NASKAH PUBLIKASI (MANUSCRIPT)

AKTIVITAS FRAKSI METHANOL, N-HEKSAN DAN N-BUTANOL DARI DAUN BOPOT (*Tabernaemontana divaricate R.Br*) TERHADAP PENGHAMBATAN MONOMIKROBA BIOFILM *Pseudomonas aeruginosa* DAN *Escherichia coli*

ACTIVITY OF METHANOL, N-HEXANE, AND N-BUTANOL FRACTIONS OF BOPOT LEAVES (tabernaemontana divaricate R.Br) ON INHIBITION OF BIOFILM FORMATION OF Pseudomonas aeruginosa AND Escherichia coli MONOSPECIES

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Aktivitas Fraksi Methanol, N-Heksan, dan N-Butanol dari Daun Bopot (*Tabernaemontana divaricate R.Br*) terhadap Penghambatan Monomikroba Biofilm *Pseudomonas aeruginosa* dan *Escherichia coli*

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Activity of Methanol, N-Hexane, and N-Butanol Fractions of Bopot Leaves (*Tabernaemontana divaricata*) on Inhibition of Biofilm Formation of *Escherichia coli* and *Pseudomonas aeruginosa* Monospecies

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Abstract: *Biofilms are known to be one of the causes of nosocomial infections and antibiotic resistance. Some bacteria that can cause nosocomial infections by forming biofilms are Escherichia coli and Pseudomonas aeruginosa. Bopot leaves are known to contain flavonoid compounds, tannins, phenolic acids that have antibiofilm mechanisms. The purpose of this study was to determine the activity of methanol, n-hexane, and n-butanol fractions of bopot leaves on the inhibition of biofilm formation of Escherichia coli and Pseudomonas aeruginosa monospecies. Extraction of bopot leaves was done by maceration method, fractionation was done by liquid-liquid extraction method using methanol, n-hexane and n-butanol solvents. Biofilm inhibition activity was carried out by crystal violet staining method which was read at a wavelength of 620 nm. The results showed that n-butanol, n-hexane and methanol fractions have the ability to inhibit biofilm formation on Escherichia coli and Pseudomonas aeruginosa bacteria with each concentration.*

Keywords: biofilm; bopot; methanol; n-hexane; n-butanol; *Escherichia coli*; *Pseudomonas aeruginosa*

1. Introduction

In developing countries such as Indonesia, infectious diseases caused by pathogenic microorganisms are a major cause of hospital-acquired morbidity and mortality. These infections are also known as nosocomial infections [1]. Some bacteria that cause nosocomial infections are known to be resistant to antibiotics because the overuse of antibiotics, especially for the treatment of disease, creates selective pressure on each bacterium, resulting in bacterial resistance [2].

One of the factors contributing to the onset of infection is biofilm formation [3]. Biofilm is a structure built by bacteria as a strategy to defend themselves against various conditions by using an extracellular matrix, mainly polysaccharides that bind to a surface [4]. Some examples of infections due to bacteria that can form biofilms are urinary tract infections (UTI) due to the

use of catheters infected with *Escherichia coli* bacteria and pneumonia due to the use of ventilators infected with *Pseudomonas aeruginosa* (VAP) [5,6].

Pseudomonas aeruginosa is a bacterium that can grow in different media and environmental conditions, in its physiological nature this bacterium has the ability to hydrolyze proteins (gammaproteobacteria) and is unable to hydrolyze carbohydrates (glucose, lactose, mannitol, maltose and sucrose) [7]. Based on a number of studies, it is known that *Pseudomonas aeruginosa* is able to form biofilms in a variety of situations and environmental conditions [8]. This biofilm formation ability is associated with the incidence of antibiotic resistance [9].

Escherichia coli is a gram-negative bacterium that can cause urinary tract infections (UTIs) and is prone to resistance [10]. These bacteria have the ability to form biofilms as a defense mechanism from unfavorable environmental conditions such as lack of nutrients, oxygen, and antibiotic use. Antibiotics and disinfectants become resistant due to biofilm-forming *Escherichia coli* [11].

