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Antibacterial activity of Mahkota Dewa (Phaleria macrocarpa) leaves extract against Propionicabacterium

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Abstract: Acne vulgaris is a chronic inflammation of the pilosebaceous unit with various clinical manifestations caused by bacterial colonization of *Propionibacterium acnes*. Various studies have been performed to improve the modality of acne vulgaris therapy with natural product, such as leaves of Mahkota Dewa (*Phaleria macrocarpa*). This experimental study aims to investigate the effect of the *Phaleria macrocarpa* leaves extract against *P. acnes* by using Disc Diffusion method and Inhibition of Biofilm formation. Research results showed that the Total Flavonoid and Tannin content were 953.10 mg QE/gr DW and 42.67 mg TAE/gr DW, respectively. The *Phaleria macrocarpa* leaves extract had a significant antibacterial effect on *P. acnes* bacteria by using disc diffusion method ($p < 0.05$), with the widest inhibition zone diameter found at a concentration of 90 ppm (19.20 mm) and the narrowest was 30 ppm (14.20 ppm). *Phaleria macrocarpa* leaves extract also significantly inhibited the formation of *P. acnes* biofilms, where the highest inhibition activity was found at a concentration of 90 ppm (71.58 ± 1.49%) and the lowest was found at a concentration of 30 ppm (58.24 ± 2.52%). *Phaleria macrocarpa* leaves extract has showed an antibacterial effect against *P. acnes* and promise a potential use of acne vulgaris therapy.

Keywords: Mahkota Dewa, Disc diffusion, Inhibitory effect, Biofilm, *Propionibacterium acnes.*

INTRODUCTION

Skin is the outermost organ that lines the outer human body. Thus, the skin can receive many external stimulations, such as touch, pain, or other harmful stimulations.[1] Globally, it was reported that the 117.4 million incident cases of acne vulgaris in 2019 among 204 countries where as China, India, Indonesia, Nigeria and the USA were the top five countries for the number of prevalent cases (more than 8·0 million).[2] Controlling acne burden by developing more effective drug and therapies is one of the most important strategy. Acne vulgaris is a chronic inflammation of pilosebaceous follicles with various clinical manifestations, including comedo, papules, pustules, and nodules. Acne vulgaris is not a life-threatening disease. However, this disease quietly affects the quality of life and reduces beauty and wellness. Acne vulgaris most commonly found among adolescents aged 15-18 years old and peaks at 17-21 years old. The predilection of acne vulgaris was the face, shoulders, neck, chest, upper back, and upper arms. [3] Some factors affect acne vulgaris, including genetic, dietary, weather, endocrine, psychological, bacterial, host immunity, and other chemicals. [3,4] There are various causes of acne vulgaris, and one of them is *Propionibacterium acnes*. It is a gram-positive bacterium that also acts as normal flora found in sebaceous glands. [5] This bacterium has a high rate of growth, especially during puberty during adolescence, due to the increase of androgen activity that stimulates the growth of sebaceous gland and leads to increased sebum production. [1,6]

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In many countries, medicinal plants have been used as the traditional medicine in any diseases. Various plants were reported for their potential effect against acne vulgaris. One of medicinal plants that potentially observed is Mahkota dewa (*Phaleria macrocarpa*) due to its phytochemical contents.[7] It's fruit has various health benefits like skin diseases, cancer, sexual dysfunction, liver and kidney disorders, hypoglycemia, hypotension, and antirheumatic. All parts of *Phaleria macrocarpa* can be used as an herb therapy, including fruit, seeds, stems and leaves.[8] The part of fruit has been reported as angiotensin converting enzyme (ACE) inhibitors.[9] Another study used ultrasound assited extraction process to optimize the potential of fruit for antioxidants and anti-gout.[10] The hexane extract of stem showed a higher α-glucosidase inhibitory activity other than flesh and leaves extracts.[11] Another study confirmed the α-glucosidase inhibitory activity may relate to bioactive compounds such as upenone, swertianolin, m-coumaric acid, pantothenic acid, and 8-C-glucopyranosyleriodictylol.[12] A comparative study of antiproliferative effects of different parts of plants included pericarp, mesocarp, seed and leaf showed that that *Phaleria macrocarpa* leaves could inhibit the proliferation of T47D cells and trigger apoptosis through caspase-3 activation and Bax/Bcl regulation. Therefore, *Phaleria macrocarpa* leaves can be used for breast cancer therapy.[13] A study on the antibacterial activity of different parts of *Phaleria macrocarpa* fruit showed a weak ability to moderate antibacterial activity against pathogenic tested bacteria (inhibition range: 0.93–2.17 cm) at concentration of 0.3 mg/disc. The anti fungi activity was only found in seed extract against *Aspergillus niger* (1.87 cm) at concentration of 0.3 mg/well.[14] However, the potential use of *Phaleria macrocarpa* leaves as antibacterial agents especially *Propionibacterium. acnes* is still unexplored recently.

