NASKAH PUBLIKASI

POTENSI FRAKSI N-HEKSANA DAN N-BUTANOL DAUN KELUBUT (*PASSIFLORA FEOTIDA L*) TERHADAP PENGHAMBATAN BIOFILM *MONOMIRCOBIAL PSEUDOMONAS AERUGINOSA* DAN *ESCHERICHIA COLI*

POTENTIAL OF N-HEXANE AND N-BUTANOL FRACTIONS OF KELUBUT LEAF (PASSIFLORA FOETIDA L.) ON BIOFILM INHIBITION OF MONOMIRCOBIALS PSEUDOMONAS AERUGINOSA AND ESCHERICHIA COLI

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Naskah Publikasi

Potensi Fraksi N-Heksana dan N-Butanol Daun Kelubut (*Passiflora Feotida L*) terhadap Penghambatan Biofilm *Monomircobial Pseudomonas Aeruginosa* dan *Escherichia Coli*

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Potential of N-Hexane and N-Butanol Fractions of Kelubut Leaf (*Passiflora foetida L.*) on Biofilm Inhibition of Monomircobials *Pseudomonas aeruginosa* and *Escherichia coli*

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Abstract

Infectious diseases are disease conditions caused by pathogenic microorganisms. Infections caused by biofilms are a significant problem because the microbial community in the biofilm matrix is highly resistant to antimicrobial agents. Pseudomonas aeruginosa is one of the gram-negative opportunistic bacteria that is often involved in biofilm formation and Escherichia coli is also one of the bacteria that can cause infections in various human bodies such as in the digestive tract and urinary tract. Kelubut leaf is one of the herbs found in Indonesia and the Southeast Asian region. This plant has a number of bioactive components that have attracted attention and many have researched especially in the pharmaceutical field. Tests to determine biofilm inhibition were carried out by microdilution testing. The minomun biofilm inhibition concentration, which causes 50% inhibition (MBIC) was determined by crystal violet staining and read using a microplate reader at 620nm. Furthermore, it was analyzed using MBIC calculation and SPSS to evaluate the mechanism of effect. The results showed that the N-Hexan fraction and N-Butanol fraction showed biofilm inhibitory activity against P.Aeruginosa bacteria were 93.76%, 70.10% and on E.Coli were 85.36%, 72.01%. N-Hexan fraction can inhibit biofilm formation for 24 hours and has no difference with Chloramphenicol positive control, N-Butanol fraction can inhibit biofilm for 24 hours but there is a difference with Chloramphenicol control.

Keywords: Fraksi N-Heksan, Fraksi N-Butanol, Daun Kelubut, Biofilm, *Pseudomonas aeruginosa, Escherichia coli*

Introduction

According to a global study in 2019 there were 7.7 million deaths caused by infections. Infectious diseases are disease conditions caused by pathogenic microorganisms such as bacteria, viruses, fungi, or parasites. According to Kim et al., (2013), reported that about 70% of treatment failures are caused by fungal or bacterial polymicrobial infections that form biofilm. Biofilms are currently considered as mediators of infection with an estimated 80% of diseases associated with biofilm formation[2]. Infections caused by biofilms are a significant problem because the microbial community within the biofilm matrix is highly resistant to antimicrobial agents. Microbes that form biofilms are usually resistant to common

antimicrobial drugs and are able to evade host cells and the immune system, which can serve as a protective barrier. Biofilms are a factor of virulence and resistance as they proliferate as the clinical infection increases in the host cell. New antimicrobial agents are now increasingly in demand[3,4].

Pseudomonas aeruginosa is a type of opportunistic gram-negative bacteria that is often associated with biofilm development. Nosocomial infections (infections that occur in healthcare facilities) are common, and it is believed that Pseudomonas aeruginosa is responsible for 10 to 20 percent of such infections. Biofilms on intravascular catheters or other implanted medical devices can increase the risk of infection and complicate therapy[5].

Escherichia coli is a type of bacteria that can cause infections in the urinary and gastrointestinal tracts, as well as other parts of the human body. A challenge in the treatment of Escherichia coli infections is antibiotic resistance, which can develop resistance to several types of antibiotics, making treatment difficult and requiring the use of stronger antibiotics or combinations of antibiotics[6].

Bacterial infections caused by biofilms are difficult to treat. To kill bacteria in biofilm form requires 1000 times the dose of antimicrobials to achieve the same results as planktonic cells. Biofilms can be controlled by utilizing chemical compounds obtained from natural materials. Using chemical compounds as prevention and treatment of infection has become a common and effective approach in society and medical practice[3].