One of the medicinal plants in Indonesia is the bopot plant (*Tabernaemontana divaricata*). The bopot plant is a plant that can be used as medicine in the roots, stems, leaves and flowers [12]. Bopot plants contain secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, saponins, phenolic acids, steroids and others with physiological and pharmacological properties [13]. Some of the chemical compounds contained in bopot plants are known to have biological activities as antimicrobial, anti-inflammatory, antioxidant, anticholinesterase, anticancer, antidiabetic, and others [14].

The purpose of this research was to determine the activity of methanol, n-hexane, n-butanol fractions of bopot leaves (*Tabernaemontana divaricata*) on the inhibition of the formation of *Escherichia coli* and *Pseudomonas aeruginosa* monospecies biofilms. Until now, there has been no research on the methanol, n-hexane, n-butanol fractions of bopot leaves (*Tabernaemontana divaricata*) as an inhibition of the formation of biofilm monospecies of *Escherichia coli* and *Pseudomonas aeruginosa*. Therefore, the author is interested in conducting research on the activity of methanol, n-hexane, n-butanol fractions of bopot leaves (*Tabernaemontana divaricata*) on the inhibition of biofilm formation of *Escherichia coli* and *Pseudomonas aeruginosa* monospecies.

2. Materials and Methods

2.1. Material

The materials used in this study are bopot leaves, *Escherichia coli*, *Pseudomonas aeruginosa*, methanol, n-hexane, n-butanol, ethyl acetate, distilled water, chloramphenicol 1%, blood agar base media, brain-heart infosion media, ethanol 96%, and crystal violet 1%.

2.2. Methods

2.2.1. Plant Determination

Determination of bopot leaves was carried out at the Faculty of Forestry Laboratory, Mulawarman University, Samarinda, East Kalimantan. Bopot leaves (*Tabernaemontana divaricata*) used in the study were obtained in Tepian Batang Village, Tanah Grogot District, Paser Regency, East Kalimantan.

2.2.2. Preparation of simplicial

Samples of bopot leaves that have been cleaned are continued by washing, chopping, and drying bopot leaves. The drying process is carried out using the sun until the simplicial is completely dry [15].

2.2.3. Production of extracts

Dried bopot leaves that have been finely ground as much as 1500 grams are macerated for 5 days using 96% ethyl acetate solvent as much as 4.5 liters, then re-macerated again with a new solvent 3 times, then filtered the results of maceration to separate the results of soluble extracts and phytates. The liquid extract is then placed in a rotary evaporator until the extract thickens slightly and then in a water bath until a thick extract is obtained.

2.2.4. Calculation of yield

Calculation of extract yield is conducted to determine the amount of bioactive content contained in the plant. Extract yield can be calculated using the formula [16]:

$$\% \text{ Yield} = \frac{\text{Weight of condensed extract}}{\text{"Weight of simplicial powder"}} \times 100\%$$

2.2.5. Preparation of methanol fraction, n-hexane fraction and n-butanol fraction of bopot leaves

Fractionation of bopot (*Tabernaemontana divaricata*) leaf extract was carried out by partition or liquid-liquid fractionation using a separatory funnel. The 10 grams of bopot leaf extract was dissolved in 100 mL of methanol until completely dissolved and then put into a separatory funnel. Then the extract was added 100 mL of n-hexane solution and mixed by shaking until two layers were formed. The lower layer is methanol solvent and the upper layer is n-hexane solvent. The two layers were separated and then each layer was further fractionated using ethyl acetate. The methanol solvent is divided into 2 layers where the methanol fraction is in the lower layer and the ethyl acetate solvent is in the upper layer. The n-hexane solvent is also divided into 2 layers where the n-hexane fraction is in the upper layer and the ethyl acetate solvent is in the lower layer. Then separated the methanol fraction and the n-hexane fraction with ethyl acetate. The results of the methanol and n-hexane fractions were evaporated with a rotary evaporator and in a waterbath to produce a condensed methanol fraction and a condensed n-hexane fraction. [17].

The chloroform extract was then liquid-liquid fractionated with N-Butanol solvent. The extract was put into a separatory funnel and dissolved with ethyl acetate, then added non-polar solvent (N-Butanol) in a ratio of 1: 1 v/v homogenized in a separatory funnel. Left to form ethyl acetate layer and N-Butanol layer. Each layer was placed in a different container. The N-Butanol layer was evaporated using a rotary evaporator to dry, then weighed and obtained the N-Butanol fraction. Next, the ethyl acetate layer was added to 100 mL of distilled water, then partitioned with N-Butanol solvent in a ratio of 1: 1 v/v, homogenized in a separatory funnel. Let it stand until the ethyl acetate layer and N-Butanol layer are formed. Each layer was placed in a different container [18].

