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Antibacterial and Antibiofilm Activity Testing of Black Betel Leaves (*Piper betle* L. var. nigra) Ethanol Extract on *Staphylococcus aureus* and *Pseudomonas aeruginosa* using Microdilution Method

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Abstract

The use of antibiotics is key to treating infected wounds. However, its effectiveness has begun to decrease due to the high bacterial resistance, which is caused by the phenomenon of biofilm. Therefore, it's necessary to discover new antibacterial and antibiofilm activity agents that can be used as alternative therapies. This study aims to test the antibacterial and antibiofilm activity of black betel leaves (*Piper betle* L. var. nigra) ethanol extract. Antibacterial and antibiofilm activity test was conducted using the microdilution method against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with concentration variations 4; 2; 1; 0.5; 0.25; and 0.125 mg/mL, also used positive control kanamycin 10 mg/mL and negative control DMSO 1%. The results showed that the black betel leaves ethanol extract had low antibacterial activity with MIC_{50} > 4 mg/mL for both bacteria and vigorous antibiofilm activity with $MBIC_{50}$ < 0.125 mg/mL for *S. aureus* and MBIC = 0.491 mg/mL for *P. aeruginosa*. The antibacterial activity of the black betel leaves ethanol extract was lower than it's antibiofilm activity, the antibacterial and antibiofilm activities of the black betel leaves ethanol extract were higher against *S. aureus* than *P. aeruginosa*.

Keywords: Antibacterial, Antibiofilm, Piper betle L. var. nigra

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Penggunaan antibiotik masih menjadi kunci sebagai terapi pada luka yang terinfeksi. Namun, efektivitasnya mulai menurun karena tingginya kejadian resistensi bakteri terhadap antibiotik yang salah satunya disebabkan oleh fenomena pembentukan biofilm. Oleh karena itu, diperlukan upaya untuk mencari dan menemukan agen antibakteri dengan aktivitas antibakteri dan antibiofilm yang dapat digunakan sebagai terapi alternatif. Penelitian ini bertujuan untuk menguji aktivitas antibakteri dan antibiofilm dari ekstrak etanol daun sirih hitam (*Piper betle* L. var. nigra) Pengujian dilakukan dengan menggunakan metode mikrodilusi terhadap bakteri *Staphylococcus aureus* dan *Pseudomonas aeruginosa* dengan variasi konsentrasi 4; 2; 1; 0.5; 0.25; dan 0.125 mg/mL, serta menggunakan kontrol positif kanamisin 10 mg/mL dan kontrol negatif DMSO 1%. Hasil penelitian menunjukan ekstrak etanol daun sirih hitam memiliki aktivitas antibakteri lemah dengan nilai MIC > 4 mg/mL untuk kedua bakteri uji dan memiliki aktivitas antibiofilm yang kuat dengan nilai MBIC < 0,125 mg/mL untuk bakteri *S. aureus* dan nilai MBIC = 0,491 mg/mL untuk bakteri *P. aeruginosa*. Aktivitas antibakteri ekstrak etanol daun sirih hitam lebih lemah dibandingkan dengan aktivitas antibiofilmnya, selain itu aktivitas antibakteri dan antibiofilm ekstrak etanol daun sirih hitam lebih tinggi terhadap *S. aureus* dibandingkan *P. aeruginosa*.

Kata Kunci: Antibakteri, Antibiofilm, Piper betle L. var. nigra

INTRODUCTION

The phenomenon of biofilm formation by pathogenic bacteria often accompanies Diabetic Foot Ulcers (DFU). Two pathogenic bacteria usually appearing in DFU cases are *Staphylococcus aureus* (17.65%) and *Pseudomonas aeruginosa* (11.76%). Both of these bacteria are known to be able to form biofilm either alone or together [1]. Bacteria that can adhere and form biofilm can cause more serious problems, and this is due to the bacterial aggregation that can have a significant effect on the self-protection of the bacteria, thereby increasing the tolerance of bacteria to exogenous pressure and creating a barrier that leads to the failure of an antibiotic agent to eradicate the bacteria [2]. Biofilm formed by pathogenic bacteria became a serious concern in public health because cases of biofilm formation by pathogenic bacteria are involved in 65-80% of all bacterial infections that occur in humans [3]. Nowadays, the use of antibiotics is still a key primary therapy in treating DFU. However, its effectiveness is starting to decline due to the high incidence of resistance to one or more than two groups of antibiotics, one of which is caused by biofilm formation [4]. Therefore, it's necessary to find and discover another agent with antibacterial and antibiofilm activity that can be used as alternative therapies. Another thing that could be done is to explore the natural compounds [5].

