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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

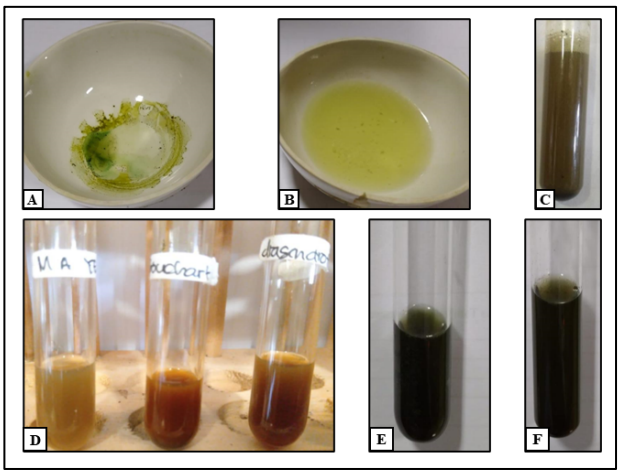

Figure 1: Phytochemical Screening of *Phaleria macrocarpa* Leaves Ethanol Extract for (A) Steroid; (B) Flavonoid; (C) Saponin; (D) Alkaloid; (E) Tannin; (F) Polyphenol.

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.

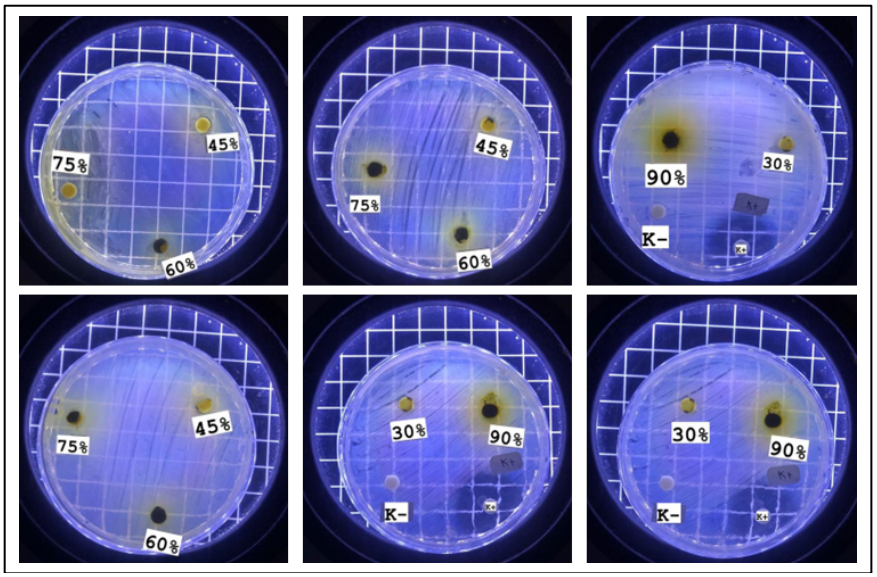

Figure 2: The Formation of Inhibition Zone of All Concentrations and Control Groups

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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Original Research

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Relationship of D-dimer, PT, APTT, and albumin with severity and mortality rate in covid-19 positive patients

Check for updates

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Abstract: COVID-19 is a global health problem that is divided into mild, moderate, and severe degrees of severity and a high mortality rate. The coagulopathy system has value in COVID-19 patients. This study aims to determine the relationship and predictive value of d-dimer, PT, aPTT, and albumin with severity and mortality in COVID-19 positive patients. The type of research used is retrospective with a cross-sectional approach. The research data were taken using a simple random sampling technique from the Medical Record Installation of Husada Utama Hospital, Surabaya in August-September 2021. Bivariate relationship data analysis used the Chi-Square test followed by a multivariate logistic regression test with cut-off values of D-dimer, PT, aPTT, and albumin each 0.5 g/mL, 14.0 sec. 36.0 seconds and 3.5 g/dL. The results of the chi-square test ($\alpha < 0.05$) showed the sig value of D-Dimer, PT, aPTT albumin with a severity level of 0.000; 0.000; 0.001; 0.001 while the value of Sig. with a mortality of 0.000; 0.047; 0.239; 0.022. The results of the multivariate logistic regression test with the degree of severity obtained the value of Sig. 0.000; 0.000; 0.021; 0.000 with a [PR] value of 16.7; 4.4; 2.7; 14.4. The results of the multivariate logistic regression test with mortality obtained the value of Sig. 0.000; 0.020; 0.273 with a [PR] value of 26.9; 2.8;1.6. There is a relationship between D-Dimer, PT, aPTT, and albumin with severity and mortality and can be used as a predictor of severity and mortality in COVID-19 patients.