This research is more focused on the leaf parts. The leaves of *Phaleria macrocarpa* have various phytochemical compounds like saponins, alkaloids, flavonoids, tannins, lignins, resins and benzophenones. These phytochemicals have a well antibacterial effect that can inhibit the growth of many bacteria, one of these bacteria was *Propionibacterium acnes*.[15,16] Another study also showed a similar results that the *Phaleria macrocarpa* leaves could inhibit biofilm formation from *Streptococcus mutans* by Congo Red Agar method with an effective concentration of 0.0009%.[17] Few studies looked for the health benefits of *Phaleria macrocarpa* leaves. The previous study only focused on investigating the health benefits of other parts of *Phaleria macrocarpa* with few numbers of bacteria. Hence, this study was performed to measure the phytochemical level of *Phaleria macrocarpa* Extract, especially tannin and flavonoid, and to investigate the inhibition biofilm formation effects of *Phaleria macrocarpa* Extract against *Propionibacterium acnes* bacteria, as one of the microorganisms that contaminated acne lesion.

MATERIAL AND METHOD

The experimental study was performed in Microbiology Laboratory, Universitas Sumatera Utara, in September-October 2022. *Phaleria macrocarpa* leaves, phytochemical reagent, aluminum chloride (AlCl3), tannic acid, quercetin, sodium carbonate, folin-cioucalteu reagent, methanol, Sodium Hydroxide (NaOH), hydrochloric acid (HCl), ether, natrium nitrite, disc diffusion, PBS, DMSO, NA, acetic acid, crystal violet, *Propionibacterium acnes* suspension, distilled water, Mueller Hinton Agar (MHA), Hydrogen peroxide (H_2O_2).

The fresh *Phaleria macrocarpa* leaves were collected from a local plantation. Then, these leaves were cleaned and dried in a drying cabinet for three days. The dried *Phaleria macrocarpa* leaves meshed into simplicial powder. The simplicial powder was macerated into 96% ethanol in a ratio of 1: 10 for three days, which was regularly stirred daily. After three days, it was filtered, and a rotary evaporator evaporated the filtrate at 40^{o}C. Phytochemical screening was performed to investigate the presence of some phytochemicals, including

flavonoid, alkaloid, triterpenoid-steroid, tannin, and saponin. Total flavonoid and tannin contents were also measured.

The concentrated *Phaleria macrocarpa* leaves extract was diluted by DMSO to form various extract concentrations. Initially, the stock solution dissolved by 0.1gram (100 mg) of the *Phaleria macrocarpa* Leaves into 100 ml of distilled water in a 100 ml volumetric flask. Then, amount of 2.25 ml, 1.825 ml, 1.5 ml, 1.125 ml, and 0.75 ml of stock solution was dissolved into 25ml distilled water to form concentrations of 30 ppm, 45 ppm, 60 ppm, 75 ppm, and 90 ppm by 25 ml volumetric flask, respectively. The negative and positive controls were 6% hydrogen peroxide solution and distilled water, respectively. A volumetric flask made the positive control by dissolving 2 ml of 30% hydrogen peroxide solution (Merck®) in 10 ml of distilled water.

