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

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Abstract

Infections caused by biofilms are significant problems because the microbial community in the biofilm matrix is highly resistant to antimicrobial agents. Pseudomonas aeruginosa and Escherichia coli are opportunistic bacteria that can cause infections in various human bodies, often involved in biofilm formation. Kelubut leaf is one of the herbs found in Indonesia and Southeast Asia. This plant has several bioactive components that have attracted attention and many have been researched, especially in the pharmaceutical field. This study aims to determine the potential of n-hexanee and n-butanol fractions in inhibiting biofilm formation from monomicrobial Pseudomonas aeruginosa and Escherichia coli. The study was conducted by extracting samples which were then fractionated and antibiofilm tests were carried out using the broth microdilution test method.

The data obtained is then calculated to determine the biofilm inhibition percentage. The results showed that the N-Hexanee and N-Butanol fractions showed biofilm inhibitory activity against P. aeruginosa bacteria at 93.76%, 70.10% and on E. coli at 85.36%, 72.01%. The n-hexanee and the n-butanol fractions can inhibit biofilm formation in monomicrobial Pseudomonas aeruginosa and Escherichia coli. Isoscoparin has a better binding affinity value, so it has the potential to inhibit biofilm formation.

Keywords: Biofilm, *Escherichia coli*, Kelubut leaves, N-hexanee fraction, N-butanol fraction, *Pseudomonas aeruginosa*.

Introduction

According to a global study in 2019, there were 7.7 million deaths caused by infections. Infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, fungi or parasites. Kim et al[16] reported that about 70% of treatment failures are caused by fungal or bacterial polymicrobial infections that form biofilm. Biofilms are currently considered mediators of infection, with an estimated 80% of diseases associated with biofilm formation[3]. Infections caused by biofilms are significant problems because the microbial community within the biofilm matrix is highly resistant to antimicrobial agents. Microbes that form biofilms, are usually resistant to standard antimicrobial drugs and can 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[11,15].

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 *Pseudomonas aeruginosa* is believed to be 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[10]. *Escherichia coli* is a type of bacteria that can cause infections in the urinary and gastrointestinal tracts and other parts of the human body. A challenge in treating *Escherichia coli* infections is antibiotic resistance, which can develop resistance to several antibiotics, making treatment difficult and requiring more potent combinations of antibiotics[27].

Bacterial infections caused by biofilms are difficult to treat. To kill bacteria in biofilm form, it 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 to prevent and treat infection has become a familiar and practical approach in society and medical practice[12].

Some herbs have been recognized to have antimicrobial properties that can be used as a substitute for formalin in some cases and are more readily available and more affordable. Kelubut plant, with the Latin name *Passiflora foetida* L., is an herb found in Indonesia and Southeast Asia.

People in certain regions have used this plant in traditional medicine for a long time. Kelubut leaves have several bioactive components that have attracted attention and have been widely researched, especially in the pharmaceutical field[14]. Based on that background, this study aims to determine the potential of n-hexanee and n-butanol fractions in inhibiting biofilm formation from monomicrobial *Pseudomonas aeruginosa* and *Escherichia coli*.

Materials and Methods

Materials: The materials used were Kelubut leaves from Samarinda, Kalimantan Timur. Other materials include: ethyl acetate, n-hexanee, 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, glass funnel, beaker glass, rotary evaporator, water bath, spatel, test tube, test tube rack, Petri dish, needle, autoclave, measuring cup, pipette, micropipette, blue tip, yellow tip, white tip, stirring rod, hotplate, incubator (lf-2b), vortex, microplate 96 wells (Iwaki®), microplate reader (HiPo Biosan), laminar airflow (LAF), microtube.

Research Method: Kelubut leaves were deposited 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.

Sample Processing: The plants were dry sorted and wet sorted and then dried by drying in the sun. When leaves are dry, they are pulverized into powder (simplisia) with a blender and stored in a tightly closed jar[17].

Extraction: Kelubut leaves were extracted with ethyl acetate using the maceration method in a ratio of 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°C to get a thick extract and then the yield is calculated[23].