Keywords: D-dimer**;** PT (*prothrombin time);* aPTT (Activated Partial *thromboplastin time);* Albumin; Severity and Mortality Rate

INTRODUCTION

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) or better known as Corona Disease (COVID-19) is known to infect humans and cause severe respiratory disease (1). The number of confirmed cases of COVID-19 infection until March 28, 2020, reached 571,678 cases, Indonesia reported the first case on March 2, 2020, and continued to grow, until September 28, 2020, there had been 498,000 cases with deaths reaching 15,884 (2).

COVID-19 is manifested by several symptoms, namely fever, cough, fatigue, mild shortness of breath, sore throat, headache, conjunctivitis, and gastrointestinal problems. The infection is transmitted and spread through the respiratory tract, through human-to-human aerosol transmission, and through contact with a contaminated environment (2,3,4). COVID-19 disease has three stages of severity according to clinical findings, namely stage 1 (mild), stage II

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(moderate), and stage III (severe) (3). Laboratory examinations, especially hematology and biochemistry, have the potential to help diagnose quickly, practically, and economically as well as to assist in disease prognosis and optimization of clinical monitoring (4, 2, 5).

D-Dimer is an important prognostic factor that was found to be higher in patients with SARS CoV-2 cases (5). Overproduction of proinflammatory cytokines, systemic inflammation, and vascular endothelial damage due to COVID-19 are the main causes of coagulation disorders and hypoalbuminemia (5). The coagulation system has significant value in COVID-19 patients because most patients have coagulopathy. Dynamic monitoring of the coagulation system parameters D-dimer, PT, and aPTT can be the key to controlling COVID-19 death to prevent thrombus or disseminated intravascular coagulation (DIC) in COVID-19 patients (6).

Low serum albumin levels are important predictors of disease morbidity and mortality (7). The mechanism of albumin reduction is caused by several factors, one of which is the presence of systemic inflammation and hypercoagulability. The liver where albumin is synthesized is also involved in the clearance of clotting factors activated by fibrinolytic products; therefore, decreased albumin may be associated with coagulopathy (9, 10). Routine hemostasis tests and albumin liver function tests can be used as additional tests for early diagnosis and monitoring the gradual progression of disease severity to prevent disease progression to death (9).

MATERIAL AND METHOD

Research Design

This study is a retrospective study with a cross-sectional approach. The study was conducted by recording medical record data of COVID-19 patients to determine the relationship between the parameters D-Dimer, PT, aPTT, albumin with mortality and severity in COVID-19 positive patients.

Population and sample research

The population of this study is medical record data from October 2020 - January 2021, positive patients for COVID-19 who were hospitalized and died at Husada Utama Hospital Surabaya. The sample size of 233 patient data was obtained from the calculation of the Isaac & Michael formula.

Research Stages

Samples of patient data that met the inclusion criteria were taken using a simple random sampling technique of 233 patient data. The data taken include patient code, gender, the value of D-dimer, PT, aPTT, albumin, and comorbid. Further stages were performing data analysis and reporting the results.

Data analysis

Data analysis was carried out using the SPSS program statistically with the Chi-Square test followed by the Logistics Multivariate Regression test and analysis of the prediction model.

Ethics Statement

This research was approved by the ethics committee of the Faculty of Medicine and Health, Universitas Airlangga Surabaya, Indonesia Number 340/HRECC.FODM/VI/2021.

RESULTS AND DISCUSSION

Based on the characteristics of 233 study subjects with positive RT-PCR results (100%) divided into moderate and severe severity groups 115 patients (49%) and 118 patients (51%) respectively and the living and dead groups were

202 (87%) and 31 (13%) respectively. Patients infected with SARS-CoV-2 will experience variable disease progression. Most COVID-19 patients are asymptomatic, experiencing mild symptoms and some will progress to severe degrees. Risk factors for disease progression that are more severe in individuals with advanced age, female sex, comorbidities, and severe pneumonia can increase mortality in COVID-19-positive patients because metabolic disorders can cause decreased immunity by impairing macrophage and lymphocyte function (12, 13). Statistical analysis for D-Dimer, PT, aPTT, and albumin with severity and mortality in COVID-19 positive patients is presented in Table 1-5.

