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Effectiveness of mahkota dewa leaves extract as a biofilm inhibitor of propionibacterium acnes growth

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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

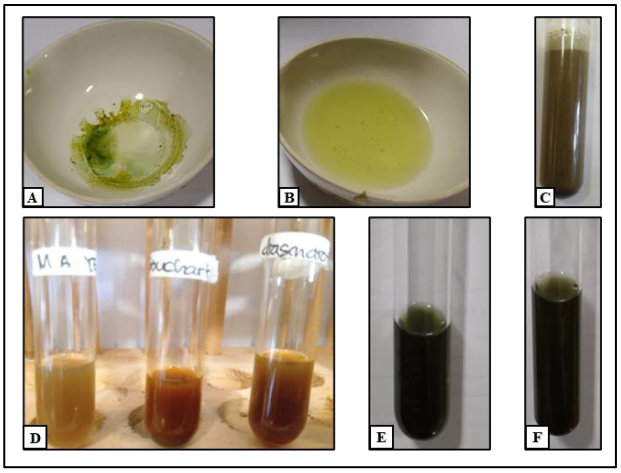

Figure 1: Phytochemical Screening of *Phaleria macrocarpa* Leaves Ethanol Extract for (A) Steroid; (B) Flavonoid; (C) Saponin; (D) Alkaloid; (E) Tannin; (F) Polyphenol.

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	
45 ppm [b]	15.70	15.60	15.90	0.003
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.

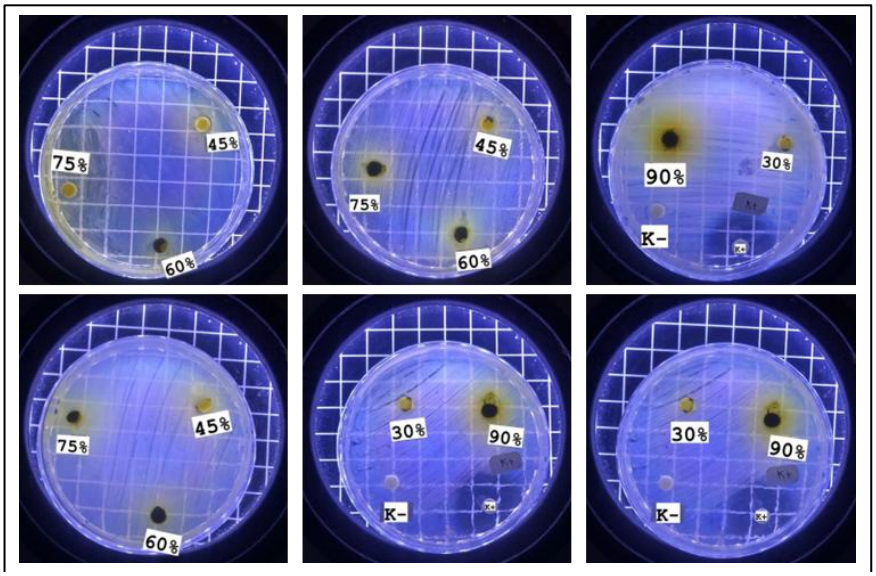

Figure 2: The Formation of Inhibition Zone of All Concentrations and Control Groups

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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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.

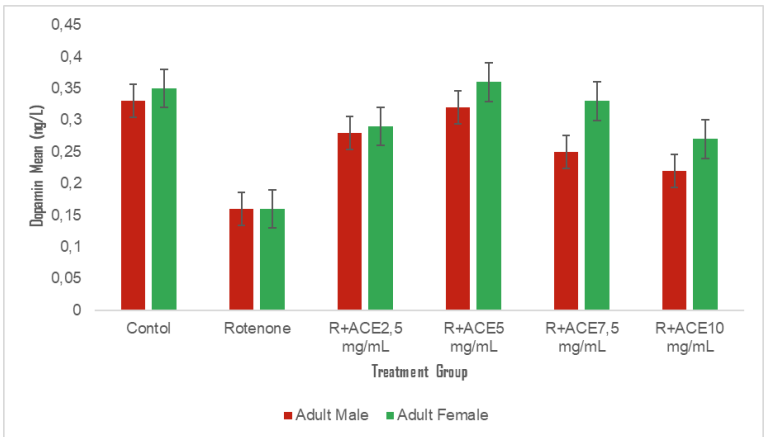

Fig 1. Dopamine levels of adult male and female zebrafish by induction of 96% ethanol extract of *A. camansi* leaves

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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Case Report

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Enucleation for retinoblastoma in a 22-month-old boy

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Abstract: Retinoblastoma is the commonest intraocular tumor in childhood. Management of retinoblastoma is quite complex depending on the stage, visual and globe saving, psychosocial factors, modality of treatment, and health infrastructure. We report a case of retinoblastoma in a child in a limited health facility without a pediatric oncologist or ophthalmology oncologist. A 22-month old boy came with complaints of leukocoria which his parents had known since the previous week. Ultrasonography of the eye shows calcification on the retina that supports Group E retinoblastoma. Bone marrow aspiration and MRI cannot be performed due to limited resources. Even though the parents only noticed the symptoms for a week and immediately brought to the ophthalmologist, the retinoblastoma stage was already in Group E. Enucleation and globe salvage were unavoidable. Enucleation was performed on the patient with histopathology finding the presence of Flexner-Wintersteiner rosette cells. After enucleation, he was referred to the oncologist for adjuvant chemotherapy. Early retinoblastoma detection is crucial in increasing the cure rate while maintaining children's vision.