The bacterial suspension was made by taking a colony of *Propionibacterium acnes* bacteria into a normal saline solution. Then, it was centrifuged by centrifugation two times. After that, the turbidity of the suspension was compared with the McFarland standard. This study used 0.5 McFarland Standard, indicating a bacterial density of 1.5 x 108 CFU/ ml. The preparation of bacterial media was performed based on the MHA manufacturer's instructions. It was made by dissolving 38 grams of MHA powder in a liter of distilled water, then heating and stirring with a magnetic stirrer until homogeneous. After that, the media was sterilized by autoclave at a temperature of 121^{o}C and a pressure of 1.5 atm for 15 minutes.

The disc diffusion assay was performed by streaking the bacteria into the surface of the MHA media with a sterile cotton swab. On the other hand, all disc papers were diffused into the various concentration of *Phaleria macrocarpa* leaves extract, negative, and positive control. These disc papers were then placed on the surface of these MHA. Finally, all Petri dishes were incubated at 30^{o}C for 24 hours and the inhibition zone was measured by a caliper.

Biofilm formation assay used Microtiter Plate Biofilm Assay methods in microplate flexible U-bottom PVC 96-well. Ten microliters of each concentration of *Phaleria macrocarpa* leaves extract, negative, and positive control were filled into each column, followed by adding 10 µL of bacterial suspension, and it was incubated for 24 hours at 37^{o}C. After 24 hours, the microplate was washed with sterile Phosphate Buffer Saline (PBS) three times. Then, it was added by 200 µL of methanol for 15 minutes, discarded, and dried. After that, it was added by 200 µL of 2% crystal violet, waited for five minutes, washed the microplate with PBS, and added 200 µL of 33% glacial acetic acid. Finally, the biofilm formation was measured by spectrophotometry at a wavelength of 570nm, and it was expressed as an absorbance or optical density (OD).

All data were analyzed by descriptive statistics, including central tendency and dispersion. Then the analysis was continued with inferential statistical analysis. Total flavonoid and tannin contents were analyzed by simple linear regression to get the standard solution curve of each standard solution. At the same time, the antibacterial data is expressed as the width of the inhibition zone and percent of biofilm formation inhibition. Data obtained was examined with the One-Way ANOVA for the analysis of variances, followed by the non-parametric Mann Whitney. The Tukey HSD Post Hoc Test was used to compare all pairs of mean treatments.

RESULTS AND DISCUSSION

The *Phaleria macrocarpa* leaves extract underwent a phytochemical analysis consisting of phytochemical screening followed by total tannin and flavonoid content measurements. The phytochemical screening results are described in Table 1 and Figure 1.

Table 1 Phytochemical screening of *Phaleria macrocarpa* Leaves Extract

Phytochemicals	Reagent	Result	Interpretation
Flavonoid	$HCl_{(aq)}$ + $Mg_{(s)}$	Orange	Positive
Alkaloid	Wegner	Yellow-colored Sedimentation	Positive
	Mayor	Brown-colored Sedimentation	Positive
	Dragendorff	Brown-colored Sedimentation in Red solution	Positive
Triterpenoid-Steroid	LP Baucardat	Reddish brown-colored	Positive
Tannin	Etanol 70% $_{(aq)}$+ $FeCl_{3(s)}$	Darkish green-colored	Positive
Saponin	Distilled Water	Foaming	Positive