Black betel is a plant widely used as a medication in Southeast Asia. Black betel (*Piper betle* L. var. nigra) is one of the types of betel plant found growing at Labanan, Berau Regency, East Kalimantan and has been widely used empirically by the local community as an antidiabetic, treat digestive disorders, hepatitis, anticholesterol, antihypertension, prevent strokes, etc [5]. Previous research on black betel leaf extract found that it has antibacterial activity against *Propionibacterium acnes* and *Streptococcus mutans* [6, 7]. In addition, other research has also showed that black betel leaf extract has antibacterial activity against *S. aureus* [8]. In this research, antibacterial and in vitro antibiofilm activity testing was carried out using the microdilution method against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Material

Sample preparation

Simplisia of black betel leaf (2.5 kg) was obtained from Berau Regency, Tanjung Redeb District, East Kalimantan. The leaves were dried in an oven and squeezed into small pieces.

Methods

Extraction

The dried leaves (2.5 kg), were extracted by ethanol 96% using the maceration method for 3 x 24 hours, then filtered using Buchner funnel and filtrate was obtained. The maceration was carried out with three repetitions. All filtrate was concentrated using a rotary evaporator at 70˚C, and crude extracts were obtained.

Media and bacteria preparation

Tryptic Soy Broth (TSB) 7.5 g dissolved in 250 mL aquadest. The media solution was then sterilized by autoclave at 121°C and 1 atm for 15 minutes. One ose of each bacterium (*S. aureus* and *P. aeruginosa*) was taken, put into 5 mL of TSB media in a conical tube, and then incubated for 16-20 hours at 37°C. After incubation, optical density measurements were conducted using a UV-Vis spectrophotometer at 600 nm wavelength [9,10].

Antibacterial activity test

Based on the protocol described by Haney [11], tubes 1-6 and 13-14 were added by bacteria. In the sample solution (tubes 1-6) a suspension of bacteria was added with a final concentration of 10^6 CFU/mL. No bacterial suspension was added in tubes 7-12 (blank). In tubes 13 (positive control) and 14 (negative control), a suspension of bacteria was added with a final concentration of 10^6 CFU/mL, while in tube 15 (media control), no bacteria were added. The amount of volume that was inserted into each well was 100 µL. After all wells on the plate were filled, the plate was closed and incubated for 16-20 hours at 37°C. After incubation, absorbance measurements were carried out using a microplate reader at 600 nm (OD_{600}) wavelength. The absorbance obtained is then inserted into the following equation 1:

$$\% \text{ inhibition} = \left(1 - \frac{(\text{Sample } OD_{600} - \text{Blanko } OD_{600})}{(\text{Average C. Bacteria} - \text{Average C. Media})}\right) \times 100\% \quad (1)$$

The antibacterial growth inhibition percentage calculation was averaged and plotted in a linear regression equation curve by entering the average inhibition percentage for all replications vs the testing concentration. Then, the dose-response curve and equation for obtaining the MIC_{50} value were obtained [11].

Antibiofilm activity test

The antibiofilm test uses the same plate as the antibacterial test, after measuring the absorbance, the plate was taken and stained with Crystal Violet (CV) 0,1% b/v. First, the contents inside the plate are discarded and rinsed using 120 µL of distilled water 3 times for each well using a multichannel pipette. Then,

the distilled water is discarded and the plate is dried. After the plate is dry, staining is carried out by adding 110 µL of 0,1% b/v CV solution to each well, then the plate is placed at room temperature for 30 minutes. After 30 minutes, the CV solution is discarded and rinsed 3 times using distilled water, then the plate is dried. Finally, add 120 µL of 96% ethanol to dissolve the CV and re-incubate at room temperature for 30 minutes with occasional shake. After incubation, absorbance measurements are conducted using a microplate reader at 595 nm (OD_{595}) wavelength [11]. The absorbance obtained is then inserted into the following equation 2:

$$\% \text{ Biofilm inhibition} = \left(1 - \frac{\text{Sample } OD_{600}\text{-Blanko } OD_{600}}{\text{(Average C. Bacteria-Average C. Media)}}\right) \times 100\% \quad (2)$$

The calculation of antibiofilm formation inhibition percentage was averaged and plotted in a linear regression equation curve by entering the average biofilm inhibition percentage for all replications vs the testing concentration, then the dose-response curve and equation for obtaining the $MBIC_{50}$ value were obtained [11].