Keywords: Retinoblastoma, leukocoria, enucleation.

INTRODUCTION

Retinoblastoma malignant tumor that arises from the retina is the most common primary intraocular malignancy in children of primitive neuroectodermal origin.[1,2] However, retinoblastoma is a rare disease with a global incidence of 1 in every 16,000–18,000 live births.[3] The incidence of retinoblastoma is similar in all populations and does not vary according to sex, ethnicity, or socio-economic status. The number of cases of retinoblastoma every year reaches 8,000 children, most of which occur before the age of 5 years.[2] Most cases occur in countries with lower socio-economic-related education levels, and 86.3% are from rural areas.[4,5] As many as 52.3% of retinoblastoma cases came from Asia, and the rest came from Africa (23.5%), Europe (12.0%), America (11.8% and Oceania (0.4%). A total of 56.8 % come from low to middle-income countries.[6]

Management of retinoblastoma in children is quite complex depending on the stage, visual saving, family psychosocial factors, culture, modality of treatment, and health infrastructure.[1] The prognosis in low and middle-income countries is often poor, even though 80% of retinoblastoma cases occur there. Children are diagnosed at an advanced stage where the visual function cannot be maintained at 38.5%, at least at stage II.[5] Although most children survive this cancer, they will lose their vision at intervals of the affected eye or need to have the eye removed.[7,8] Although about 50% of cases are referred to an ophthalmologist within one week after symptoms are detected at the primary care center, one-third of patients still present after eight weeks or even 24 weeks.[7,9] The importance of family awareness of the possibility of retinoblastoma is associated with earlier diagnosis and higher

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rates of globe salvage in patients with retinoblastoma.[10,11] We report a case of retinoblastoma in a child in a limited health facility without a pediatric oncologist or ophthalmology oncologist.

CASE REPORT

ZAH, a 22-month-old boy, was admitted to the Ophthalmologist department of Dr. Ramelan Navy Hospital Surabaya on January 24th, 2023. His parents notice his child's eye looks unusual. His parents complained shiny white spot-like eye cat on the right eye for one week ago (Figure 1). Parents stated if their child had eye complaints. The child has never experienced complaints of red eyes, watery eyes, purulent eyes, and swollen eyes. Parents also never complained of decreased vision. The child has never complained of decreased vision, such as hitting furniture when playing or having difficulty doing activities in the afternoon or less light. Parents stated that the right and left eyeballs movements were balanced or there were no abnormalities. The movement of the right eyeball looks different after a white spot appears in the eye. There was no history of head and eye trauma. Parents never noticed abnormalities in their child's eyes until the last week after a white spot appeared in the middle of the eyeball. Parents also never brought complaints to health workers regarding eye complaints.

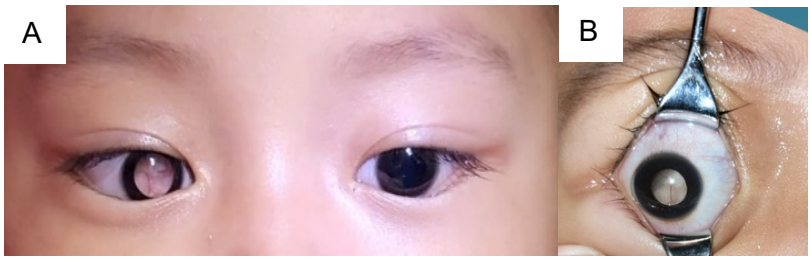

Figure 1. Child's eye looks A) The shiny white spot like eye cat of the right eye on the first visit, B) Leukocoria of the right eye

He was born spontaneously, fully termed, and assisted by a local midwife. His birth weight was 3100 grams. He did not get any prior medication for a specific disease. His family had no history of retinoblastoma or other malignancy. He had complete immunization status according to the government. When a child comes to a health worker in primary health care for immunizations, the officer never says if the child's eyes have abnormalities. The patient never had an eye examination after birth or a vision screening during a visit to primary health care. The patient was given breastmilk and combined formula milk until now. His growth and development were within normal limits until now.

Physical examination revealed good consciousness. Pulse rate was 110 times per minute, and respiration rate was 28 times per minute, axillary temperature was 36.6° C, blood pressure was 100/70 mmHg. His body weight was 11.3 kg. We did not find abnormalities on the ear, nose, throat, or neck examination. There were no palpable lymph nodes or nuchal rigidity. The chest examination revealed no precordial bulging. The Ictus cordis was noticeable on the 5th intercostal space on the left midclavicular line. There was no thrill. The heart sound for first and second heart sounds was normal, without a murmur. The movement of both sides of the chest was symmetrical. Vesicular respiratory sounds were noted, without wheezing or rales. The abdomen examination relieved no hepatomegaly and no splenomegaly. The upper and lower extremities showed no deformity. There was no edema, no cyanosis, and the capillary refill time was normal. The physiological reflexes of the patella and Achilles tendon were normal. Motor strength in the extremities was normal.

