minimum Killing Concentration value was obtained at 12,5%. The Minimum Killing Concentration aims to determine the activity of antibacterial which can be observed by inoculating the preparation from the test tube on Mannitol Salt Agar media. The water fraction is the most active fraction against *Pseudomonas aeruginosa* and *Staphylococcus aureus* compared to ethanol extract from ashitaba leaves, ethyl acetate fractions, n-hexane and water. The higher the polarity index value, the more polar a solvent is, the higher the polarity level, the higher the yield obtained.[19] The polar nature of water and its polarity index value and the highest yield value result in the water fraction being more effective in extracting antibacterial compounds such as flavonoids, saponins and tannins, which causes the water fraction to be the most active fraction with testing on biofilms having the smallest IC_{50} and EC_{50} values compared to the n-hexane fraction and ethyl acetate fraction.

CONCLUSION

The most active fraction of ashitaba leaves in inhibiting the formation and degrading *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms is the water fraction. The most

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active fraction of ashitaba leaves has antibacterial activity against *Staphylococcus aureus* with an MBC value of 12.5%. The location of the most active fraction of ashitaba leaves against bacteria and *Staphylococcus aureus* is on the bacterial cell wall.

Further research can be carried out on antibiofilm testing against other bacteria and development in the form of a preparation formulation.

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