Data analysis

Data were analyzed using data processing software and presented as each group's averages and standard deviation (SD). The data were tested for normality using Shapiro-Wilk, if the result is data normally distributed ($p > 0.05$), then the data can be continued testing using the one-way ANOVA test. From this test, it can be seen whether there is a significant difference between the testing groups or not, and if the data is not normally distributed, then the data can be analyzed using the Mann-Whitney test.

RESULTS AND DISCUSSION

Phytochemical profile identification

Black betel leaves simplisia (2.5 kg) were macerated using ethanol 96% with three repetitions. After being concentrated using a rotary evaporator, 413.98 g (16.56% yield) crude extract was obtained. The results of the phytochemical profile analysis of the black betel leaves ethanol extract were carried out using the Liquid Chromatography – High-Resolution Mass Spectrometry (LC-HRMS) method and 48 compounds were successfully identified. There were 9 compounds with best match confidence index above 90% that assumed it's present in the mixture was trustworthy (**Table** 1).

Two major compounds namely (6E)-7-(2H-1,3-benzodioxol-5-yl)-1-(piperidin-1-yl)hept-6-en-1-one and 5,7-dihydroxy-2-phenyl-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-8-(3,4,5-trihydroxyoxan-2-yl)-4H-chromen-4-one were identified as major compound in the sample with retention times 12.35 minutes (peak area 32.71%) and 6.60 (peak area 23.09%), but the confidence index were under 75%, so it was assumed that those compounds that had been identified were not valid. Meanwhile, 37 other compounds only have average peak area under 1% and also came with small confidence index, so it was not count either.

Table 1. Phytochemical profile analysis

No	Chemical Compounds	Formula Molecules	Retention time (Rt)	% area	Confidence index (%)	Groups
1	DL-Tryptophan	$C_{11}H_{12}N_2O_2$	2.355	0.21	97.1	Amino acid
2	Dibenzyl amine	$C_{14}H_{15}N$	5.709	2.12	93.4	Alkaloid
3	4-Indolecarbaldehyde	C_9H_7NO	6.383	0.27	92.2	Alkaloid
4	(-)-Caryophyllene oxide	$C_{15}H_{24}O$	10.334	0.16	94.4	Sesquiterpene
5	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	13.02	0.65	97.1	Sulfonate
6	α-Linolenic acid	$C_{18}H_{30}O_2$	14.594	2.73	99.2	Fatty acid
7	9(Z),11(E),13(E)-Octadecatrienoic Acid methyl ester	$C_{19}H_{32}O_2$	16.394	3.56	98.4	Ester, Fatty acid
8	Methyl palmitate	$C_{17}H_{34}O_2$	17.167	0.44	97.9	Fatty acid
9	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	17.567	2.29	93.4	Plasticizer

From all compounds with the highest confidence index α-Linolenic acid and 9(Z),11(E),13(E)-Octadecatrienoic acid methyl ester have known for it's bioactivity as antibacterial [12, 13]. Based on previous research related to the phytochemical screening of black betel leaves (*Piper betle* L. var. nigra), it was found that this plant contains secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins, tannins, and steroids with bioactivity such as antibacterial, anti-inflammatory, antioxidant etc [6, 14]. GC-MS analysis on black betel leaf ethanol extract also showed that it's contained compounds including phytol, patchouli alcohol, palmitic acid, 4-decenoic acid, and 9,12-octadecadienoic acid [5].

Antibacterial activity

Antibacterial testing was conducted using the microdilution method based on a linear turbidity factor to bacterial growth. This is seen based on the absorbance value, which is linear to the test concentration, so when the concentration value is higher, the antibacterial activity is more significant.

The antibacterial inhibition value was determined based on the absorbance value, which is calculated using Equation 1. Antibacterial test was using concentration variations of 0.125; 0.25; 0.5; 1; 2; and 4 mg/mL against *S. aureus* and *P. aeruginosa*, the inhibition value that have been obtained between all variations concentration for both bacteria were close without significant differences. So, this shows that the black betel leaf ethanol extract has low potential and relatively weak as an antibacterial.

The test concentration is linear to the inhibition value. In contrast, the absorbance value is inverse to the inhibition value, where if the test concentration is more significant, the absorbance value obtained will be smaller. The inhibition value received will be more excellent, so the antibacterial activity is more potent.

Table 2 show that inhibition values were obtained in concentration variations of 0.125; 0.25; 0.5; 1; 2; and 4 mg/mL on *S. aureus* in the range of 12 ± 2% - 23 ± 6%. The highest inhibition value was obtained at the test concentration of 0.25 mg/mL which was 23 ± 6%, while at the test concentration of 4 mg/mL only obtained an inhibition value of 13 ± 6%. Meanwhile, in the positive control 10 mg/mL kanamycin gave an inhibition value of 79 ± 2%, and in the negative control 1% DMSO had no inhibition.