The right-eye examination showed orthophoria and normal eyeball movement. The anterior segment of the right eye showed visual activity and had no light perception. There were no abnormalities on the palpebra, conjunctiva, sclera, or cornea. Posterior segment examination of the right eye with direct ophthalmoscope showed positive fundus reflexes (Figure 2). We found leukocoria in the right eye. There were no abnormalities on examination of the left eye, including palpebra, visual field, orbital movement, or anterior and posterior segment. Posterior segment examination of the left eye with direct ophthalmoscope showed fundus reflexes were normal.

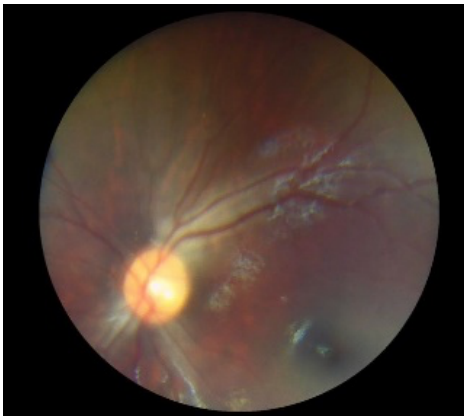

Figure 2. Segment posterior of the left eye

Complete blood count, kidney function, liver function, and albumin levels were within normal limits. The hemoglobin level was 13.5 g/dL, the leukocyte count was $8.6x10^9$/L, and the platelet count was $324x10^9$/L. Ocular ultrasound of the right eye demonstrated a solid mass with calcification in the right eye (Figure 3). The size mass was 1.46 x 1.62 cm. The tumor has occupied more than 50% of the right eyeball. There were no retinal detachment and calcification of the lens. Ocular ultrasound of the left eye showed no retinal detachment or calcification of the lens. Corpus vitreous was normal. We did not perform bone marrow aspiration, head-CT scan and head MRI. Based on anamnesis, clinical manifestation, and ocular ultrasound, the patient was diagnosed with retinoblastoma of the right eye Group E based on International Classification of Retinoblastoma (ICRB). Management of this patient was enucleation of the right eye.

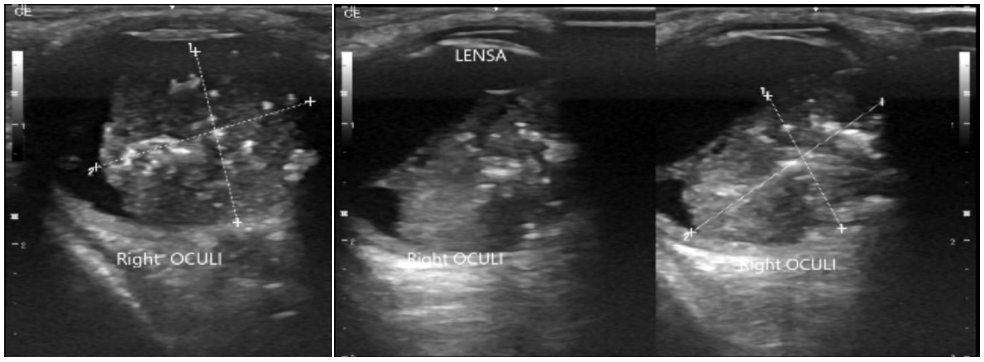

Figure 3. A solid mass in the posterior segment of the right eye

The patient had enucleation on January 27th, 2023, with the removal of the entire globe of the right eye and its intraocular contents, with preservation of all other periorbital and orbital structures (Figure 4). The right eye globe has been taken by anatomical, pathological examination.

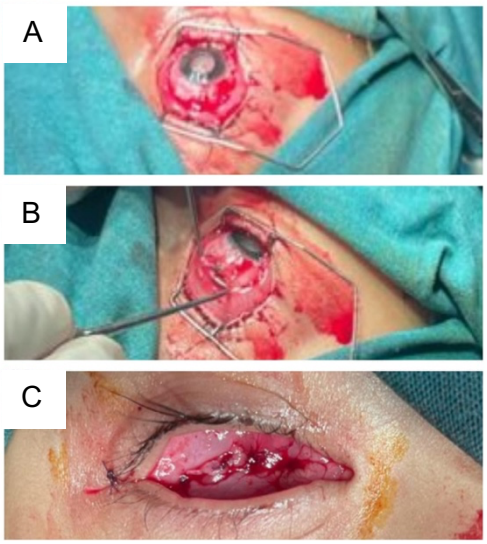

Figure 4. Enucleation A) Eyelids are retracted with a lid speculum, B) Tenon's fascia away from the globe using curved Stevens scissors in the four quadrants between the rectus muscles, C) Conjunctiva was closed and sutured (bottom)

January 31st, 2023, the patient was controlled to the ophthalmologic department. The physical examination showed a palpebral hematoma of the right eye. We give amoxicillin, paracetamol, eye ointnment, and a warm compress. The patient was controlled for two more weeks.

February 14th, 2023, the patient was controlled to the ophthalmologic department with histopathology examination result. Histopathology finding showed that the tumor tissue was a proliferation of anaplastic cells with a monotonous round nucleus, small size, hyperchromatic, thin cytoplasm, and slightly formed Flexner-Wintersteiner rosette (Figure 5). Retinoblastoma was also found at the end of the optic nerve and was therefore referred to the pediatric oncologist for adjuvant chemotherapy.