Some herbal have been recognized to have antimicrobial properties that can be used as a substitute for formalin in some cases and are more easily available and more affordable. Kelubut plant with the Latin name Passiflora foetida L. is one of the herbs found in Indonesia and Southeast Asia. This plant has been used in traditional medicine by people in certain regions for a long time. Kelubut leaves have a number of bioactive components that have attracted attention and have been widely researched, especially in the pharmaceutical field. [7].

Matherial and Methods

Materials : Materials used were Kelubut Leaves from Samarinda, Kalimatan Timur. Other matherials include the following: ethyl acetate, N-Hexan, N-Butanol, Pseudomonas aeruginosa and Escherichia coli bacteria, Blood Base Agar (BBA) media, Brain Heart Infusion (BHI) media, Aquadest, Crystal violet 1%, ethanol 96%, Nystatin, Chloramphenicol 1%, DMSO.

Apparatus: Analytical balance, blender, corong kaca, beaker glass, erlenmeyer, *rotary evaporator*, persolvent cup, *waterbath*, spatel, test tube, test tube rack, petri dish, ose needle, autoclave, bunsen, measuring cup, pipette, micropippette, blue tip, yellow tip, white tip, stirring rod, hotplate, incubator (lf-2b), vortex, *microplate 96 wells* (Iwaki®), *microplate reader* (HiPo Biosan), *laminar air flow* (LAF), *microtube*.

Research Method Kelubut leaves were determined in Herbarium Mulawarman, Laboratory Ecology and Conservation Tropical Forest Biodiversity, Faculty of Forestry, Mulawarman University Samarinda with the document number 69/UN17.4.08/LL/2023.

Processing The plants were dry sorted and wet sorted and then dried by drying in the sun. After the kelubut leaves are dry, they are pulverized into powder (simplisia) with a blender and stored in a tightly closed jar [8].

Extraction Kelubut leaves were extracted with ethyl acetate by maceration method in a ratio 1:3. Maceration was carried out for 15 days at a constant temperature and periodic stirring was done. Every five days the macerate was filtered with a cloth to separate it from the pulp. After that, the extract will be concentrated using a *vacuum rotary evaporator* at 55^{o}C to get a thick extract, and then the yield is calculated. [9].

Fractionation The result of thick ethyl acetate extract, measure as much as 5 grams. Then mix it with 100 mL of boiling water in an erlenmeyer flask. After the contents of the mixing container are put into a separatory funnel, 100 mL of N-Hexane is added and the mixture is stirred for 1 minute. Let stand for a while after shaking to produce two different stages. Fractionation was carried out repeatedly with the addition of N-Hexan until the fraction obtained changed color to clear. The resulting fraction is concentrated until it becomes a thick extract. Then the same was done for fractionation using N-Butanol solvent.[10]

Microbial Preparation *Pseudomonas aeruginosa* and *Escherichia coli* bacteria were cultured in BBA media and incubated at 37ºC for 24 hours. Then, Pseudomonas aeruginosa and Escherichia coli bacteria were re-cultured into liquid media, namely BHI and incubated at 37°C for 24 hours. The optical density (OD600) of the microbial cultures was adjusted to the standard of (0,5 *Mc Farland* ~1,5 x 10^8 CFU/ml) [3].

Biofilm Inhibition The microdilution technique was used to test biofilm inhibition. A 96-well polystyrene microtiter plate with a flat bottom was used for the test, and concentration levels of 25%, 50%, and 100% were used. 100µL of Pseudomonas aeruginosa and Escherichia coli bacterial suspensions were put into the microplate wells and 100µL of N-Hexan & N-Butanol fraction solutions of kelubut leaves with concentrations (25%, 50%, 100%) were added. Furthermore, as a control medium, a bacterial suspension was given without any microbial growth, and as a growth control, a bacterial suspension was used. To compare the test findings, a microbiological culture containing 100 µL of chloramphenicol was used as a positive control in the wells. After that, it was cultured for 24 hours at 37°C in an incubator. To remove residual water, the microplate was then dried at room temperature and cleaned three times using distilled water. To color the growing biofilm, 125 µL of 1% crystal violet solution was applied to each well. For fifteen minutes, the microplate was incubated at room temperature. After the incubation period, each well received 200 µL of 96% ethanol, and the microplate was thoroughly cleaned three times using distilled water to remove residual crystal violet 11. The use of crystal violet as a dye increases precision and enables quantitative detection of biofilms. The basic dye called crystal violet binds to polysaccharides and negatively charged compounds in the extracellular matrix. As a result, the biofilm matrix and living and dead cells can be stained with crystal violet[11]. The reading of biofilm degradation results was carried out using a 620nm Optical Density (OD) microplate reader. The OD value is then used to calculate the biofilm inhibition [3],[12].