On the other hand, inhibition value range of 5 ± 1% - 12 ± 1% was obtained in *P. aeruginosa*. The most significant inhibition value was obtained at a test concentration of 0.125 mg/mL which was 12 ± 1%, while at the largest test concentration 4 mg/mL only obtained an inhibition value of 5 ± 1%. Then, in the positive control 10 mg/mL kanamycin gave an inhibition value of 93 ± 1%, and in the negative control 1% DMSO had no inhibition. The inhibition value of both bacteria up to 4 mg/mL concentration did not reach the 50% inhibition value (MIC_{50}). In general, an extract can be assumed to have good antibacterial activity if it has an $MIC_{50} \leq 1$ mg/mL, while the MIC_{50} value obtained for both bacteria was higher than 4 mg/mL, so this shows that the black betel leaf ethanol extract has relatively weak antibacterial activity against both bacteria [15].

The inhibition value that has been obtained between all variations concentration for both bacteria were close and were statistically analysis. **Figure** 1 showed that there was no significant difference ($p > 0.05$) in antibacterial activity and inhibition values between all concentration variations against both bacteria (*S. aureus* and *P. aeruginosa*), but there was a significant difference ($p < 0.05$) between the percentage value of the concentration variations against the positive control and negative control for both bacteria.

Table 2. Antibacterial inhibition value

Bacteria Types	Testing Groups	Concentrations	Inhibition Value (%)				% Inhibition ± SD	MIC_{50}
S. aureus	Black Betel Extract	4 mg/mL	21	9	9	15	13 ± 6	> 4 mg/mL
		2 mg/mL	10	12	11	13	12 ± 2	
		1 mg/mL	16	14	20	19	17 ± 3	
		0.5 mg/mL	18	19	26	20	21 ± 3	
		0.25 mg/mL	15	24	31	21	23 ± 6	
		0.125 mg/mL	18	20	23	22	21 ± 2	
	Negative Control (DMSO 1%)	1%	0	0	0	0	0	-
	Positive Control (Kanamycin)	10 mg/mL	86	78	77	77	79 ± 2	-
P. aeruginosa	Black Betel Extract	4 mg/mL	5	4	6	6	5 ± 1	> 4 mg/mL
		2 mg/mL	9	10	9	13	10 ± 2	
		1 mg/mL	6	7	8	9	8 ± 1	
		0.5 mg/mL	10	11	9	9	10 ± 1	
		0.25 mg/mL	11	11	11	10	11 ± 0	
		0.125 mg/mL	12	13	11	10	12 ± 1	
	Negative Control (DMSO 1%)	1%	0	0	0	0	0	-
	Positive Control (Kanamycin)	10 mg/mL	94	93	92	92	93 ± 1	-