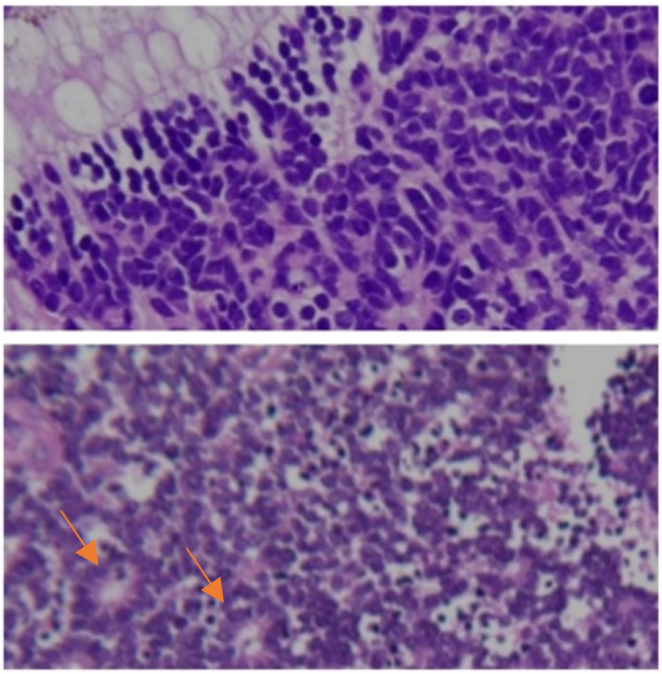

Figure 5. Anatomical pathological of the right eye after enucleation showed Flexner-Wintersteiner rosette (arrow)

RESULTS AND DISCUSSION

Retinoblastoma is a tumor that develops in the retina. In this case, the patient was a 22-month-old boy, whose parents complained of a shiny white spot like a cat's eye on the right eye a week ago. A cohort study of 4351 cases in 153 countries reported that the median age at diagnosis was 30.5 months, and 54.6% were male.[8] The cumulative incidence of Retinoblastoma was higher in boys than girls. The mortality rate in boys was 3.4 times more than in girls.[12] The incidence and mortality rates for Retinoblastoma and other malignant cancers in children are higher for boys than for girls, including acute lymphoblastic leukemia, lymphoma, renal carcinoma, and rhabdomyosarcoma.[13] After all confounding variables, including social aspects controlled, sex differences in the incidence, mortality, and survival of retinoblastoma were associated with the X-linked chromosome in boys.[12] The X chromosome in women plays a role in apoptosis, especially in the activation of p53 as a tumor suppressor gene[14] and estrogen elaboration).[15]

In this case, from ocular examination showed leukocoria of the right eye. Thus, the patient has unilateral leukocoria without family history of retinoblastoma or other malignancies. Once the tumor develops, a white pupillary reflex known as leukocoria is the first readily observed sign, which is noticed by the family and described as a glow, glint, or cat's-eye appearance. The main symptoms of retinoblastoma from large-scale studies were leucoria (62.8%), strabismus (10.2%), and proptosis (7.4%).[8] However, in this case we found no other symptoms other than leukoria. In this case, the parents started to panic and stress when the child had leukoria. However, primary healthcare professionals may have difficulty detecting the most common symptom (leukoria). They may not be aware that retinoblastoma is the underlying pathology of eye symptoms that are more common in infants and young children.[7] About 20% of cases of retinoblastoma in Argentina are diagnosed within 24 weeks of the onset of symptoms.[9] A one-third of patients were diagnosed at an advanced stage, 29.5% at stage II and 4.2% at stage III/IV.[9] About 50% of children were referred to an ophthalmologist within one week from the first consultation with primary healthcare professionals; a quarter of delayed referral was more prolonged than eight weeks.[7] The proportion of parents presenting to a retinoblastoma treatment center at an early stage with leukocoria or strabismus alone mainly comes from low-income countries.[6] There is a significantly increased risk of delayed diagnosis in younger patients, those with squint rather than leukoria, and those who first present to a health visitor rather than a general practitioner. The risk of local tumor invasion increases significantly with diagnostic delays.[6,7,9] Primary health professionals need education about the importance of ocular symptoms, especially squint, in pediatric patients.

We did not find a family history of cancer including retinoblastoma in either the paternal or maternal families. Retinoblastoma could be familial or sporadic, bilateral or unilateral, and heritable or nonheritable. The incidence of retinoblastoma is related to the interaction of many factors, for example, where the parents live in city, the presence of pets during pregnancy mainly cat and dog, and exposure to hazardous chemicals from the father six months before pregnancy (paint, leather equipment, decoration, electronic accessories, benzene, formaldehyde and other heavy metals.[16] Cases of nonheritable retinoblastoma have unilateral tumors, unlike heritable retinoblastoma, which often develops bilaterally and multifocally. The most cases about two-thirds of all cases were unilateral retinoblastoma.[17]

The diagnosis of retinoblastoma is established based on clinical examination, ophthalmology, and support and confirmed by histopathology. In this case, USG showed a solid mass with calcification in the right eye without retinal detachment, calcification of the lens, or vitreous seeding. Based on anamnesis, clinical manifestations, and USG, the patient was diagnosed with unilateral

retinoblastoma stage E of the right eye. The first classification for intraocular retinoblastoma was Reese and Ellsworth (RE) in the 1960s to predict eye survival after external beam radiotherapy. The R-E classification system was no longer appropriate and substituted by an International Intraocular Retinoblastoma Classification (IIRC) in 1990 after introducing intravenous chemotherapy.[17] The IIRC classification categorizes tumors from the A-E group, depending on size, location, and additional features, including the presence of 'seeded' retinoblastoma (small colonies of cancer cells in the vitreous) and/or retinal detachment. The Minister of Health of the Republic of Indonesia has issued National Guidelines for Medical Services for the Treatment of Retinoblastoma in 2022. The retinoblastoma classification system refers to the International Classification of Retinoblastoma (ICRB), which divides retinoblastoma into Groups A to Group E.