Fractionation: The result of thick ethyl acetate extract measures as much as 5 grams. Then, mix it with 100 mL of boiling water in an Erlenmeyer flask. When the contents of the mixing container are put into a separatory funnel, 100 mL of n-hexanee are added and the mixture is stirred for 1 minute. Stand for a while after shaking to produce two different stages. Fractionation was carried out repeatedly with the addition of n-hexane 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[13].

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 microbial cultures' optical density (OD600) was adjusted to the standard of (0,5 Mc Farland ~1,5 x 10^8 CFU/ml)[11,26].

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-hexane and n-butanol fraction solutions of samples 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. A microbiological culture containing 100 µL of chloramphenicol was used as a positive control in the wells to compare the test findings. After that, it was cultured in an incubator for 24 hours at 37°C. Then, residual water was removed and the microplate was dried at room temperature and cleaned three times using distilled water.

On staining, biofilms are formed by adding 125 µL of 1% crystal violet solution 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[6,18,22]. Using crystal violet as a dye increases precision and enables quantitative detection of biofilms. The basic crystal violet dye 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[6]. Biofilm inhibition results were read using a 620nm optical density (OD) microplate reader. The OD value is then used to calculate the biofilm inhibition[11,12,26].

Preparation of Protein Molecules: The target proteins for *Pseudomonas aeruginosa* are proteins with PDB IDs 5OE3 and 2UV0 and for *Escherichia coli* bacteria, they are 1QJ8 and 4JVI. Receptor data for *Pseudomonas aeruginosa* and *Escherichia coli* bacteria was obtained from the Protein Data Bank (PDB). The protein structure data was cleaned using Discovery Studio 2024, removing any additional water molecules and ligands. The data was ultimately visualized with PyMol. The cleaned protein files were then saved in .pdb format for further analysis[5].

Preparation of Ligans: The ligands contained in *Passiflora foetida* are based on previous literature, searched for structures and information related to these ligands through the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). We also downloaded from the same site different compounds reported to be presented in this *Passiflora foetida* extract like Pantothenic acid (CID:6613), Dihydroxycoumarin (CID: 5281417), Phaselic acid (2-O-Caffeoylmalic acid) (CID: 6124299), Isololiolide (CID: 11019783), Vicenin-2 (Apigenin-6,8-di-C-glucoside) (CID: 3084407), Loliolide (CID: 100332), Isoorientin (Luteolin-6-C-glucoside) (CID: 114776), Vitexin-2"-O-rhamnoside (CID: 5282151), Isoscoparin (Scoparin) (CID: 442611) and Isorhamnetin-3-O-glucoside (CID: 5318645). The results of

the chemical components are then downloaded in the form of .pdb data. The data was then minimized using PyRX and then docking analysis was carried out [25].

Site specific binding and visulalization: Molecular docking analysis or installation between ligand and protein files have been prepared using PyRx software with Autodock Vina Wizard. In this program, energy minimization is carried out for all ligands to be tested. Next, the location of the grid box for the mooring location is determined. The next stage is to run a docking analysis. The results of the docking analysis are predictions of the binding energy between the ligand and the protein, along with the optimal conformation and location of the interaction. The data was then exported in .pdb format for analysis using PyMol and Discovery Studio regarding the type of bond formed at the interaction site. The test ligands analyzed are the test ligands with the most negative binding energy values[8].

Data Analysis: The percentage of biofilm inhibition was counted using the formula:

$$\% \text{ Biofilm Inhibition} = \frac{(OD\ growth\ control - OD\ sample)}{OD\ growth\ control} x\ 100\%$$

Statistical Analysis: Data was analyzed using the SPSS for Windows program. The 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 standard if a significance value greater than 0.05 is found. 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[1].

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, proceed to the Man Withney test[4].