Data Analysis

The percentage of inhibition and degeneration concentration was counted using the formula below:

$$\% \text{ Inhibition} =$$

Statistical Analysis Analysis using the SPSS for Windows program, *One way Analysis of Variance* (ANOVA) test was used to test the research data. With a data significance level of 0.05, this ANOVA is a parametric test that compares the average differences of two or more treatment groups using a numerical data scale. The *Shapiro Wilk* test is used to determine whether the data distribution is normal. Data is said to be normal if a significance value greater than 0.05 is found. While the data is not normally distributed if <0.05. The Lavene test was used in the sample homogeneity test, and the findings that showed homogeneous data had a significance value >0.05[13].

The *Kruskal Wallis* test will be used to assess the data of this study if it does not meet the

requirements of the one-way ANOVA test. After using one-way ANOVA, *Tukey's post hoc* test was conducted. However, if *Kruskal Wallis* is used in data analysis, then proceed to the Man Withney test[14].

Results and Discussion

Kelubut leaves (Passiflora foetida L.) that have been determined are dried then mashed and macerated extraction is carried out as much as 1000 grams for 5 days using 96% ethyl acetate solvent in a ratio of 1: 3 and repeated 3 times. Extraction of dried leaves (1000gram) produced 25.58 grams of ethyl acetate extract (13.04%). Then the liquid-liquid fractionation process was carried out with N-Hexan solvent which obtained a thick extract of 13.04 grams and N-Butanol as much as 5.17 gram. Biofilms caused by bacterial and fungal infections are one of the health problems that about 70% result in treatment failure. Infections caused by biofilms are a significant problem because the microbial community within the biofilm matrix is highly resistant to antimicrobial agents. Microbes that form biofilms are usually resistant to common antimicrobial drugs and are able to evade host cells and the immune system, which can serve as a protective barrier. In this study, we evaluated the potential of N-Hexane and N-Butanol fractions of kelubut leaves in the inhibition of monomicrobial biofilm formation of Pseudomonas aeruginosa and Escherichia coli. The results showed that the N-Hexan and N-Butanol fractions of kelubut leaves could inhibit 50% of biofilm formation (Figure 1 and Figure 2).

The concentration of chloramphenicol used in this study was 1%. Chloramphenicol is an antibiotic that is useful against a wide variety of bacteria. Chloramphenicol is a bacteriostatic antibiotic that at high enough concentrations can also kill bacteria. Its function is to block the formation of peptide bonds by attaching to ribosomes, preventing protein synthesis 15. The use of positive controls has the aim of comparing the results of inhibition of biofilm formation [15,16].

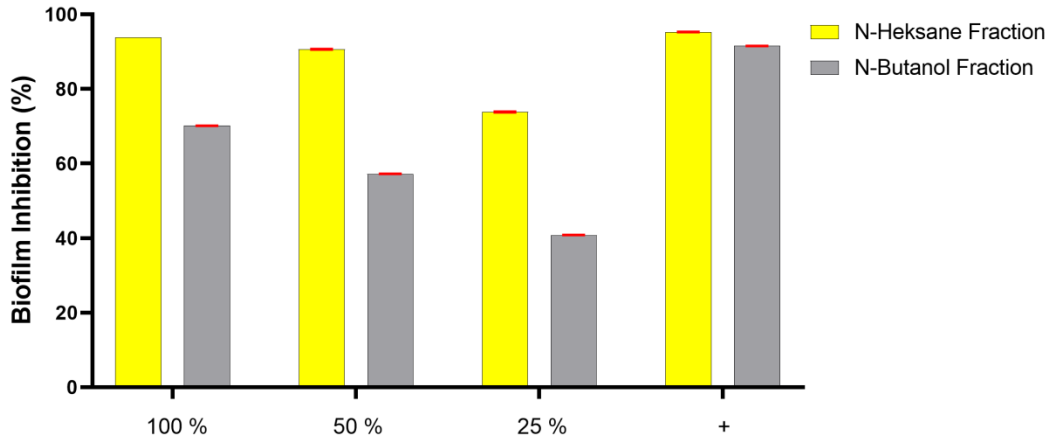

Fig. 1: Biofilm inhibition activity monomicrobial *Pseduomonas aeruginosa*

In this result, it was reported that the N-Hexan fraction of kelubut leaves provided greater biofilm formation inhibitory activity compared to the N-Butanol fraction. The N-Hexan fraction gave an activity of 93.76% while the N-Butanol fraction gave an activity of 70.10% at 100% concentration. The activity of the N-Hexan fraction was almost the same as the positive control, namely chloramphenicol 1% at 95.27% (Figure 1). These results

indicate that concentrations of 100% and 50% of the N-Hexan fraction and N-Butanol fraction of kelubut leaves are able to inhibit the formation of Pseudomonas aeruginosa monomicrobial biofilms above 50%.