Ultrasonography, CT-scan, and MRI are imaging modalities for head and neck tumors in children. The currently recommended diagnostic evaluation for retinoblastoma is ultrasonography and MRI.[18–20] However, in this patient, Ct-scan and MRI were not performed. The examination supplementing the diagnostics is the ultrasound (USG) of the eyeball.[18,21,22] USG of the eyeball assessing the size or location of the tumor, internal features of the malignant tumor (including the presence of calcifications), and possible extra-ocular propagation. Pathognomonic ultrasound for retinoblastoma is a retinal mass with hyperechoic calcification.[18] The growth pattern of retinoblastoma can be endophytic, exophytic, and infiltrative. Endophytic retinoblastoma is a white mass penetrating the internal limiting membrane and may be accompanied by vitreous seeding.

The initial step in these patients was enucleation, followed by histopathological examination to identify high-risk histopathological features and tumor differentiation. The treatment chosen for retinoblastoma depends on the type of retinoblastoma (intraocular or extraocular) and globe involvement (unilateral or bilateral). In addition to primary management, supportive therapy should be given, including nutritional support and infection management. The objective of managing a child with retinoblastoma is the survival of the patients, globe salvage, and vision salving.[23] In 2006, the Intraocular Classification of Retinoblastoma scheme successfully predicted the outcome of intravenous chemotherapy.[17] For retinoblastoma in groups A–C, the globe salvage in ≥ 90% of eyes. Group E retinoblastoma underwent primary enucleation.[17] Enucleation is the surgical procedure that involves removing the entire globe and its intraocular contents, preserving all other periorbital and orbital structures.[24] The management of retinoblastoma has experienced rapid development, but enucleation is still the main choice at an advanced stage. Enucleation is usually performed for advanced Group D and Group E retinoblastoma. Enucleation was also indicated for extraocular extension (orbital cellulitis, neovascular glaucoma, intraocular hemorrhage, tumor in the anterior chamber, optic nerve or choroid involvement).[23] Globe salvage is an eyeball rescue that does not require enucleation.[25] The average globe salvage reached 93% in Group A and only 19% (95% CI 5-50%) in Group E.[25] In this case, globe salvage is no longer possible because retinoblastoma is already in stage E. We must explain to the parents that vision cannot return.

Before enucleation, the patient or parents must be given informed consent to explain the indications, risks, benefits, and possible complications.[24,26] In this case, Histopathology finding refers to retinoblastoma, with a monotonous round nucleus and Flexner-Wintersteiner rosette. Retinoblastoma on microscopic examination reveals small hyperchromatic cells, a high nuclear-to-cytoplasmic ratio, necrosis, and multifocal calcification. Tumor differentiation was classified as well differentiated (>50% known as Homer- Wright rosette) or poorly differentiated (<50% known as Flexner-Wintersteiner rosette).[21,27] The incidence of high-risk retinoblastoma in eyes that have undergone primary enucleation reaches 77.5%,

especially in poor and undifferentiated cells, more in children over two years of age.[28]

We plan chemotherapy after enucleation in our patient. Primary enucleation followed by adjuvant chemotherapy will improve survival, reducing the occurrence of metastasis in children with locally advanced retinoblastoma and histopathologic high-risk characteristics.[29–33] This patient will be given adjuvant chemotherapy with a regimen of vincristine, etoposide, and carboplatin by a pediatric oncologist. Systemic chemotherapy for retinoblastoma is chemo reduction of large tumors (neoadjuvant chemotherapy) or reduces the risk of relapse, metastasis, or recurrence after surgical enucleation (adjuvant chemotherapy).[34] The patient requires adjuvant chemotherapy. Retinoblastoma requires combination therapy which is not only with enucleation but can be followed by chemotherapy and/or radiotherapy.[23,30,31,35] Combination therapy can provide more effective results and a better prognosis. Meta-analyses report that overall survival in children with retinoblastoma is 79% (74-84%), and globe salvage is 22% (14-32%).[8] Poor prognosis (lower globe salvage, metastasis related mortality, treatment failure) are more common in countries with low incomes and in rural areas, and access to health services is limited, including in Southeast Asia.[8,36]

The World Health Organization (WHO) Guide for Effective Programs in Cancer Control emphasizes the early diagnosis of retinoblastoma, with the target population being children with white reflexes and strabismus as early symptoms. Screening for early detection of retinoblastoma has developed in various developing countries, but it is necessary to emphasize screening newborns through examination of the red reflex.[10,35,37] Early retinoblastoma detection is crucial in increasing the cure rate while maintaining children's vision.[38] The late referral will delay diagnosis, although retinoblastoma may be more aggressive at an older age. Health workers, especially pediatricians, and ophthalmologists, need to educate and increase parents' understanding of retinoblastoma's early signs and symptoms. Parents can have symptoms of depression (73%), anxiety (64%), and stress (100%) as a psychological impact of retinoblastoma and a decrease in the child's quality of life.[11]