Table 1. Characteristics of D-Dimer, PT, aPTT, and Albumin in COVID-19 positive patients

Examination Parameters	**Result Characteristics**		
	Minimum	Maximum	Average ±SD
D-Dimer (µg/mL)	0.02	180.9	3.1
PT (second)	9.6	66.2	14.6
aPTT (second)	20.8	271.2	36.1
Albumin (g/dL)	2.3	5.2	3.9

Table 2. Relationship of D-Dimer with severity and mortality

		Severity		***p-value***
		Moderate	**Severe**	
D-Dimer (µg/mL)	≤ 0.5	78	6	
	> 0.5	37	112	0. 000
Total		115	118	
		Mortality		***P-values***
		Living	**Died**	
D-Dimer (µg/mL)	≤ 0.5	83	1	
	> 0.5	118	31	0. 000
Total		201	32	

Table 3. Relationship between PT and Severity and Mortality in COVID-19 Positive Patients

		Severity		***p-value***
		Moderate	**Severe**	
PT (second)	≤ 14	74	44	
	> 14	41	74	0.000
Total		115	118	
		Mortality		***p-value***
		Living	**Died**	
PT (second)	≤ 14	107	11	
	> 14	94	21	0.047
Total		201	32	

Table 4. Relationship of aPTT with Severity and Mortality in COVID-19 Positive Patients

	Severity		***p-value***
	Moderate	**Severe**	

aPTT (second)	≤ 36	93	73	
	> 36	22	45	0.001
Tota		115	118	
		Mortality		***p-value***
		Living	**Died**	
aPTT (second)	≤ 36	146	20	
	> 36	55	12	0.239
Total		201	32	

P: Uji Chi-Square

Table 5. Relationship of Albumin with Severity and Mortality in COVID-19 Positive Patients

		Severity		***p-value***
		Moderate	**Severe**	
Albumin (g/dL)	>3.5	108	68	
	≤ 3.5	7	50	0.001
Total		115	118	
		Mortality		***p-value***
		Living	**Died**	
Albumin (g/dL)	> 3.5	157	19	
	≤ 3.5	44	13	0.022
Total		201	32	

Statistical analysis of bivariate logistic regression of D-Dimer, PT, aPTT and albumin with severity and mortality using the chi-square test in this study can be seen in table 6.

Table 6. Bivariate logistic regression test of D-dimer, PT, aPTT and Albumin with severity and mortality

Parameter	***P-value***	
	Severity	Mortality
D-Dimer	0.000	0.000
PT	0.000	0.047
aPTT	0.001	0.239
Albumin	0.001	0.022

Statistical analysis using multivariate logistic regression test D-Dimer, PT, aPTT and albumin with degrees of severity and mortality in the study can be seen in Tables 7 and 8.

Table 7. Multivariate logistic regression test of D-Dimer, PT, aPTT and Albumin with severity

	Sig.	**Exp(B)**	**95% C.I. for EXP(B)**	
			Lower	**Upper**
D-Dimer	0.000	16.720	7.418	37.689

Albumin	0.000	14.487	5.057	41.504
PT	0.000	4.398	1.989	9.726
aPTT	0.021	2.699	1.161	6.272
Constant	0.000	0.065		

Table 8. Multivariate logistic regression test of D-Dimer, PT and Albumin with mortality

			95% C.I. for EXP(B)	
	Sig.	**Exp(B)**	**Lower**	**Upper**
D-Dimer	0.000	26.797	6.118	117.374
PT	0.034	2.659	1.076	6.57
albumin	0.280	1.616	0.676	3.864
aPTT	0.789	1.135	0.447	2.882
Constant	0.000	0.007		

The PR value for risk factors or the predicted value of D-dimer > 0.5 g/mL in COVID-19 patients will have a 16-fold risk of increasing the severity and a 30-fold risk of increased mortality in Covid-19 positive patients. PT values > 14 seconds will have a 4-fold increased risk of severity and a 3-fold risk of increased mortality in COVID-19 positive patients. APTT values > 36 will have a 3-fold risk of increasing the severity of COVID-19-positive patients. Albumin values <3.5 g/dL will have a 14-fold risk of increasing the severity of COVID-19 positive patients.

Increasing D-dimer and thrombotic complications have been widely reported in COVID-19 patients. Several studies have been conducted to investigate the relationship between D-dimer measurements and disease severity (11). High levels of D-dimer are an indication of the occurrence of thrombosis due to pulmonary capillary endothelial injury that contributes to the death of severe COVID-19 patients (12). Prolongation of PT >3 seconds or aPTT >5 seconds from the reference value is a marker of coagulopathy and a predictor of thrombotic complications in COVID-19 patients (13). Albumin is a protein that exerts an important homeostatic effect which is a predictive marker for risk in critically ill patients with COVID-19 (14). Hypoalbuminemia in inflammation due to SARS-CoV-2 virus infection is associated with thrombosis and poor disease prognosis (15). The coagulation pathogenesis of COVID-19 is a coagulopathy leading to intravascular coagulation (DIC) which is considered a major contributing factor to death (16).