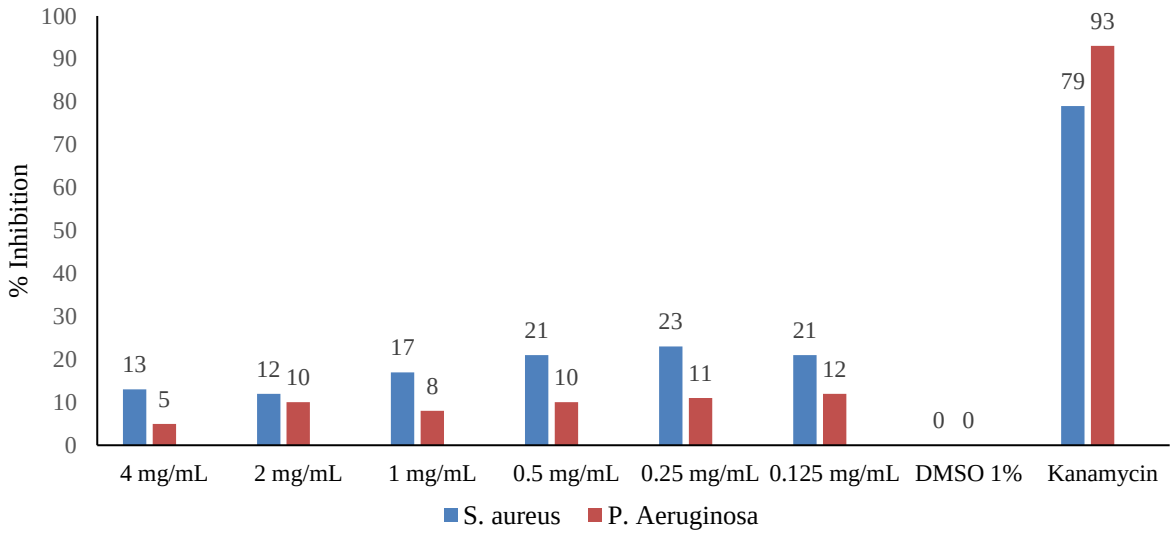

Figure 1. Antibacterial inhibition value

Other researchers have also reported the antibacterial activity of black betel leaf extract using different methods. Aprilia [6] reported that black betel leaf extract at concentration variations of 5%, 10%, 15%, and 20% w/v had an average inhibition zone diameter respectively of 15.1; 16.9; 17.5; and 18.59 mm against *Propionibacterium acne*. Other research by Prasetya [7] showed that black betel leaf extract with concentration variations of 0.5% b/v and 1% b/v had antibacterial activity against *Streptococcus mutans* and *Streptococcus sanguinis*. At a concentration of 0.5% b/v, the average inhibition zone diameter was 18.2 ± 1.8 mm (*S. mutans*) and 9.9 ± 1.8 mm (*S. sanguinis*). In comparison, at a concentration of 1% b/v, the average inhibition zone diameter was 19.6 ± 1.9 mm (*S. mutans*) and 12.3 ± 1.1 mm (*S. sanguinis*). In addition, other studies also showed that black betel leaf extract tested against *Escherichia coli* and *Staphylococcus aureus* bacteria had minimum inhibitory concentration (MIC) values respectively 0.015; 0.02; and 0.027 mg/mL [8].

Antibiofilm Activity

Antibiofilm testing was conducted using the microdilution method based on the turbidity factor of the dissolved biofilm (**Table** 3 and **Figure** 2). **Table** 3 shows that increasing test concentration causes a decrease in biofilm inhibition value, indicating increased antibiofilm activity against both bacteria. The biofilm formation inhibition value is determined using equation 2. **Figure** 2 shows that the inhibition values are linear to the variation concentration. At the highest concentration (4 mg/mL).

This data shows that antibacterial activity tests using different methods and different types of bacteria can give different results. The inhibition value was 88 ± 3% for *S. aureus* and 70 ± 3% for *P. aeruginosa*, while at the smallest concentration (0.125 mg/mL), the inhibition value was 54 ± 2% for *S. aureus* and 48 ± 3% for *P. aeruginosa*. These data indicate that the antibiofilm activity of black betel leaf extract is higher against *S. aureus* than *P. aeruginosa*.

Statistical analysis showed there was no significant difference ($p > 0.05$) in the absorbance values between all concentration variations against *S. aureus*, but there was a significant difference ($p < 0.05$) between the absorbance of the concentration variations against the positive control (kanamycin 10 mg/mL) and negative control (DMSO 1%). Meanwhile, for *P. aeruginosa* there was a significant difference ($p < 0.05$) in the absorbance values between all concentration variations as well as against the positive control and negative control.

Based on the inhibition value for *S. aureus* at a concentration of 4 mg/mL (88 ± 3%), there was a significant difference ($p < 0.05$) against the concentration of 0.125 mg/mL (54 ± 3%), but no significantly different ($p > 0.05$) with other test concentrations. Statistical analysis also showed a significant difference ($p < 0.05$) with the positive control (64 ± 1%). These data indicate that the extract with a concentration of 4 mg/mL provides higher antibiofilm activity than the positive control. Meanwhile, the statistical analysis of the inhibition value for *P. Aeruginosa* showed that at a concentration of 4 mg/mL, there was no significant difference ($p > 0.05$) with all concentration variations and the positive control. Thus, this indicates that the ethanol extract of black betel leaves has vigorous antibiofilm activity against *S. aureus* than *P. Aeruginosa.*