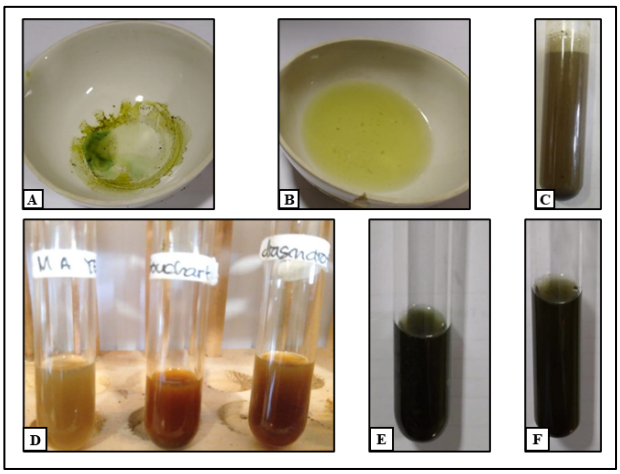

Figure 1: Phytochemical Screening of *Phaleria macrocarpa* Leaves Ethanol Extract for (A) Steroid; (B) Flavonoid; (C) Saponin; (D) Alkaloid; (E) Tannin; (F) Polyphenol.

Based on Table 1 and Figure 1, phytochemical screening showed that the *Phaleria macrocarpa* leaves extract had some phytochemicals, including flavonoid, alkaloid, triterpenoid-steroid, tannin, and saponin. After that, the phytochemical analysis was continued to measure the total flavonoid and tannin content described in Table 2.

Table 2 Total Flavonoid and Tannin Content of *Phaleria macrocarpa* Leaves Extract

Phytochemicals	Value
Total Flavonoid Content (mg QE/ gr DW)	953.10
Total Tannin Content (mgTAE/ gr DW)	42.67

Based on Table 2, the total flavonoid and tannin contents were 953.10 mg QE/ gr DW and 42.67 mg TAE/ gr DW, respectively. Then, the analysis can be continued to evaluate the antibacterial activity of *Phaleria macrocarpa* Leaves extract.

The antibacterial activity of *Phaleria macrocarpa* leaves extract was evaluated in two different methods: disc diffusion assay and biofilm formation assay. The disc diffusion assay was expressed as the Width of the Inhibition Zone in millimeters, and the width of the Inhibition zone was described in Table 3.

Table 3 Comparison of Antibacterial Activity in All Concentration Based on Disc Diffusion Assay

Concentration	Width of Inhibition Zone (mm)			*P-Value*
	Median	Min	Max	
30 ppm [a]	14.20	14.00	14.30	0.003
45 ppm [b]	15.70	15.60	15.90	
60 ppm [c]	16.80	16.50	17.00	
75 ppm [d]	18.00	17.90	18.30	

90 ppm [e]	19.20	19.00	19.20
Positive Control [e]	20.30	20.00	20.50
Negative Control [d]	6.00	6.00	6.00

P-value was obtained from the Kruskal-Wallis; Different superscripts in the same column show a significant difference based on the Mann-Whitney

Based on Table 3, it can be seen that there was a significant difference in the diameter of the inhibition zone in all concentrations (P value < 0.05). The widest inhibition zone was found in the negative control group (20.30 mm), then followed by the 90 ppm (19.20 mm), 75 ppm (18.00 mm), 60 ppm (16.80 mm), 45 ppm (15.70 mm), and the narrowest inhibition zone was found in 30 ppm, that was 14.20 mm. Meanwhile, the positive control group did not show any clear zone as the inhibition zone. Hence the inhibition zone in the positive control group in Table 1 was expressed as the wide of the disc diffusion (6 mm). Then, the antibacterial assay was continued to the biofilm formation assay. The formation of clear zone as the inhibition zone in petri dishes were described in Figure 2.