Severe inflammatory conditions due to COVID-19 infection cause severe disruption of coagulation system hemostasis, decreased platelet count, prolonged prothrombin and activated partial thromboplastin time (PT/aPTT), increased fibrin degradation products such as D-Dimer and decreased albumin (17). COVID-19 patients with severe severity show blood clotting disorders which are characterized by increased D-dimer, prolonged PT and aPTT so that monitoring of blood clotting function in COVID-19 patients can be used as a predictor of severity and mortality and is useful for early diagnosis, prevention. and treatment in COVID-19 positive patients (14).

The limitation of this study is that this study is a retrospective one. The secondary data collection was carried out due to the high risk of COVID-19 so data collection for D-dimer, PT, aPTT, and albumin was not evenly distributed on a

regular basis in COVID-19 patients. Data were taken from only one data of D-dimer, PT, aPTT and albumin at the onset of an increase.

CONCLUSION

There is a significant relationship between D-dimer, PT, aPTT, and albumin with the severity and mortality of COVID-19 patients. Parameters D-dimer, PT, aPTT, and albumin can be used as predictors of severity and mortality in COVID-19 patients. Suggestions for further research need to do a serial examination of D-dimer, PT, aPTT, and albumin in order to find out how the pattern of increase and decrease in D-dimer, PT, aPTT and Albumin values in COVID-19 positive patients.

AUTHORS' CONTRIBUTIONS

Budi Santosa: concept and design, writing original and revising manuscript, analysis and interpretation of data, supervision and final approval of the version to be published.
Amellya Octifani: concept and design, methodology, laboratory analysis, administration, and research permission.
Junaedi Wibawa: concept and design, writing original and revising manuscript, analysis and interpretation of data, supervision and final approval of the version to be published.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this research are available from the corresponding author on reasonable request

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Original Research

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Kluwih (Artocarpus camansi) leaves extract effects in zebrafish models of Parkinson's disease

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Abstract: Parkinson's disease is a condition that affects the central nervous system in the brain and is brought on by a lack of dopamine. Uncontrollable tremors, uncoordinated movement, and stiffness characterize Parkinson's disease. Until now, the medication for Parkinson's disease is limited to relieve the symptoms and maintain the quality of life; thus, the progression of the disease can be delayed. In order to search for alternative therapy from herbs, *Kluwih* (*Artocarpus camansi*) has been used traditionally to relieve convulsants. This research aims to observe 96% ethanol extract of *A. camansi* leaves in dopamine and locomotor activity in adult male and female zebrafish (*Dario rerio*). The *A. camansi* extract concentration was 2.5; 5; 7.5; and 10 mg/ml for 28 days. Zebrafish locomotion was observed for 5 minutes on days 0; 7; 14; 21; and 28. ELISA measured the observations of dopamine after 28 days. The 96% ethanol extract of *A. camansi* leaves at 5 mg/ml can increase dopamine levels after induced with rotenone, but the dopamine level decreased at 7,5 and 10 mg/ml. The maximal concentration to increase locomotor activity is also at 5 mg/ml, along with dopamine concentration. Our findings revealed that 5 mg/ml of 96% ethanol extract of *A. camansi* leaves was the optimal dosage to stimulate dopamine release and enhance locomotor activity.

Keywords: Kluwih, Zebrafish, Parkinson, Rotenone, Dopamine.

INTRODUCTION

In the last 25 years, the prevalence of Parkinson's Disease has doubled increase globally. Disability and death due to Parkinson's are rapidly rising more than any other neurological disorder. According to the latest figures, in 2019, Parkinson's disease caused 5.8 million years of life with disability and resulted in 329,000 deaths, an increase of more than 100% since 2000.[1] This disease occupies the second position as the most common cause affecting individuals over 60, and it is estimated that by 2030, it will continue to increase by more than two-fold, in line with the increasing population of early aging.[2]

Parkinson's disease is uncommon in individuals younger than 50s, but its prevalence increases and peaks at ages 60 to 75. While the preference is roughly the same for both sexes, men are more susceptible to its effects with a ratio of 3:2.[3] Parkinson's is a progressive neurodegenerative disease characterized by the loss of neurons in the substantia nigra resulting in decreased dopamine production and accumulation of Lewy bodies (LB) due to the formation of α-synuclein aggregates. The LB formation impairs the ubiquitin-proteasome degradation