Table 3. Antibiofilm Inhibition Value

Bacteria Types	Testing Groups	Concentrations	Biofilm Inhibition Value (%)				% Biofilm Inhibition ± SD	$MBIC_{50}$
S. aureus	Black Betel Extract	4 mg/mL	93	88	85	85	88 ± 3	< 0.125 mg/mL
		2 mg/mL	74	72	68	73	71 ± 3	
		1 mg/mL	71	73	68	68	70 ± 3	
		0.5 mg/mL	72	74	73	72	73 ± 1	
		0.25 mg/mL	69	72	76	67	71 ± 4	
		0.125 mg/mL	53	53	55	56	54 ± 2	
	Negative Control (DMSO 1%)	1%	0	0	0	0	0	-
	Positive Control (Kanamycin)	10 mg/mL	65	65	64	63	64 ± 1	-
P. aeruginosa	Black Betel Extract	4 mg/mL	74	72	68	66	70 ± 3	0.491 mg/mL
		2 mg/mL	67	72	73	60	68 ± 6	
		1 mg/mL	67	68	55	57	62 ± 7	
		0.5 mg/mL	54	51	49	48	50 ± 3	
		0.25 mg/mL	52	48	52	43	48 ± 4	
		0.125 mg/mL	51	49	45	46	48 ± 3	
	Negative Control (DMSO 1%)	1%	0	0	0	0	0	-
	Positive Control (Kanamycin)	10 mg/mL	82	80	80	79	80 ± 1	-

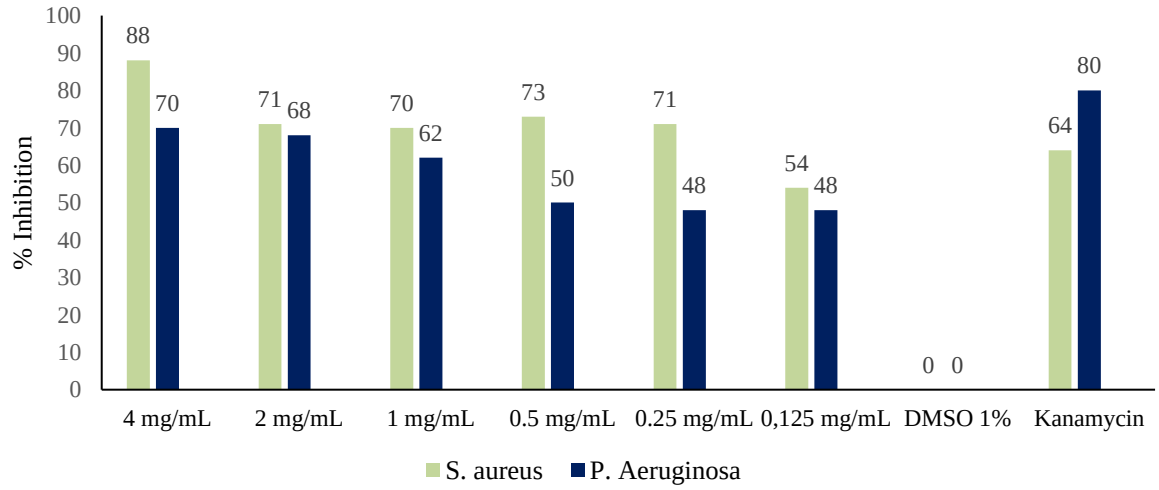

Figure 2. Antibiofilm Inhibition Value

Furthermore, the determination of the $MBIC_{50}$ value was carried out based on the regression equation of the relation between concentration variations and the biofilm formation inhibition percentage. The $MBIC_{50}$ for *S. aureus* was lower than 0.125 mg/mL, and $MBIC_{50}$ for *P. aeruginosa* was 0.491 mg/mL.

Based on the phytochemical profile analysis result, α-Linolenic acid and 9(Z),11(E),13(E)-Octadecatrienoic acid methyl ester was included to fatty acid group, which assumed to be the compounds that responsible for the antibacterial bioactivity. Research by Yuyama [16] showed that α-Linolenic acid could inhibit biofilm formation of gram-positive bacteria, especially against *Staphylococcus aureus*. Other research by Jung [17] also showed that α-Linolenic acid have antibiofilm activity against *Streptococcus mutans* by reduced viability in a dose dependent manner and reduced biofilm accumulation during initial and mature biofilm formation. Another possible mechanism was fatty acids inhibit biofilm development by affecting the adhering surface, changing cell membrane fluidity, reducing EPS, forming fimbriae or hypha, and modulating quorum sensing systems. Being natural molecules, fatty acids

have great potential to attenuate microbial biofilm formation and virulence without generating drug resistance [18].