Retinoblastoma is a cancer in children which is still a challenge in terms of globe salvage and chemotherapy.[11] Early detection of retinoblastoma should have been carried out earlier by parents and first-line health workers. Identification failure will cause the child to come with an advanced condition. Delay in diagnosis will result in globe salvage failure, and the child must lose his sight. A general ophthalmologist can perform surgery. The limitations of this case report are the absence of head imaging examinations such as MRI and bone marrow aspiration. The next step is adjuvant chemotherapy. However, cancer service centers are unavailable everywhere, so he must be referred immediately.

CONCLUSION

In our case, a 22-month-old boy with a shiny white spot-like cat eye on the right eye (unilateral leukocoria) without a family history of retinoblastoma. Ocular ultrasound showed a solid mass with calcification in the right eye, which refers to intraocular retinoblastoma stage E of the right eye. The management is enucleation, and the histopathology finding refers to retinoblastoma (Flexner-Wintersteiner rosette). He was sent to the pediatric oncologist for further chemotherapy to provide a better prognosis.

AUTHORS' CONTRIBUTIONS

All authors contributed equally from conception, design, data extraction, and statistical analysis to interpretation of data.

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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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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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Potential of aloe vera gel as an alternative inductor in platelet aggregation test

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Abstract: Platelet aggregation tests are crucial for evaluating platelet function, with inductors being substances that stimulate the aggregation process. The use of ADP (adenosine diphosphate) as an inductor has the disadvantage of being costly and difficult to obtain. Aloe vera, a plant native to Africa, has been widely used for wound healing and therefore has the potential to serve as an alternative inductor agent. This study aims to evaluate the effectiveness of Aloe vera gel as a platelet inductor compared to the ADP reagent using the Velaskar method. This research began with the preparation of fresh Aloe vera to obtain Aloe vera gel. Venous blood was then collected from 16 participants for the platelet aggregation test. The test was divided into two groups: the ADP group (addition of ADP) and the Aloe vera group (addition of Aloe vera gel). The final step involved analyzing the results of platelet aggregation statistically using the Independent Sample T-test. The average percentage of platelet aggregation with ADP and Aloe vera gel was 88.2 ± 5.6% and 85.7 ± 5.4%, respectively. Independent Sample T-test analysis showed no significant difference between the percentage of platelet aggregation with ADP and Aloe vera gel. In conclusion, Aloe vera gel has the potential to be an alternative platelet inductor. Some benefits of Aloe vera gel include its ability to induce platelets similarly to ADP, its application not affecting the morphology of erythrocytes, and its economical and practical nature (as it does not require elaborate preparation). However, a disadvantage is that pure Aloe vera gel contains several components that may affect its performance as an inductor.

Keywords: Aloe vera, Adenosine Diphosphate, Alternative inductor, platelet aggregation

INTRODUCTION

Hemostasis is the process of stopping bleeding spontaneously from blood vessels that have acquired vascular injury[1]. Platelets play a major role in hemostasis through formation and stabilization of the platelet plug[2]. Disruption of platelet function can cause myocardial infarction or commonly known as a heart attack[3]. According to the World Health Organization (WHO), cardiovascular disease is the leading cause of mortality worldwide, accounted for 17.3 million deaths in 2013. It is predicted to reach 23.6 million by 2030[4]. The highest prevalence of acute myocardial infarction in Indonesia is East Nusa Tenggara (4.4%), while in Central Java it reaches 0.5%[5].

Platelet aggregation test is one of the tests to evaluate platelet function. Several methods were used for the test, one of them was the method introduced by Velaskar DS and Chitre in 1982. The Veslaskar method was applied using a peripheral blood smear, with the principle of aggregation visible when the smear is formed, free platelets and aggregated platelets can be counted separately on smear[6].

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Platelet aggregation test is very sensitive assay because influenced by several factors, such as the concentration of an inductor addition[7]. Inductors are substances that stimulate the aggregation process. The strength of the inductor influences platelet responsiveness. Weak inductors are adenosine diphosphate (ADP) and epinephrine, moderate inductors are thromboxane A 2 (TxA2), while powerful inductors are thrombin and collagen. ADP is the most commonly used inductor for the Velaskar method[8].

Aloe vera is a plant from Africa which has been widely used for wound healing[9]. This plant is divided into two parts, namely the mucilage gel and the exudate (mucus) part, which is composed of yellow sap (yellow mucus) and colorless mucin gel. Aloe vera contains several substances such as tannins, flavonoids, saponins, and anthraquinones that play a role in the blood clotting coagulation[10].

Aloe vera contains several substances that play a role in blood coagulation, including tannins, flavonoids, saponins, and anthraquinones[10]. According to Sukeksi and Rizqy (2021), the tannin present in the ethanol extract of betel leaves has the potential to substitute ADP since it has astringent effects. The astringent effect is the ability to form complexes with macromolecules, particularly proteins[11].