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process, causing the failure of adenosine triphosphate (ATP) production, which results in mitochondrial dysfunction.[4] People with Parkinson's have mitochondrial dysfunction resulting in a malfunction of calcium ion regulation. More percentage of calcium in the body has a more toxic effect on neurons with α-synuclein aggregates accumulation.[5] This condition also activates the formation of free radicals due to oxidative stress that occurs when an imbalance between reactive oxygen species (ROS) production and cellular antioxidant activity. Increasing ROS production can inhibit the tyrosine hydroxylase (TSH) enzyme by decreasing dopamine levels.[6,7] Furthermore, the dopamine levels are inadequate to stimulate dopamine receptors in the striatum basal ganglia. It affects disturbances in locomotor activity characterized by slowed movements, tremors, stiffness, or balance problems.[8]

On the other hand, Zebrafish (*Danio rerio*) can be used as an experimental animal model for Parkinson's disease because it has a unique ventral telencephalon similar to the human brain striatum.[5,9] Another advantage of zebrafish is that many genes and proteins are similar to humans, transparent and large embryos.[10] While inducing Parkinson's disease in zebrafish models commonly use rotenone as a pesticide with neurotoxin.[11] Rotenone can penetrate cells, causing complex mitochondrial dysfunction and triggering the formation of oxidative stress,[12] which leads to dopaminergic and degenerative damage to peripheral motor nerves.[5]

Treatment of synthesis of Parkinson's disease uses several approaches, likes: 1) dopamine agonists such as levodopa, monoamine oxidase-b inhibitors such as selegiline, 2) anticholinergics such as trihexyphenidyl and N-methyl-D-aspartate (NMDA), 3) antagonists such as amantadine. All treatment is known to relieve and overcome the symptoms of Parkinson's due to improving the Activity of Daily Living (ADL) and Quality of Life (QQL), but they do not stop dopamine degradation.[3,8] This condition encourages research to develop better neuroprotective therapy strategies as supportive therapies for Parkinson's disease. The supportive therapy should be highly effective for the disease remedy and common side effects for patients. It could use herbs; thus, one of the herbs that have the potential to be developed as a supporting herb for Parkinson's therapy is *kluwih* (*A. camansi*). *Kluwih* (*A. camansi*) has traditionally been reported to be used to treat seizures.[13] *Kluwih* is a plant rich in compounds such as stilbenoids, aryl benzofurans, and flavonoids.[14] Flavonoid group from *A. camansi* leaves could inhibit the activity of the acetylcholinesterase (AChE) enzyme, anticholinergic, and antioxidant, which is effective against Alzheimer's disease. In this study, we wanted to investigate the effect of 96% ethanol extract of *A. camansi* leaves on the expression of dopamine levels and motility (locomotor) activity in adult male and female zebrafish, which had been induced by rotenone.[15]

MATERIAL AND METHOD

Zebra Fish

Adult male and female zebrafish, wild-type strain blackfish, were obtained from Tulungagung cultivators in East Java, Indonesia. Zebrafish identification was obtained from Airlangga University, Faculty of Fisheries and Maritime Affairs, Surabaya, East Java, with identification number 074/ULMKILP/UA.FPK/12/2022. The zebrafish has been ages group into three different groups, such as (1) early adulthood (3 - 6 months) has 0.42 ± 0.04 g in mass body and 28.4 ± 0.75 mm for a length, (2) middle adulthood (7 - 9 months) has mass and length body at 0.62 ± 0.09 g and 31.6 ± 1.17 mm, and (3) late adulthood (> nine months) calculates in 0.08 g for mass and 30.6 ± 0.95 mm for length body. This research took late adulthood zebrafish to figure out elderly human from the 60s until the 75 as the most preferred in Parkinson's patients. Acclimatization was carried out for seven days, and maintenance was according to standard procedures approved by the research ethics committee of Airlangga University (No: 3.KEH.159.11.2022).

Chemical Material
The chemicals used included ethanol 96% (Merck), rotenone (Sigma R 8875), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and Tween 80 (Sigma-Aldrich), 2N HCl, chloroform, NH4OH, dragendorf reagent, Mayer reagent, 10% NaCl, FeCl3 reagent, gelatin, chloroform, CH3COOH, concentrated H2SO4.