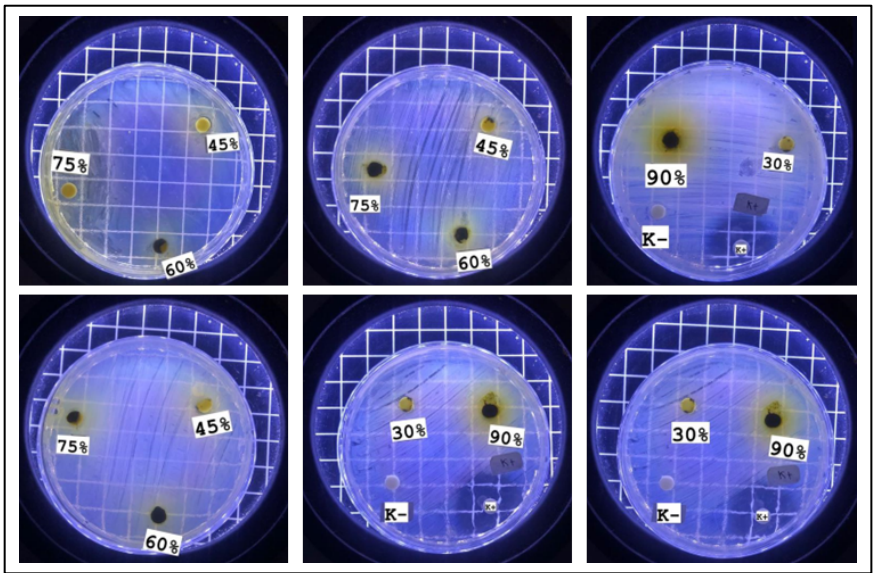

Figure 2: The Formation of Inhibition Zone of All Concentrations and Control Groups

Biofilm formation assay in *Propionibacterium acne* was performed by measuring the opacity of the broth media. The opacity was expressed as Optical Density in 600nm (OD600), obtained by spectroscopy. This OD600 was then compared to the control value to obtain the percentage of biofilm formation inhibition. The OD600 value in all concentrations is described in Table 4.

Table 4 Comparison of OD600 Biofilm in All Concentrations of *Phaleria macrocarpa* Leaves Extract

Concentration	OD600 Biofilm		*P-Value*
	Mean	SD	
30 ppm[d]	0.174	0.003	
45 ppm[c]	0.144	0.006	
60 ppm[bcd]	0.161	0.017	
75 ppm[ab]	0.120	0.002	< 0.05
90 ppm[a]	0.119	0.009	
Kontrol Positif[a]	0.115	0.012	
Kontrol Negatif[e]	0.234	0.006	

P-Value was obtained from the One Way ANOVA; Different superscripts in the same column show a significant difference based on the Tukey HSD Post Hoc Test

Based on Table 4 above, the OD600 value of all groups showed some significant differences (P value < 0.05). The OD600 value is inversely proportional to the concentration of *Phaleria macrocarpa* leaves extract. The lowest concentration showed the highest OD600 value among the other concentration of *Phaleria macrocarpa* leaves extract. However, the lowest concentration of *Phaleria macrocarpa* leaves did not show a higher OD600 value than the negative control group, which did not receive any treatment. Meanwhile, the OD600 value at the highest test concentration (90 ppm) showed the lowest value compared to the lower concentration of *Phaleria macrocarpa* leaves extract. However, the OD600 value was not lower than the positive control group, which received 30% Hydrogen Peroxide. The higher OD600 value indicates higher bacterial growth and biofilm formation. The biofilm formation inhibition was expressed as a percent, and the percentage of biofilm formation inhibition was described in Table 5.

Table 5 Comparison of Biofilm Inhibition Activity in All Concentration of *Phaleria macrocarpa* Leaves Extract

Concentration	Percentage of Biofilm Formation Inhibition (%)		*P value*
	Mean	**SD**	
30 ppm[a]	58.24	2.52	
45 ppm[ab]	65.41	3.01	
60 ppm[a]	61.20	5.58	
75 ppm[b]	71.29	1.25	< 0.05
90 ppm[b]	71.58	1.49	
Positive Control [b]	72.25	3.96	
Negative Control [c]	43.74	3.75	

P-Value was obtained from the One Way ANOVA; Different superscripts in the same column show a significant difference based on the Tukey HSD Post Hoc Test