Results and Discussion

Kelubut leaves (*Passiflora foetida* L.) are dried, 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 (1000 grams) produced 25.58 grams of ethyl acetate extract (13.04%). Then, the liquid-liquid fractionation process was carried out with n-hexane solvent, which obtained a thick extract of 13.04 grams and n-butanol as much as 5.17 grams. Biofilms caused by bacterial and fungal infections are one of the health problems that result in about 70% treatment failure. Infections caused by biofilms are significant problems because the microbial community within the biofilm matrix is highly resistant to antimicrobial agents. Microbes that form biofilms are usually resistant to standard antimicrobial drugs and can evade host cells and the immune system, which can serve as a protective barrier. In this study, we evaluated the potential of n-hexanee and n-butanol fractions to inhibit the monomicrobial biofilm formation of *Pseudomonas aeruginosa* and *Escherichia coli*. The results showed that the n-hexane and n-butanol fractions 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 can also kill bacteria at high enough concentrations. Its function blocks the formation of peptide bonds by attaching to ribosomes, preventing protein synthesis. Positive controls aim to compare the results of inhibition of biofilm formation[2,7].

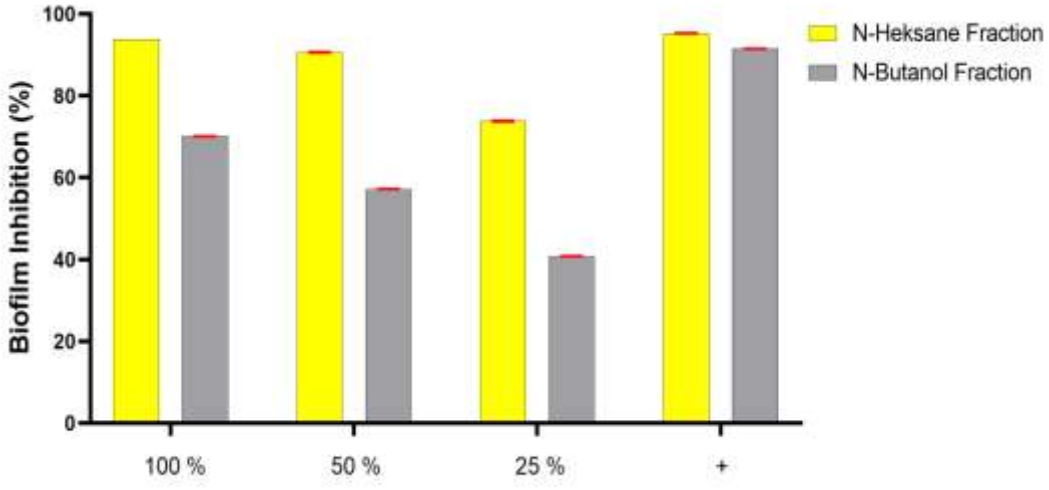


Fig. 1: Biofilm inhibition activity monomicrobial *Pseudomonas aeruginosa*

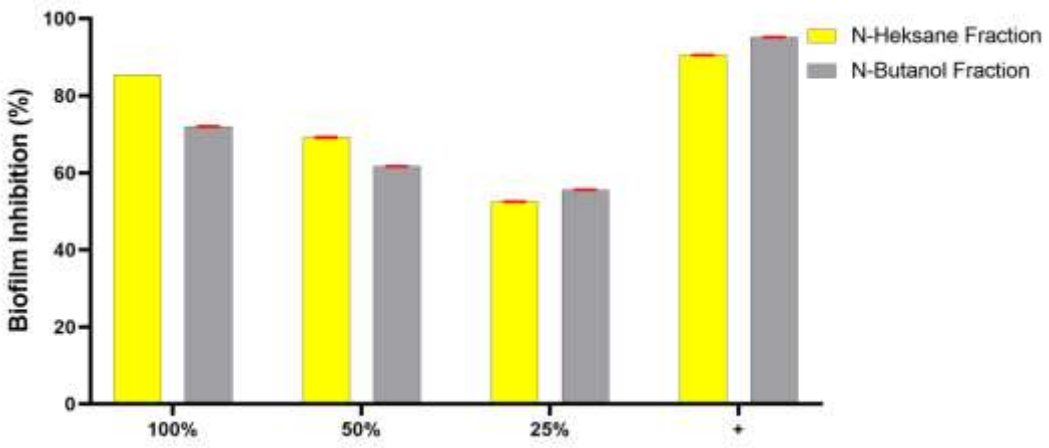


Fig. 2: Biofilm Inhibition activity monomicrobial *Escherichia coli*

In this result, it was reported that the N-hexanee fraction provided more significant biofilm formation inhibitory activity than the N-butanol fraction. The n-hexane 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-hexane 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-hexane and n-butanol fractions can inhibit the formation of *Pseudomonas aeruginosa* monomicrobial biofilms above 50%.

The study results in figure 2 show that the 100% n-hexane 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%, the n-hexane fraction has a biofilm formation inhibitory activity of 52.48% and at 25%, the N-butanol fraction has 55.95%. Biofilm inhibition mechanisms and planktonic inhibition mechanisms are different. In biofilms, bacteria together form a group or community to produce a more complex and robust defense, while in planktonic, 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[9].