Extraction
Kluwih plants (*A. camansi*) were obtained and tested for termination at UPT Herbal Laboratory Materia Medika Batu, Malang, East Java, with letter of determination number 074/124/102.20-A/2022. *A. camansi* leaves dry powder (200gr) was extracted with 96% ethanol with a volume ratio of 1:10 and macerated for 3x24. The liquid extract was concentrated into a viscous extract using a Rotavapor® apparatus. The concentrated extract was made for several dosages, such as 2.5 mg/ml; 5mg/ml; 7.5 mg/ml; and 10 mg/ml.

Phytochemical Screening of *A. camansi* Leaves Extract
Phytochemical screening was carried out on the ethanol extract of *A. camansi* leaves, according to Sogandi & Amelia, 2020 which included testing the flavonoids, alkaloids, steroid-triterpenoids, phenolics, tannins, and saponins.

Rotenone and *A. camansi* Treatment
Zebrafish were induced to set Parkinsonis' disease model by adding 5 µg/L rotenone (Sigma R8875) to 2L of water in a 25 x 16.5 x 12.5 cm aquarium. The pool water is reversed every two days; thus, the concentration of rotenone in the aquarium is retained. Pool water temperature is maintained in the range of 24-25.5℃ with the darkest cycle of 14:10 (Khotimah et al., 2015). The zebrafish are fed thrice daily with Tetra Bit and Color Tropical Flakes; Tetra Sales; Blackburg, Germany. The sample consisted of a 96% ethanol extract of *A. camansi* leaves in several dosages (2.5; 5; 7.5; and 10 mg/ml) given concurrently with rotenone for 28 days.

Analysis of Dopamine Levels with ELISA
Zebrafish were anesthetized by immersion in ice water (5 parts ice to 1 part water at 0-4℃ for 30 seconds). The brain part of the fish head is carried by dissecting with the help of a dissecting microscope; then, the pure brain is extracted to obtain protein. The results of zebrafish brain extraction were tested for dopamine levels using the ELISA method (Fish Dopamine KIT Brand Biassay Technology Laboratory (BTLab) Cat.No EA0018FI).

Motility Observation
The locomotor activity test method was carried out by vertically dividing the aquarium into three zones (right sideline, middle line, and left side). Three vertical lines are drawn at identical intervals on the tank. Simple observations were made in this test to determine the locomotor activity of adult male and female zebrafish. Fish movements are captured in a 5-minute, then observed using the Tracker Video Analysis and Modeling software.

Data analysis
All data groups obtained from each treatment were analyzed using SPSS version 29 with one-way ANOVA ($p < 0.05$) for statistical analysis. These results are expressed as the mean ± SD for each treatment group.

RESULTS AND DISCUSSION

Kluwih (*Artocarpus camansi*) is a species of the Moraceae family found in Indonesia, India, Malaysia, Africa, Australia, Brazil, and many other countries. Traditionally, breadfruit (*A. camansi*) has been effective in seizure treatment.[13] The flavonoid group from *keluwih* leaves has been reported to inhibit the activity of the enzyme acetylcholinesterase (AChE), anticholinergic affected, and high antioxidant against Alzheimer's disease effectively.[15] This study used breadfruit (*A. camansi*) as an antiparkinsonian agent for zebrafish rotenone-induced.

Phytochemical screening was carried out in this study to determine the class of compounds found in *A. camansi* leaves extract. The results of the phytochemical screening test showed that the 96% ethanol extract of *A. camansi* contained flavonoids, alkaloids, tannins, steroid-triterpenoids, and phenolic compounds (Table 1). Furthermore, dopamine levels and locomotor activity tests were evaluated on adult zebrafish of different sexes.

Table 1. Phytochemical Screening of 96% Ethanol Extract of *A. camansi* Leaves

Phytochemical	Annotation	Result
Flavonoids	+	Orange red precipitation
Alkaloids	+	White and orange precipitation
Tannins	+	Green brownish change in color
Steroid	+	No blue-greenish ring
Triterpenoids	+	Brownish ring
Phenolic	+	Dark blue and greenish blue color
Saponin	-	No foam

Dopamine Concentration

To determine the effect of rotenone and *A. camansi* extract (ACE) on dopamine levels, after 28 days, dopamine levels were tested using the ELISA method from adult male and female zebrafish brains.