Phytochemical screening of black betel leaves (*Piper betle* L. var. nigra) was reported to contain secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins, tannins, and steroids [5]. In this study, phytochemical analysis result using LC-HRMS showed that black betel leaf contains alkaloid and sesquiterpene compounds, which several plants alkaloids can inhibit biofilm formation and disperse bacterial biofilms including imidazoles, isoquinolines, piperidines, and pyrrolidines [19]. As said before, major compound in black betel leaf from this study based on the phytochemical analysis result was piperidines with peak area 32.71%, but the confidence index was only 73.9%. The low confidence index might be caused by the failure of detection. Sesquiterpene also known to have antibiofilm activity, (-)-Caryophyllene oxide have antibiofilm activity against *P. aeruginosa*, *P. fluorescens*, and *S. maltophilia* with the most prominent binding affinity [20]. Other research by Yoo and Jwa [21] showed that caryophyllene reduced the expression of *gtf* genes for *S. mutans*. Therefore, caryophyllene may have anti-biofilm activity and the inhibitory effect on biofilm related factor. Flavonoids are well known as antibacterial and antibiofilm agents against a wide range of pathogenic microorganisms [22]. Flavonoid compounds such as 6-aminoflavone (6-AF), 3,2-dihydroxyflavone (3,2-DHF), and 2,2-dihydroxy-4-methoxybenzophenone (DHMB) have been reported as inhibitors of biofilm formation. Flavonoids can disrupt preformed biofilm and suppress virulence properties, including motility, cell hydrophobicity, and aggregation. Flavonoids interfere with metabolic activities, cell division, and membrane permeability to exert antibiofilm effects [23]. Other research showed that terpenoid compounds such as carvacrol, thymol, and eugenol have antibacterial activity and effectively reduce biofilm formation [24, 25].

This study revealed the antibiofilm potential of black betel leaves ethanol extract against *S. aureus* and *P. aeruginosa*. Black betel leaves can be used as candidates to develop as an antibiofilm agent for effectively treating infectious bacterial diseases accompanied by biofilm formation.

CONCLUSION

Black betel leaf extracts up to a concentration of 4 mg/mL showed weak antibacterial activity against both *S. aureus* and *P. aeruginosa*, with bacterial growth inhibition percentage lower than 50%. The antibiofilm test showed vigorous antibiofilm activity where at a concentration of 4 mg/mL the biofilm formation inhibition percentage was 88 ± 3% for *S. aureus*, which was higher than the positive control, and the $MBIC_{50}$ < 0.125 mg/mL. Meanwhile, vigorous antibiofilm activity was also obtained for *P. aeruginosa* at a concentration of 4 mg/mL (70 ± 3%) with $MBIC_{50}$ = 0.491 mg/mL.

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REFERENCES

[1] A. C. Afonso, D. Oliveira, M. J. Saavedra, A. Borges, and M. Simões, "Biofilms in Diabetic Foot Ulcers: Impact, Risk Factors and Control Strategies," *IJMS*, vol. 22, no. 15, p. 8278, 2021.

[2] C. Rizzo *et al.*, "Antibiofilm Activity of Antarctic Sponge-Associated Bacteria against *Pseudomonas aeruginosa* and *Staphylococcus aureus*," *JMSE*, vol. 9, no. 3, p. 243, 2021.

[3] C. Liu, J. Lu, L. Lu, Y. Liu, F. Wang, and M. Xiao, "Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1," *Bioresource Technology*, vol. 101, no. 14, pp. 5528–5533, 2010.

[4] G. Boschetti *et al.*, "Antimicrobial Resistance Patterns in Diabetic Foot Infections, an Epidemiological Study in Northeastern Italy," *Antibiotics*, vol. 10, no. 10, p. 1241, 2021.

[5] M. Rizki and A. Fernandes, "Profil Fitokimia Dan GC-MS Daun Sirih Hitam (*Piper betle L.*) Dari Sekitar KHDTK Labanan, Kabupaten Berau," *Majalah Farmasi dan Farmakologi*, vol. 25, no. 1, pp. 11–14, 2021.

[6] F. E. Aprilia, M. Ardana, and H. Kuncoro, "Pengaruh Lama Penyimpanan Ekstrak Etanol Daun Sirih Hitam (*Piper betle L. var Nigra*) Terhadap Aktivitas Antibakteri," *Proceeding of*

Mulawarman Pharmaceuticals Conferences, 2021.

[7] F. Prasetya *et al.*, "Novel Amides Derivative with Antimicrobial Activity of *Piper betle var. nigra* Leaves from Indonesia," *Molecules*, vol. 26, no. 2, p. 335, 2021.

[8] Junairiah, N. Matuzahroh, N. I. Zuraidassanaaz, and L. Sulistyorini, "Antifungal and Antibacterial Activity of Black Betel (*Piper betle L. var Nigra*) Extract," *Bioscience Research*, vol. 14, no. 4, pp. 750–755, 2017.

[9] Z. Ye *et al.*, "Effects of daphnetin on biofilm formation and motility of *pseudomonas aeruginosa*," *Front. Cell. Infect. Microbiol.*, vol. 12, p. 1033540, 2022.