2.2.6. Microbial preparation

The microbes to be cultured is *Escherichia coli* and *Pseudomonas aeruginosa*. The bacteria were each cultured in blood agar base media and incubated at 37 ^{o}C for 24 hours. After incubation, the bacteria were re-cultured in Brain-Heart Infosion (BHI) media and incubated for 24 hours. Then the bacteria in the BHI media were transferred into a centrifuge tube and put into a centrifuge to get the bacterial precipitate. The precipitated bacteria were transferred to new BHI media and vortexed to homogenize. After homogeneous, the Optical Density (OD) of each bacterium was measured using a spectrophotometer and adjusted to the McFarland standard of 0.5 or equivalent to 1.5 x 10^8 CFU/ml.

2.2.7. Test of biofilm formation inhibitory activity

Escherichia coli and *Pseudomonas aeruginosa* monospecies testing was conducted as follows:

The 110 µL of test bacterial suspension and 90 µL of bopot leaf fraction are put into 96-well microplate. In the 96-well microplate there was also a positive control containing 110 µL of test bacterial suspension and 90 µL of chloramphenicol suspension, negative control containing 110 µL of test bacterial suspension and 90 µL of media, and media control. Then incubated at 37^{o}C for 24 hours. After incubation, the 96-well microplate was washed with sterile distilled water. Then staining was done using 1% crystal violet solution which was added to the 96-well microplate as much as 200 µL and incubated for 15 minutes at room temperature. The dye was washed with sterile distilled water and allowed to dry at room temperature. After drying, 200 µL of 96% ethanol was added to the 96-well microplate and incubated for 15 minutes at room temperature. Then the 96-well microplate was measured using a microplate reader at an Optical Density (OD) of 595nm [19].

Based on Famuyide [20] the inhibition value can be classified as having high antibiofilm activity if the percentage value is ≥ 50%, while the percentage of 0-49% is categorized with weak antibiofilm activity. The percentage of inhibition for each concentration of methanol fraction of bopot leaves calculated using the formula below :

$$\% \text{ Inhibition} = \frac{\text{OD Control negatif - OD Test sample}}{\text{OD Control negatif}} \times 100\%$$

Ket: OD = Optical Density

2.2.8. Data analysis

The data analyzed is Optical density (OD) data. If the data is normally distributed and homogeneous, the data will continue with the one way ANOVA analysis method. However, if the data is not normally distributed and homogeneous, the data will be analyzed by the Kruskall-Wallis method.

3. Results and Discussion

3.1. Plant determination results

Determination of bopot leaves conducted at the Faculty of Forestry Laboratory, Mulawarman University, Samarinda, East Kalimantan. The bopot leaves (*Tabernaemontana divaricata*) used in the research were obtained in Tepian Batang Village, Tanah Grogot District,

Paser Regency, The results of determination declare that leaves used in this research correctly the leaves of the bopot plant.

3.2. Extraction result

The dried bopot leaves that have been refined are 1500 grams macerated for 5 days using 96% ethyl acetate solvent totaling 4.5 liters and then filtered so that obtained liquid extracts. The advantage of the maceration method is not destructive to active substances that are not heat resistant and the work procedure is quite simple [21]. Then the liquid extract was rotary evaporated until the extract thickened slightly and then in a waterbath until a thick extract of 97.42 was obtained. It can be seen on Table 1 below the calculation of yield ethyl acetate extract of bopot leaves.

Tabel 1. Extraction result and yield value

Simplicial	Condensed Extract	Yield (%)
1500 gram	97,42 gram	6,49

3.3. Fractionation result

The condensed ethyl acetate extract obtained was then fractionated. Fractionation is a method of separation an organic compound based on the solubility of the compounds in the two solvent or more that is not mixed, usually between a water solvent and an organic solvent. [22]. The solvents used in this research are methanol, n-hexane, n-butanol. Can be seen in table 2 below the results of the condensed fraction obtained.