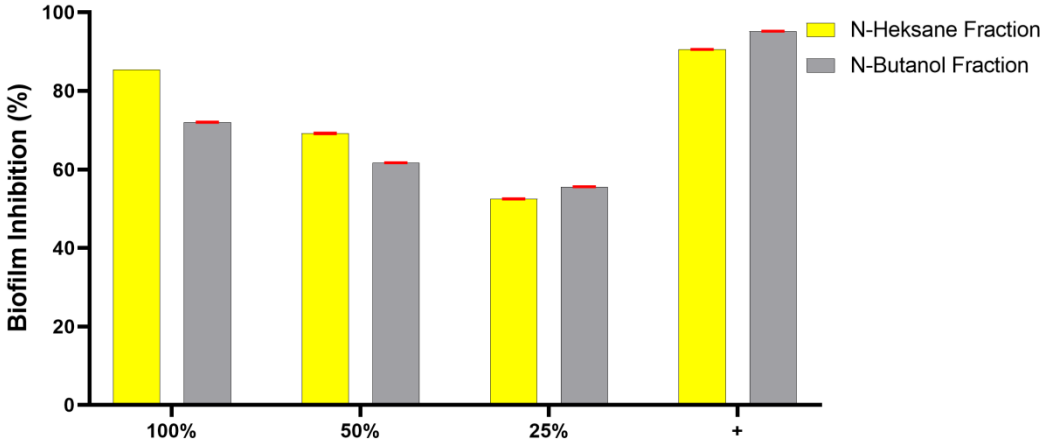

Fig. 2: Biofilm Inhibition activity monomicrobial *Escherichia coli*

The results of the study in Figure 2 show that the 100% N-Hexan fraction provides biofilm formation inhibitory activity against Escherichia coli monomicrobials of 85.36% and at 100% N-Butanol fraction concentration provides activity of 72.01%. At a concentration of 25% N-hexan fraction has a biofilm formation inhibitory activity of 52.48% and at 25% N-Butanol fraction of 55.95%.

Biofilm inhibition mechanism and planktonic inhibition mechanism are different. In biofilms, bacteria together form a group or community so as to produce a more complex and stronger defense, while in plactonic bacteria are only a single cell and live freely so that antimicrobial agents can cause more damage naturally to defense cells and reach target cells[17].

The results of the biofilm inhibition test presentation above show an increase in the percentage of biofilm inhibition against Pseudomonas aeruginosa and Escherchia coli bacteria along with an increase in the concentration used. This means that the higher the concentration used, the higher the biofilm inhibition produced. An increase in the potential inhibition of biofilm formation was found at a concentration of 100% N-Hexan fraction against Pseudomonas aeruginosa, there was no significant difference ($p>0.05$) when compared to the positive control, which means there is no significant difference in the inhibition of biofilm formation. At a concentration of 100%, the N-Butanol fraction found a significant difference ($p<0.05$) when compared to the positive control, which means it has a difference in inhibiting the formation of Pseudomonas aeruginosa biofilm. At a concentration of 100% N-Hexan fraction against Escherichia coli when compared to the positive control did not have a significant difference ($P>0.05$) which means it does not have a meaningful difference. At a concentration of 100%, the N-Butanol fraction has a significant difference ($p < 0.05$) when compared to the positive control, which means it has a significant difference.

According to Miquel et al., (2016) Plants have bioactive chemicals that can inhibit the process of bacterial attachment to solid surfaces and the formation of extracellular polymer (EPS) scaffolds. This may result in anti-biofilm action. Kelubut leaves contain alkaloids, steroids, tannins, saponins, coumarins, tyrosine, glycine, and flavonoids.