Based on Table 5, it can be seen that there was a significant difference in the percentage of biofilm inhibition against Propionibacterium acne among all concentrations of extract (P value < 0.05). The concentration extract change did not significantly affect the biofilm formation inhibition activity, according to the Post Hoc Test Tukey HSD. The lowest biofilm inhibition activity was found in the negative control (43.74 ± 3.75%), followed by the 30 ppm (58.24 ± 2.52%), 60 ppm (61.20 ± 5.58%), 45 ppm (65.41 ± 3.01%), 75 ppm (71.29 ± 1.25%), 90 ppm (71.58 ± 1.49%), and the highest was in the positive control group (72.25 ± 3.96%). The two lowest concentrations (30 ppm and 45 ppm) showed no significant difference in biofilm inhibition activity. It was similar to the two highest concentrations (75 pp and 90 ppm). Thus, the best inhibition biofilm formation was found in the two highest *Phaleria macrocarpa* leaves extracts (75 pp and 90 ppm), that was as well as the positive control group.

It can be obviously seen that the *Phaleria macrocarpa* leaves ethanol extract contains various phytochemical compounds, including flavonoids, alkaloids, triterpenoids, steroids, tannins, and saponins. The total flavonoid and tannin content from *Phaleria macrocarpa* leaves ethanol extract were 953.10 mg QE/gr DW and 42.67 mg TAE/gr DW, respectively. These compounds showed an antibacterial activity against *Propionibacterium acne* by inhibiting the growth and biofilm formation. This most potent antibacterial effect was observed from the width of inhibition zone and percentage of biofilm inhibition in the highest concentration extract, that were 19.20 mm and 71.58%, respectively.

Phytochemical analysis of the current study also showed a similar result to some previous studies. Salih et al. (2016) reported that *Phaleria macrocarpa* leaves aqueous-methanol extract has several phytochemicals such as alkaloids, saponins, flavonoids, tannins, reduced-sugars, terpenoids, cardiac glycosides, and phenols. However, Salih et al. also reported that the *Phaleria macrocarpa* leaves aqueous-methanol extract did not contain steroids according to the Lieberman-

Burchard test, while in this analysis, steroids were detected by a similar method. The difference in the results of this study was due to the difference in the solvent used in this study with the previous study performed by Salih et al. (2016).[18]

In a previous study conducted by Salih et al. (2016), the extraction process was performed by diluted methanol with distilled water in a ratio of 3:4. Meanwhile, in the current study, the solvent used was 96% ethanol. The dilution of solvent by distilled water increased the polarity of the solvent, while steroids are compounds with low polarity. Therefore, adding distilled water to the solvent will reduce the effectiveness of the solvent in pulling steroids from dry simplicia. The best solvents for extracting steroids are solvents with semi-polar to non-polar polarities, such as ethyl acetate or n-hexane. However, this study focuses on exploring the benefits of the phytochemical content that tends to be polar in the *Phaleria macrocarpa* leaves extract. Hence, this study was focused on analyzing the total content of polar compounds present in *Phaleria macrocarpa* leaves extract, including flavonoids and tannins, while none of the previous studies looked for either total flavonoid or tannins content. [18–20]

Various studies have also been performed to analyze the antibacterial activity of the *Phaleria macrocarpa* Leaves. Othman et al. (2014) reported that the *Phaleria macrocarpa* Leaves extract with various solvents, including methanol, ethyl acetate, dichloromethane, and n-hexane, have some antibacterial effects against various gram-negative and gram-positive bacteria**.** Furthermore, Othman et al. demonstrated the antibacterial effect of *Phaleria macrocarpa* Leaves extract against gram-positive bacteria like *Bacillus subtilis* and *Staphylococcus aureus* and gram-negative bacteria like *Escherichia coli* and *Pseudomonas putida*. These antibacterial effects were expressed as a clear zone formation in the media. The width of the clear zone for *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas putida* were 6.3-7.3 mm and 6.1-7.3 mm, 6.0 – 6.4 mm, and 6.0-6.3 mm, respectively. On the other hand, Othman et al. also analyzed the antibacterial activity using the dilution method to determine the MIC value of the *Phaleria macrocarpa* Leaves Extract against bacteria *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas putida*, which ranged between 900-1800 g/ml.[21]