The use of the platelet aggregation test Velascar method plays an important role as a screening test prior to examining platelet aggregation using the Aggregometry method. Furthermore, the platelet aggregation test Velascar method also serves as an assessment of platelet aggregation function in small to medium-sized laboratories or health centers that do not have facilities for testing platelet aggregation function using the Agregometric method. Providing the significance of this test, the use of ADP reagents becomes critical. The use of ADP in the laboratory has affordability and availability limitations[12,13]. On the other hand, *Aloe vera* gel shows potential as a natural alternative inductor agent. The use of *Aloe vera* gel in platelet aggregation tests has never been reported. The objective of this study is to investigate the potential of *Aloe vera* gel as a new platelet inductor.

MATERIAL AND METHOD

Materials

Fresh *Aloe vera* leaves, ADP reagent (Helena laboratories), 70% ethanol (Merck), methanol (Merck), and Wright-Geimsa stain (Merck), Analytical balances (O'hauss), centrifuge (Gemmy PLC03), blender (Philips), glass object (Sail brand), micropipette (Human), white-tip and yellow-tip (Axygen), filter paper (Whatman), glass funnel (Herma), microscope (Olympus CX23), Syringe 3 mL (BD).

Aloe vera Gel Preparation

Fresh *Aloe vera* leaves are collected from plants 8 months old and 40-70 cm long. Remove the outer skin of the aloe vera leaf and thoroughly wash it with water. After peeling the skin, the visible aloe vera gel is cut into 2×3 cm sizes, then put in a mixed until smooth and filtered. Filtered Aloe vera gel is ready to be used for platelet aggregation test.

Samples for Analysis

All samples were taken from the 16 subjects (normal invidious). A clean venipuncture was used to collect 0.9 ml of blood into a syringe containing 0.1 ml of 3.8% sodium citrate, mixed and delivered into a sil-iconized glass tube. (Note: One drop of sample was transferred to a glass slide, spread to produce a thin smear, and immediately dried in air to measure the so-called initial aggregation (no inductor addition).

Platelet aggregation test

Inductor agent addition

A amount of the sample (0.2 mL) was transferred to a new siliconized glass tube. 0.02 mL of the appropriate agent was added to the sample. ADP group (ADP addition) and *Aloe vera* group (addition of *Aloe vera* gel). Simultaneously, a stop watch was started. For 10 seconds, the tube was vigorously agitated. (No more agitation was provided to the tube until the test was completed).

Smears preparation

The smears were made at various time intervals, as shown below. ADP group: thin smears were made from 0.01 ml of mixed blood with inductor at the exact 180 second after addition of ADP. *Aloe vera* group: thin smears were made from 0.01 ml of mixed blood with inductor at the exact 60, 120, 180, 240, 300 second after addition of aloe vera gel. All smears were rapidly dried in air and labeled.

Fixation dan staining

The smears were fixed in methanol for 10 minutes and stained by the Wright-Geimsa stain for 20 minutes.

Examination

The smears were observed using an ordinary light microscope with an oil-immersion objective lens after being washed and dried. All platelets encountered in such a linear examination (lateral zone) were counted differentially: the number of free platelets against the number of platelets in aggregation. Both these numbers were recorded and added together. The platelet aggregation percentage was calculated and rounded to the nearest whole value.

Data analysis

The data of platelet aggregation percentages were analyzed using the Statistical Package for the Social Sciences (SPSS) software 16.0. The data of platelet aggregation percentages were analyzed using the Statistical Package for the Social Sciences (SPSS) software 16.0. The Mann-Whitney test was used to analyze data normality, and the Independent Sample T-test was used to compare means. P-value smaller than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The potential of *Aloe vera* gel as a new inductor agent was evaluated using the percentage of platelet aggregation in citrate blood samples with the addition of ADP and *Aloe vera* gel (Graph 1). visible image of the aggregation smears using ADP and *Aloe vera* gel is shown in Figure 2.

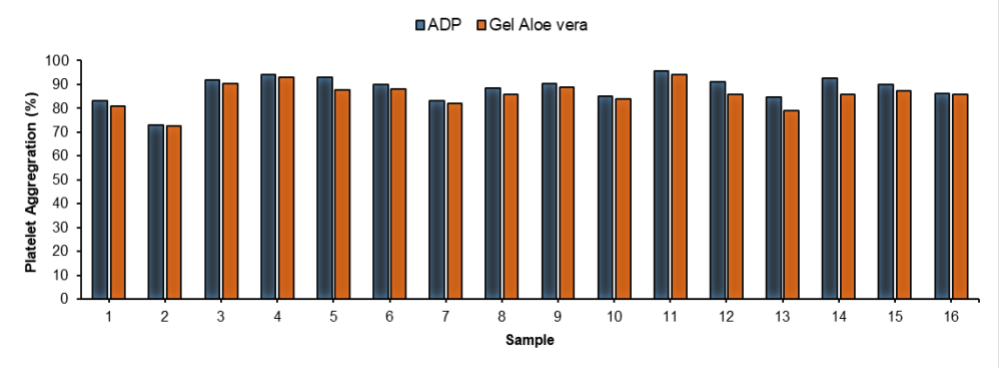

Figure 1. Comparison of platelet aggregation percentage in 16 samples using adenosine diphosphate (ADP) and *Aloe vera* gel

The percentage of platelet aggregation with ADP was higher than *Aloe vera* gel with a mean of 88.2 ± 5.6% and 85.7 ± 5.4%, respectively. The highest percentage of platelet aggregation was found in platelet aggregation smears with

ADP, at 95.6%, while the lowest value was found in platelet aggregation smears with *Aloe vera* gel, at 72.4%. statistical analysis of the Independent Sample T-test (p-value > 0.05) showed no significant difference between the percentage of platelet aggregation with ADP and *Aloe vera* gel. Microscopic observations also showed a similar pattern. Platelet aggregation smears with *Aloe vera* gel produced the same microscopic appearance as the ADP. Platelets aggregated are clearly seen, not covered by latex with normal erythrocyte morphology surrounding.