However, in this study, N-Hexan and N-Butanol fractionation was carried out so it is possible that the compounds of interest in this N-Hexan fractionation are steroids/triterpenoids and saponins. Steroids/triterpenoids prevent the production of new proteins that accumulate and modify the constituent elements of bacterial cells, thus inhibiting bacterial development[19]. By attaching to the bacterial biofilm layer, saponins work by reducing the number of bacterial cells thereby disrupting their permeability and making their cell walls brittle and eventually resulting in death[20]. N-Butanol fractionation allows the attraction of polar compounds, namely alkaloids, flavonoids, and tannins. Alkaloids have the ability to inhibit the synthesis of peptidoglycan in bacterial cells thus inhibiting the formation of the cell wall layer properly, while flavonoids can interfere with energy transduction in the bacterial cytoplasmic membrane, inhibiting the production of ATP which is essential for bacterial life. Flavonoids can also inhibit bacterial motility by inhibiting flagellum synthesis and inhibiting enzymes involved in bacterial movement, and tannin compounds can cause cell damage by denaturing proteins[21].

Conclusion

In conclusion, the available data show that the N-Hexan fraction and N-Butanol fraction of kelubut leaves (Passiflora foetida L.) have inhibitory activity onomicrobial biofilm formation Pseduomonas aeruginosa and Eschericihia coli. N-Hexan fraction is the most active fraction as inhibition of biofilm formation with the highest percentage of inhibition against Pseudomonas aeruginosa 93.76% and against Escherichia coli 85.36%. The N-Hexan fraction when compared with the positive control chloramphenicol 1% did not have a significant difference ($p>0.05$) in inhibiting biofilm formation of Pseduomonas aeruginosa and Escherichia coli monomicrobials. The N-Butanol fraction also has inhibitory activity of biofilm formation with a percent inhibition of 70.10% on Pseudomonas aeruginosa bacteria and a percent inhibition of 72.01% on Escherichia coli bacteria. However, the N-Butanol fraction has a significant difference when compared to the positive control of chloramphenicol 1%.

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SURAT KETERANGAN ARTIKEL PUBLIKASI

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Menyatakan bahwa artikel ilmiah yang berjudul "Aktivitas Fraksi N-butanol Dari Daun Kelubut (*passiflora foetidal L*) Terhadap Uji penghambatan Mikroorganisme Dan Uji Biofilm *Pseudomonas aeruginosa* dan *Escherichia coli*".

Demikian surat keterangan ini dibuat untuk dapat dipergunakan sebagaimana mestinya.

Wassalamu'alaikum Wr. Wb

Samarinda, 14 Desember 2023

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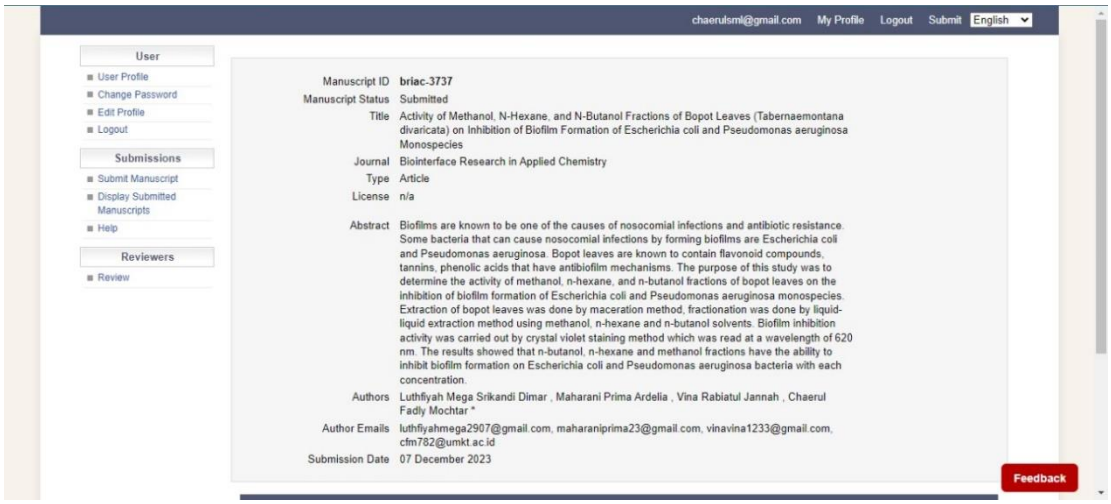
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Title Activity of Methanol, N-Hexane, and N-Butanol Fractions of Bopot Leaves (Tabernaemontana divaricata) on Inhibition of Biofilm Formation of Escherichia coli and Pseudomonas aeruginosa Monospecies
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Type Article
License n/a
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.
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