The results of the biofilm inhibition test presentation show an increase in the percentage of biofilm inhibition against *Pseudomonas aeruginosa* and *Escherichia coli* bacteria, along with an increase in the concentration used. This indicates that the higher is the concentration, the higher is the biofilm inhibition produced. An increase in the potential inhibition of biofilm formation was found at a concentration of 100% n-hexane 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. Compared to the positive control, a concentration of 100% n-hexane fraction against *Escherichia coli* did not have a significant difference ($P>0.05$), meaning it does not have a meaningful difference. At a concentration of 100%, the N-butanol fraction has a significant difference ($p<0.05$) compared to the positive control, which means it has a considerable difference.

According to Miquel et al [21], plants have bioactive chemicals that can inhibit the process of bacterial attachment to solid surfaces and the formation of extracellular polymer (EPS) scaffolds. Kelubut leaves contain alkaloids, steroids, tannins, saponins, coumarins, tyrosine, glycine and flavonoids.

However, in this study, n-hexane and n-butanol fractionation was carried out, so the compounds of interest in thin n-hexane fractionation may be 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[24].

By attaching to the bacterial biofilm layer, saponins reduce the number of bacterial cells, 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 can inhibit the synthesis of peptidoglycan in bacterial cells, thus properly inhibiting the formation of cell wall layers.

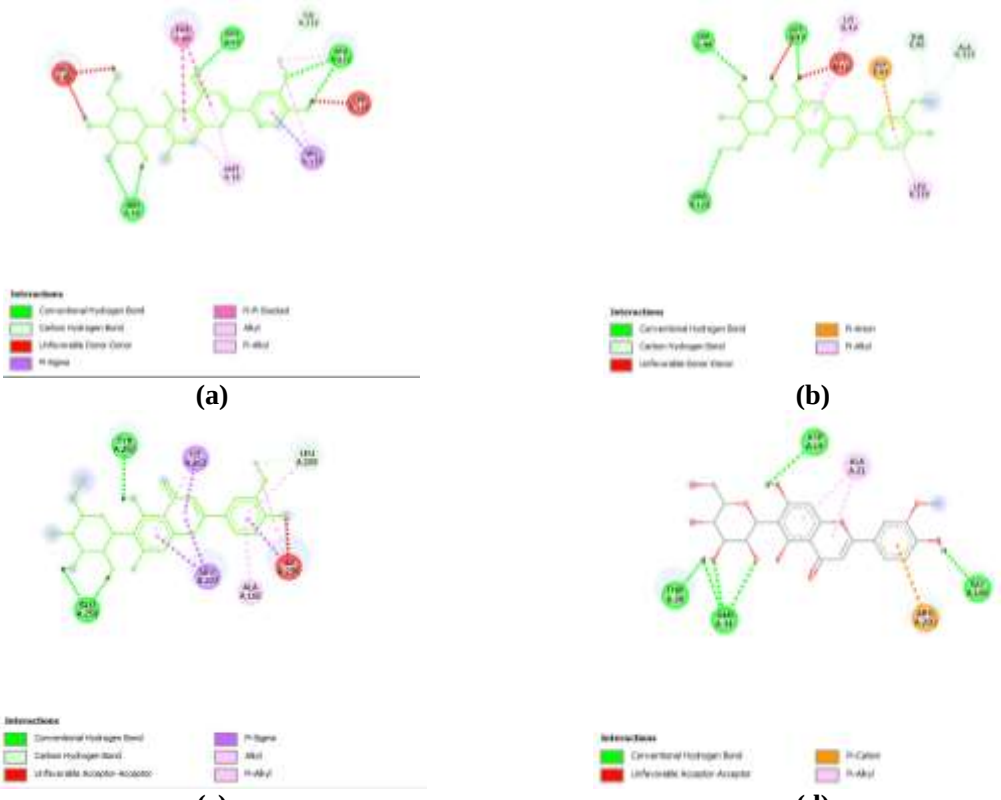
At the same time, flavonoids can interfere with energy transduction in the bacterial cytoplasmic membrane, inhibiting the production of ATP which is essential for bacterial life. Flavonoids can also inhibit bacterial motility by inhibiting flagellum synthesis and inhibiting enzymes involved in bacterial movement. Tannin compounds can cause cell damage by denaturing proteins[19].