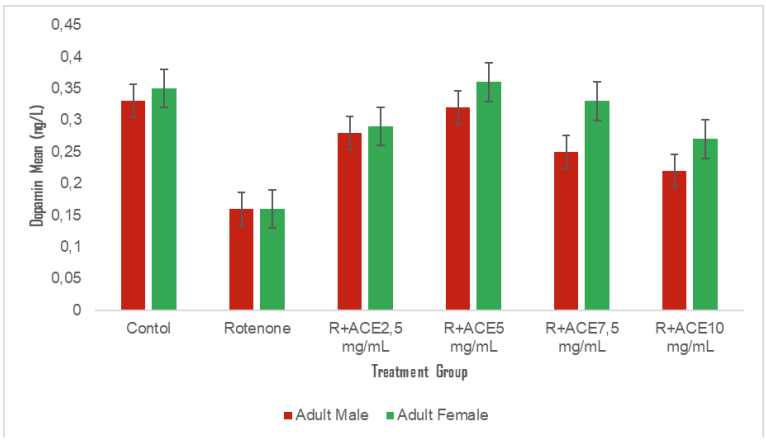

Fig 1. Dopamine levels of adult male and female zebrafish by induction of 96% ethanol extract of *A. camansi* leaves

Figure 1 shows that rotenone-induced significantly reduced dopamine levels compared to the control group ($p<0.05$). Giving 96% ethanol extract of *A. camansi* leaves levels of 2.5 and 5 mg/L to adult male and female zebrafish can increase dopamine levels. Improving the concentration of *A. camansi* leaves extract, which is higher than 5 mg/L, is not followed by an increasing dopamine level, otherwise decreasing dopamine levels. Hence, a concentration of 5 mg/ml is the maximum concentration in protecting neurons from rotenone-induced damage and is also a concentration to keep dopamine levels stable.

Dopamine (DA) is a brain hormone that acts as a neurotransmitter that regulates movement or motor systems, maintaining mood, memory, sleep, and cognitive processes.[16] Decreasing dopamine levels, cause increased Parkinson's symptoms with decreased motor activity and anxiety.[2]

Decreasing dopamine levels related with decrease locomotor activity is affected by the neurotoxin rotenone, which has a highly lipophilic structure. It encourages the molecules to quickly penetrates and cross the blood-brain barrier (BBB), then enters the central nervous system (CNS) and reach the position of the dopaminergic neurons.[17] Apart from the lipophilicity aspect, from a structural perspective, rotenone has a similar structure to dopamine, driving it easier to penetrate the dopaminergic nervous system of zebrafish. The improved dopamine level after 96% ethanol extract of *A. camansi* leaves treatment induced by flavonoid compound as antioxidant agent linked with locomotor effect.

Locomotor Activity Assessment (*Tank Test*)

The effect of rotenone-induce and 96% ethanol extract of *A. camansi* leaves treatment from 28 days onward on locomotor activity in adult male and female zebrafish was evaluated by motility observation with a tank test every seven days.

Table 2. The Motility of Male Adult Zebrafish for each group

Day	Mortality means					
	Control	***Rotenone***	**2,5**	**5**	**7,5**	**10**
0	436,23± 3,25	459,40± 3,67*	472,57± 2,67**	486,80± 3,95**	437,73± 2,60**	420,70± 2,42**
7	459,17± 3,65	458,23± 4,83	409,77± 3,18**	463,73± 3,98**	429,03± 3,91**	436,73± 10,93**
14	472,23± 3,07	363,37± 2,95*	426,03± 4,27**	456,47± 4,74**	389,17± 3,50**	399,83± 7,35**
21	463,17± 2,83	335,83± 3,73*	412,57± 3,15**	561,73± 1,84**	456,83± 4,05**	413,20± 2,75**
28	500,97± 3,26	316,60± 4,00*	578,87± 53,77**	572,73± 2,80**	536,27± 3,80**	518,73± 60,63**

*Each value is expressed as the mean ± SD. The significant difference compared to the control (without treatment) ($p<0.05$).
** Each value is expressed as the mean ± SD. The significant difference compared to rotenone ($p<0.05$).

In Table 2, the group of adult male zebrafish that received rotenone was shown to experience a decrease in locomotor activity compared to the control group ($p<0.05$). The 96% ethanol extract of *A. camansi* leaves treatment of 2.5 and 5 mg/ml in the adult male zebrafish group decreased motility activity. It affected rotenone induction, compared to higher concentrations of 7.5 and 10 mg/ml did not cause increased locomotor activity. The concentration of 5 mg/ml is the optimal concentration to maintain the locomotor activity of male zebrafish, and this is in line with the maximum dopamine level of 5 mg/ml. The decreasing dopamine levels were aline with decreasing adult male zebrafish locomotor activity.