Tabel 2. Extraction result and yield value

Sample	Condensed Extract	Result (gram)
97.42 g ethyl acetate extract of bopot leaves	Metanol	9,20
	N-Heksan	13,04
	N-Butanol	7,63

3.4. Activity of methanol, n-hexane, and n-butanol fractions of bopot leaves on inhibition of Escherichia coli biofilm formation

Fractions of bopot leaves obtained were each made into 3 concentrations, namely 100%, 50%, and 25%. The three concentrations of each fraction were tested on *Escherichia coli* bacteria using the Microtitter Plate Assay (MPA) method.

The results of the percentage inhibition of *Escherichia coli* biofilm formation from methanol, n-hexane, n-butanol, and positive control (chloramphenicol) fractions are seen in Figure 1. The results showed greater the concentration, greater the percentage of inhibition produced. The increase in concentration is directly proportional to the increase in the amount of bioactive compounds so that greater activity of the sample in inhibiting biofilm.

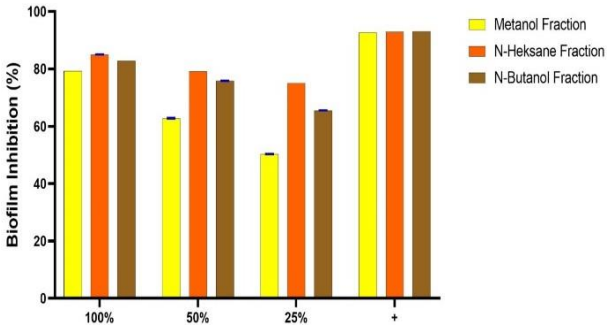

Figure 1. Percentage inhibition of *Escherichia coli* biofilm formation

Based on Figure 1, it is known that the methanol fraction at concentrations of 100%, 50%, and 25% respectively has inhibitory activity against *Escherichia coli* biofilm formation with a percentage 79.19%, 62.80%, and 50.34%. In the n-hexane fraction, each concentration of 100%, 50%, and 25% respectively has inhibitory activity against *Escherichia coli* biofilm formation with a percentage 85.01%, 79.13%, and 74.98%. And the n-butanol fraction at each concentration of 100%, 50%, and 25% respectively has inhibitory activity on *Escherichia coli* biofilm formation with a percentage of 82.75%, 75.82% and 65.51%.

The n-hexane fraction at each concentration had the greatest percentage of biofilm inhibition against *Escherichia coli* from methanol and n-butanol fractions. Researchers assume that the levels of bioactive compounds in the n-hexane fraction are more than the levels of bioactive compounds from the methanol and n-butanol fractions so the methanol and n-butanol fractions have a percentage below the n-hexane fraction. From the results of the three fractions, it can be concluded that bopot leaves have potential as an antibiofilm agent because the results of the percentage inhibition of *Escherichia coli* biofilm formation obtained ≥ 50%.

One of the bioactive compounds contained in bopot leaves is phenolic acid and tannin. Phenolic acids known to inhibit *Escherichia coli* bacterial biofilm formation by reducing Cell Surface Hydrophobicity (CSH). Based on the hydrophobicity test, phenolic acids can affect cell surface hydrophobicity (CSH) which is indicated by lower hydrophobicity resulting in less bacterial adhesion and biofilm [23].

Tannin compounds are known to inhibit *Escherichia coli* attachment by disrupting receptors on the surface of bacteria by binding to bacterial adhesin proteins so that receptors on the surface of bacteria are disrupted which results in inhibition of protein synthesis in forming cell walls and decreasing bacterial attachment and forming biofilms [3].

The optical density (OD) data that has been obtained is then carried out a statistical test in the form of the One Way Anova test, previously a normality test and homogeneity test must be carried out to ensure that the data obtained is normally distributed and homogeneous. If what is obtained is not normally distributed, then do the data transformation, if it is still not normal, the Kruskal Wallis test is carried out and followed by the Mann-Whitney test to compare each treatment group.

The optical density (OD) data of the methanol fraction and n-hexane fraction showed that the data were normally distributed but not homogeneous, so the Kruskal wallis test was carried out and continued with the mann-whitney test. The results of the mann-whitney test of the two fractions showed that there was a significant difference (p value ≤ 0.05) between all concentration groups with the negative control group and all concentration groups with the positive control group, but there was no significant difference between the 50% and 25% concentration groups (p value > 0.05).