Another study by Yosie et al. (2011) demonstrated the weak antibacterial effect of *Phaleria macrocarpa* Leaves Hexane Extract against some bacteria, including *Pseudomonas aureus, Bacillus cereus*, and *Streptococcus ubellis*. Furthermore, Yosie et al. (2011) also reported that the best antibacterial effect was found in the *Phaleria macrocarpa* Leaves ethyl acetate extract against some bacteria, including *Escherichia coli, Pseudomonas aureginosa, Klebsiella pneumonia, Bacillus cereus, Staphylococcus aureus*, and *Streptococcus ubellis* bacteria. Another type of *Phaleria macrocarpa* Leaves extract, methanol extract, also showed a good antibacterial effect, although it was not as good as the other type of *Phaleria macrocarpa* extract against some bacteria, including *Pseudomonas aureginosa, Bacillus cereus*, and *Staphylococcus aureus*.[22]

Previous studies investigated the antibacterial effect of *Phaleria macrocarpa* leaves extract against typical bacterial and atypical bacteria like MRSA (*Methicillin-Resistant Staphylococcus aureus*). Hestiyani and Handini (2020) reported that *Phaleria macrocarpa* Leaves hydroethanolic extract has an antibacterial effect against the MRSA bacteria, which can be seen from clear zone formation by disc diffusion assay. The average width of the inhibition zone at 6% and 40% *Phaleria macrocarpa* leaves hydroethanolic extract were 6mm and 9mm, respectively.[23]

This study also investigated the antibacterial effects of the *Phaleria macrocarpa* Leaves by different methods. This method was a biofilm inhibition assay against *Propionibacterium acne*. Biofilm is a defense mechanism bacterium in the dormant phase to offend external obstacles, such as antibiotics, biocides, and other chemical compounds. Hence, it plays an important role in antibiotic

resistance by various bacteria. Meanwhile, in the industrial sector, biofilm is associated with biofouling, pipe corrosion, and friction resistance. Biofilms are formed on the surface of bacterial cells and embedded in an exopolysaccharide matrix that holds various ions, nutrients from outside the sequestered bacterial cells, and extracellular enzymes (β-lactamases, proteases, and polysaccharides). This biofilm later acts as a diffusion barrier and reaction sink. Thus, it contributes to antibiotic resistance for bacteria by reducing antibiotic penetration into the intracellular compartment. [24,25]

Based on the results of the current study, it can be seen that the *Phaleria macrocarpa* Leaves ethanol extract is enriched by some phytochemicals. These phytochemicals have high polarity and contribute to antibacterial by inhibiting biofilm formation. In addition, other studies also reported many other mechanisms that have the potential to support the antibacterial activity of the *Phaleria macrocarpa* Leaves extract. The alkaloid in *Phaleria macrocarpa* Leaves extract can inhibit bacterial growth by inhibiting the protein and DNA formation in bacteria cells. Meanwhile, the flavonoid content of *Phaleria macrocarpa* Leaves extract also destroys bacterial cell walls. Saponins are soap-like compounds that have antiseptic activity and can disturb bacterial metabolism. Finally, tannins disturb the ability of bacteria to adhere to body tissues (adhesion) and inhibit some enzymes in the bacterial transport process.[23] Furthermore, Othman et al. also identified some derivates of flavonoids from the *Phaleria macrocarpa* Leaves ethanol extract, including kaempferol, myricetin, naringin, quercetin, and rutin.[21]

CONCLUSION

Phaleria macrocarpa leaves ethanol extract has an antibacterial effect against *Propionibacterium acne*s. *Phaleria macrocarpa* leaves ethanol extract showed an antibacterial effect as good as the positive control at the 75-90 ppm concentration. The mechanism of action from the antibacterial effect of *Phaleria macrocarpa* leaves ethanol extract inhibited biofilm formation in the *Propionibacterium acne*s growth process.

AUTHORS' CONTRIBUTIONS

Rut Indah Susilo prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Ali Napiah Nasution and Maya Sari Mutia reviewed and supervised the manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data in this investigation are available from the corresponding author on reasonable request

DISCLOSURE STATEMENT

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