Table 1
Docking score of different ligands with *Pseudomonas aerginosa* and Escherichia coli protein

S.N.	Ligan	*Pseudomonas aeruginosa*		*Escherichia coli*	
		5OE3	2UV0	1QJ8	4JVI
1.	Pantothenic acid	-5,5	-6,8	-5,1	-4,9
2.	Dihydroxycoumarin	-9,3	-7,7	-6,9	-6,8
3.	Phaselic acid (2-O-Caffeoylmalic acid)	-7,6	-6,6	-5,9	-6,0
4.	Isololiolide	-7,1	-6,8	-5,9	-5,8
5.	Vicenin-2 (Apigenin-6,8-di-C-glucoside)	-9,8	-7,9	-6,8	-7,1
6.	Loliolide	-6,5	-7,1	-5,8	-6,0
7.	Isoorientin (Luteolin-6-C-glucoside)	-9,7	-9,3	-7,3	-7,3
8.	Vitexin-2"-O-rhamnoside	-10,0	-7,8	-6,7	-7,4
9.	Isoscoparin (Scoparin)	-9,3	-9,8	-6,8	-7,5
10.	Isorhamnetin-3-O-glucoside	-9,5	-7,4	-6,5	-6,9

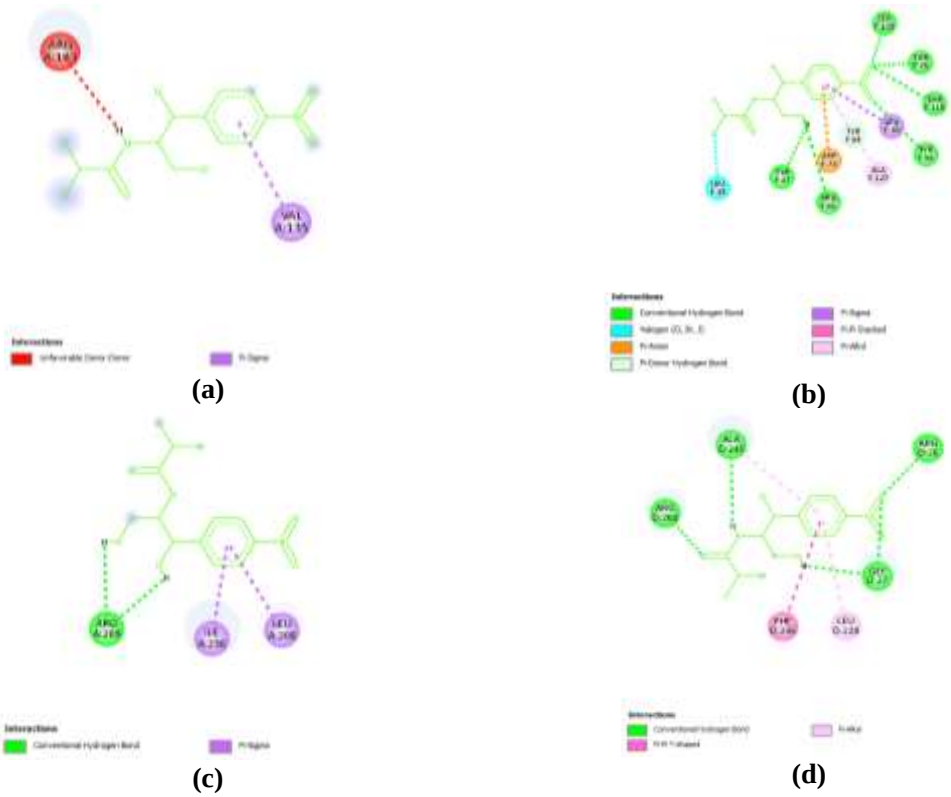
Table 2
Docking score of different Chloramphenicol with *Pseudomonas aerginosa* and *Escherichia coli* protein