[10] R. Zoraghi *et al.*, "Identification of Pyruvate Kinase in Methicillin-Resistant *Staphylococcus aureus* as a Novel Antimicrobial Drug Target," *Antimicrob Agents Chemother*, vol. 55, no. 5, pp. 2042–2053, 2011.

[11] E. F. Haney, M. J. Trimble, and R. E. W. Hancock, "Microtiter plate assays to assess antibiofilm activity against bacteria," *Nat Protoc*, vol. 16, no. 5, pp. 2615–2632, 2021.

[12] D. Kusumah, M. Wakui, M. Murakami, X. Xie, K. Yukihito, and I. Maeda, "Linoleic acid, α-linolenic acid, and monolinolenins as antibacterial substances in the heat-processed soybean fermented with *Rhizopus oligosporus*," *Bioscience, Biotechnology, and Biochemistry*, vol. 84, no. 6, pp. 1285–1290, 2020.

[13] I. Prost *et al.*, "Evaluation of the Antimicrobial Activities of Plant Oxylipins Supports Their Involvement in Defense against Pathogens," *Plant Physiology*, vol. 139, no. 4, pp. 1902–1913, 2005.

[14] F. Prasetya, M. Arifuddin, and A. Ibrahim, "Identifikasi Senyawa Marker Dominan Ekstrak Daun Sirih Hitam dan Kuantifikasi Berdasarkan Perbedaan Lokasi Tanam: Identification of Dominant Marker Compounds of Black Betel Leaf Extract and Quantification Based on Differences in Planting Locations," *J. Sains. Kes.*, vol. 3, no. 2, pp. 147–157, 2021.

[15] N. Aligiannis, E. Kalpoutzakis, S. Mitaku, and I. B. Chinou, "Composition and Antimicrobial Activity of the Essential Oils of Two *Origanum* Species," *J. Agric. Food Chem.*, vol. 49, no. 9, pp. 4168–4170, 2001.

[16] K. T. Yuyama, M. Rohde, G. Molinari, M. Stadler, and W.-R. Abraham, "Unsaturated Fatty Acids Control Biofilm Formation of *Staphylococcus aureus* and Other Gram-Positive Bacteria," *Antibiotics*, vol. 9, no. 11, p. 788, 2020.

[17] J.-E. Jung, S. Pandit, and J.-G. Jeon, "Identification of linoleic acid, a main component of the *n* -hexane fraction from *Dryopteris crassirhizoma*, as an anti-*Streptococcus mutans* biofilm agent," *Biofouling*, vol. 30, no. 7, pp. 789–798, 2014.

[18] P. Kumar, J.-H. Lee, H. Beyenal, and J. Lee, "Fatty Acids as Antibiofilm and Antivirulence Agents," *Trends in Microbiology*, vol. 28, no. 9, pp. 753–768, 2020.

[19] M. E. Skogman, J. Kujala, I. Busygin, R. Leino, P. M. Vuorela, and A. Fallarero, "Evaluation of Antibacterial and Anti-biofilm Activities of Cinchona Alkaloid Derivatives against *Staphylococcus aureus*," *Natural Product Communications*, vol. 7, no. 9, p. 1934578X1200700917, 2012.

[20] N. L. Vuković *et al.*, "The antimicrobial and antibiofilm potential of the *Piper nigrum L.* essential oil: in vitro, in situ, and in silico study," *Industrial Crops and Products*, vol. 209, p. 118075, 2024.

[21] H.-J. Yoo and S.-K. Jwa, "Inhibitory effects of β-caryophyllene on Streptococcus mutans biofilm," *Archives of Oral Biology*, vol. 88, pp. 42–46, 2018.

[22] F. Farhadi, B. Khameneh, M. Iranshahi, and M. Iranshahy, "Antibacterial activity of flavonoids and their structure–activity relationship: An update review," *Phytotherapy Research*, vol. 33, no. 1, pp. 13–40, 2019.

[23] O. S. Faleye, J.-H. Lee, and J. Lee, "Selected flavonoids exhibit antibiofilm and antibacterial effects against Vibrio by disrupting membrane integrity, virulence and metabolic activities," *Biofilm*, vol. 6, p. 100165, 2023.

[24] H.-C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice, and S. Kjelleberg, "Biofilms: an emergent form of bacterial life," *Nat Rev Microbiol*, vol. 14, no. 9, pp. 563–575, 2016.

[25] T. Yamaguchi, "Antibacterial effect of the combination of terpenoids," *Arch Microbiol*, vol. 204, no. 8, p. 520, 2022.