Furthermore found a similar result, which is more of 5 µg/L rotenone inducing a lack of locomotor activity in zebrafish.[18] It seems that rotenone, a naturally occurring toxin and a widely used pesticide that inhibits the reduced form of nicotinamide-adenine dinucleotide dehydrogenase in mitochondria, imitates the neuropathological, neurochemical, and behavioral characteristics of Parkinson's disease in vertebrates.[19] Even though this rotenone effect depends on several factors, such as temperature, pH, sunlight, depth of the aquarium, and the

presence of organic debris,[20] the reduction in zebrafish movement could be attributed to a decline in the velocity of motor nerve conduction.[21]

Table 3. The Mortality of Female Adult Zebrafish for each group

Day	Mortality means					
	Control	***Rotenone***	**2,5**	**5**	**7,5**	**10**
0	545,03± 4,17	522,53± 4,44*	519,07± 2,44	526,03± 2,48	519,97± 3,76	469,20± 78,53
7	535,90± 3,58	525,97± 2,63	457,17± 3,36**	509,20± 3,37**	478,43± 3,20**	377,07± 53,92**
14	496,83± 3,76	489,57± 2,97*	577,50± 1,81**	359,73± 57,27**	447,10± 3,42**	432,57± 3,00**
21	593,00± 2,31	458,93± 2,93*	415,40± 3,06**	553,00± 2,89**	586,40± 2,15**	348,30± 2,95**
28	575,53± 2,85	432,97± 2,55*	429,87± 53,77**	592,73± 1,10**	535,80± 4,03**	435,03± 1,16**

*Each value is expressed as the mean ± SD. The significant difference compared to the control (without treatment) ($p<0.05$).
** Each value is expressed as the mean ± SD. The significant difference compared to rotenone ($p<0.05$).

In this research, we observed different motility between adult male and adult female zebrafish. Table 3 contains the motility of adult female zebrafish with rotenone-induced resulting in decreased motility. The increase of motility activity in adult female fish began to be seen as stable in 5 mg/ml *A. camansi* extract after being induced by rotenone. Along with increasing the concentration of leaves extract of 7.5 and 10 mg/ml group concentration was not followed by an increase in locomotor activity in adult female zebrafish. This pattern was also observed in adult male zebrafish, which had no increasing activity despite increasing extract concentrations. From these results, it can be seen that the maximum concentration to maintain dopamine levels in both adult male and female fish is 5 mg/ml. In addition, this research shows that male zebrafish have lower dopamine with lower locomotor activity than female zebrafish. It relates to the research on humans with Parkinson's disease that adult males have high risk than adult females.[22]

Several research has evaluated that 96% ethanol extract of *A. camansi* leaves dominated by a flavonoid, which is related to this research finding.[13,23,24] Moreover, flavonoid has significantly affected Parkinson's disease ailment, proven by clinical research by Gao *et al.* (2012).[25] The research evaluated 438 men and 367 women who developed PD during 20–22 years of follow-up consuming flavonoid-rich foods, resulting in a lower risk of Parkinson's disease. This result is supported by the flavonoid activity as an antioxidant that overcomes ROS levels in the brains of patients caused by mitochondrial damage accumulation.[26]

In several animal studies, flavonoids have been found to possess anti-inflammatory, antioxidant, and antidepressant properties.[27,28] These effects are believed to result from their ability to regulate neurotransmitter levels in the brain by interacting with transcription factors, enzymes, and kinases or by modifying neurotransmitters themselves.[29] Flavonoids also appear to inhibit the production of reactive oxygen and nitrogen species, which can lead to mitochondrial DNA damage and lipid peroxidation due to their potent antioxidant properties. When combined with endogenous scavengers, flavonoids have a synergistic and additive effect.[30] They can interfere with more than three free radical-producing systems at

a time and ultimately enhance the action of endogenous antioxidants, reducing cellular damage.

CONCLUSION

The 96% ethanol extraction of *kluwih* (*A. camansi*) leaves treatment can increase dopamine levels and locomotor activity in adult male and female zebrafish. The maximum of *A. camansi* leaves extract in male and female zebrafish to maintain stable dopamine levels is 5 mg/ml. The results of the phytochemical screening showed that the *A. camansi* leaves extract positively possesses flavonoids, alkaloids, tannins, steroid-triterpenoids, and phenolics. Subsequent research continues to determine the active fractions and subfractions of the ethanol extract of *kluwih,* which can increase dopamine levels and locomotor activity.

AUTHORS' CONTRIBUTIONS

Marisca Evalina Gondokesumo: prepared the samples, designed the protocols, executed the protocols, wrote the manuscript, submit and revision the manuscript. Krisyanti Budipramana: reviewed and supervised the manuscript. Putu Dea Angelita Putri and Ni Putu Diah Nopitasari: data collection. Martanty Aditya and Liza Yudistira Yusan: data analytic and visualization statistically. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this research are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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