Positive Control	*Pseudomonas aeruginosa*		*Escherichia coli*	
	5OE3	2UV0	1QJ8	4JVI
Chloramphenicol	-7,2	-8,9	-5,5	-6,1



(a) **(b)** **(c)** **(d)**

Fig. 3: 2D interactions of amino acids of Isoscoparin with (a) 1QJ8 protein, (b) 2UV0 protein, (c) 4JVI protein and (d) 5OE3 protein



(a) **(b)** **(c)** **(d)**

Fig. 4: 2D interactions of amino acids of Chloramphenicol with (a) 1QJ8 protein, (b) 2UV0 protein, (c) 4JVI protein and (d) 5OE3 protein

From the results of the *in silico* study, it is known that the isoscoparin compound has a relatively higher binding affinity as an inhibitor of the bacterial biofilm *Pseudomonas aeruginosa* and *Escherichia coli*. The binding affinity value obtained by isoscoparin, when compared with chloramphenicol as a positive control, has a greater value. The binding affinity obtained by isoorientin is -9.3 and -9.8 for each target protein in the *Pseudomonas aeruginosa* bacteria. For the target protein in the *Escherichia coli* bacteria, it is -6.8 and -6.9. Meanwhile, the positive control chloramphenicol has a binding affinity of -7.2 and -8.9 for *Pseudomonas aeruginosa* bacteria and -5.5 and -6.1 for *Escherichia coli* bacteria.

It can be seen that the amino acid bonds formed between isoscoparin and each target protein have several similarities with the bonds between amino acids formed from chloramphenicol and the target protein. The same amino acid bonds are ARG133, VAK135, UNK1, LEU208 and ILE236. This explains that the mechanism of action of chloramphenicol is similar to the isoscoparin ligand, so this ligand has the potential as an antibiotic in inhibiting biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* bacteria.

Conclusion

In conclusion, the available data show that the n-hexane fraction and n-butanol fractions of Kelubut leaves (*Passiflora foetida* L.) have inhibitory activity in monomicrobial biofilm formation *Pseudomonas aeruginosa* and *Escherichia coli*. N-hexane fraction is the most active fraction as it inhibits biofilm formation with the highest percentage of inhibition against *Pseudomonas aeruginosa*, 93.76% and against *Escherichia coli*, 85.36%. The n-hexane fraction when compared with the positive control chloramphenicol 1%, did not have a significant difference ($p>0.05$) in inhibiting *Pseudomonas aeruginosa* and *Escherichia coli* monomicrobial biofilm formation.

The N-butanol fraction also has an inhibitory activity of biofilm formation with a percent inhibition of 70.10% on *Pseudomonas aeruginosa* bacteria and 72.01% on *Escherichia coli* bacteria. However, the n-butanol fraction significantly differs from the positive control of chloramphenicol 1%.

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