The optical density (OD) data of the n-butanol fraction showed that the data were normally distributed and homogeneous. The results of the one-way ANOVA test showed that there were significant differences between the treatment groups in the biofilm attachment prevention test ($p \leq 0.05$).

3.5. Activity of methanol, n-hexane, and n-butanol fractions of bopot leaves on inhibition of Pseudomonas aeruginosa biofilm formation

The fractions of bopot leaves obtained were each made into 3 concentrations, namely 100%, 50%, and 25%. The three concentrations of each fraction were tested on *Pseudomonas aeruginosa* bacteria using the Microtitter Plate Assay (MPA) method.

The results of the percentage inhibition of *Pseudomonas aeruginosa* biofilm formation from methanol, n-hexane, n-butanol, and positive control (chloramphenicol) fractions are seen in Figure 2. The results showed greater the concentration, greater the percentage of inhibition produced. The increase in concentration is directly proportional to the increase in the amount of bioactive compounds so that the greater the activity of the sample in inhibiting biofilms.

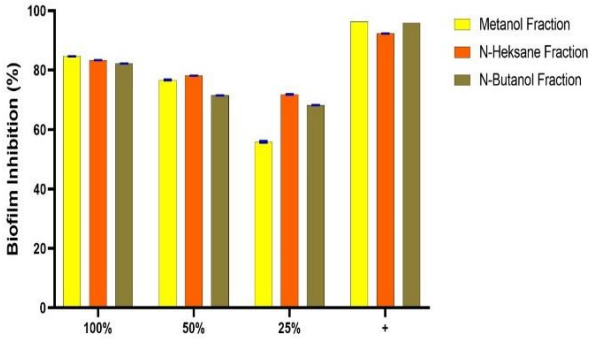

Figure 2. Percentage inhibition of *Pseudomonas aeruginosa* biofilm formation

Based on Figure 2, it is known that the methanol fraction at concentrations of 100%, 50%, and 25% respectively has inhibitory activity against *Pseudomonas aeruginosa* biofilm formation with a percentage 84.66%, 76.69%, and 55.92%. In the n-hexane fraction, concentrations of 100%, 50%, and 25% respectively have inhibitory activity against *Pseudomonas aeruginosa* biofilm formation with a percentage 83.39%, 78.17%, and 71.83%. And the n-butanol fraction at concentrations 100%, 50%, and 25% respectively had inhibitory activity on *Pseudomonas aeruginosa* biofilm formation with a percentage of 82.24%, 71.52% and 68.21%.

The methanol fraction at 100% concentration had the highest percentage between the n-hexane and n-butanol fractions, but at 50% and 25% concentrations the percentage of the n-hexane fraction was higher than the methanol and n-butanol fractions. From the results of the three fractions, it can be concluded that bopot leaves have potential as an antibiofilm agent because the results of percentage inhibition of *Escherichia coli* biofilm formation obtained ≥ 50%.

The content of bioactive compounds in bopot leaves is tannins and flavonoids. Tannins and flavonoids work by binding to one of the bacterial adhesion proteins used as bacterial surface receptors [22]. It is known that *Pseudomonas aeruginosa* have two type of adhesion proteins which are adhesion proteins found on pili and those found on the cell surface [24]. The presence of tannin and flavonoid compounds contained in bopot leaves reduces the adhesion of *Pseudomonas aeruginosa* bacteria.

The optical density (OD) data that has been obtained is then carried out a statistical test in The One Way Anova test, previously a normality test and homogeneity test had to be carried out to ensure the data obtained was normally distributed and homogeneous. The results of the one-way ANOVA test of the methanol, n-hexane, and n-butanol fractions showed that there were significant differences between the treatment groups in the biofilm attachment prevention test ($p \leq 0.05$).

4. Conclusions

Based on the results of this research, it is concluded that the methanol fraction, n-hexane fraction and n-butanol fraction of bopot leaves (*Tabernaemontana divaricata*) have inhibitory activity on biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa* bacteria.

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Conflicts of Interest

The authors declare no conflict of interest.

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Menyatakan bahwa "Aktivitas Fraksi N-Heksane dari Daun Bopot (*Tabernaemontana divaricata R.Br*) Terhadap Penghambatan Monomikroba Biofilm *Pseudomonas aeruginosa* dan *Escherichia coli*" telah di submit pada jurnal Briac pada tahun 2023.

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