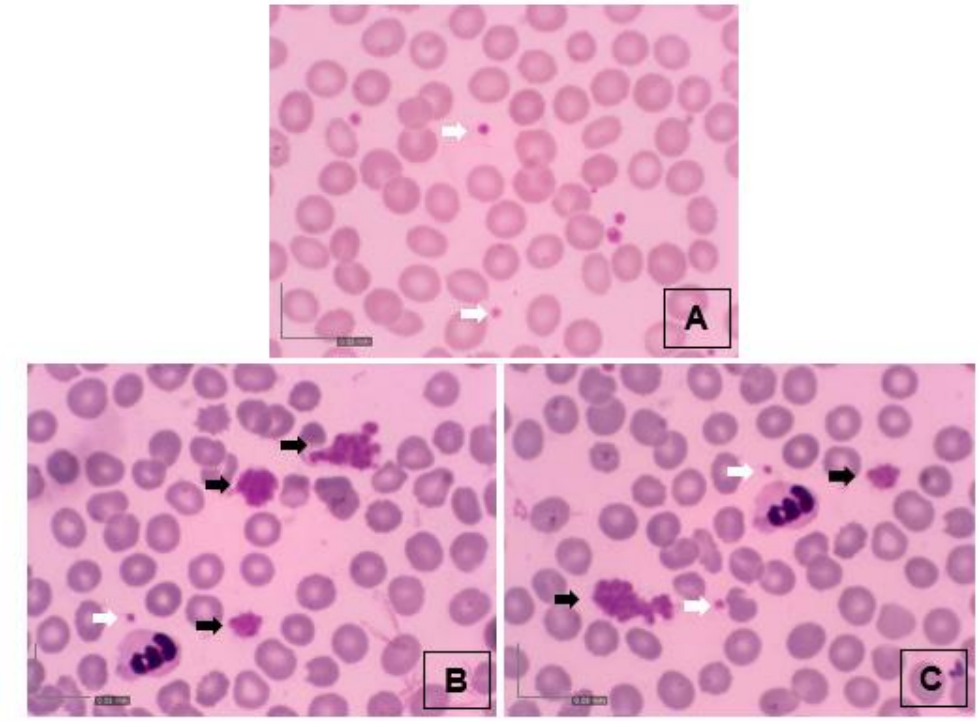

Figure 2. Microscopic observation of platelet aggregation smears using Giemsa staining (1000× magnification); (A) Initial aggregation, (B) ADP inductor, (C) *Aloe vera* gel, black arrow: platelets aggregate; white arrow: free platelets.

Both of these results showed that aloe vera gel has the same potential as ADP to stimulate platelets and aggregation formation. The concentration of 10L of *Aloe vera* gel added is equivalent to the concentration of 10L of ADP, where 10L of the ADP reagent has been determined in the procedure. The ability of *Aloe vera* gel as an inductor does not affect the morphology of erythrocytes, thus, making Aloe vera gel as an ideal inductor agent.

Aloe vera gel contains arachidonic acid, an unsaturated fatty acid that can stimulate platelets to aggregate through the Cyclooxygenase enzyme[14,15,16,17]. The cyclooxygenase-1 enzyme catalyzes the transformation of arachidonic acid into the intermediate product Prostaglandin H2 (PGH2). PGH2 is further metabolized to Thromboxane A2 (TxA2), which is found in platelets. Thromboxane A2 is a potent inductor platelet that stimulates platelet aggregation. Thromboxane A2 not only stimulates platelet aggregation but also shows strong vasoconstrictive effects[18,19]. *Aloe vera* gel contains the amino acid tryptophan which is the precursor of serotonin. Serotonin or 5-hydroxytryptamine (5-HT) is a substance released by platelets that play a role in hemostasis by enhancing the effect of contraction by norepinephrine, histamine, or angiotensin II. This effect is expected to enhance platelet function during hemostasis[20].

Platelet aggregation is the process when platelets adhere to one another. Platelet aggregation is divided into two stages: primary aggregation (reversible aggregation) and secondary aggregation (irreversible aggregation). The primary aggregation phase occurs when thromboxane A2 (TXA2) synthesis increases, leading to platelet aggregation and vasoconstriction. Besides TXA2, ADP is also

an aggregation inducer which with the P2Y12 receptor then induces changes in the shape of platelets from discs to oval pseudopods[21,22].

Furthermore, ADP promotes the expression of the calcium-GPIIb/IIIa complex on the platelet surface and binds to fibrinogen to create bridge-like linkages. This binding will facilitate platelet aggregation in the primary aggregation phase. The secondary aggregation phase occurs when levels of ADP, serotonin, and epinephrine increase, causing irreversible platelet aggregation[21,23].

A previous study on alternative inductors was conducted using betel leaf ethanol extract. There was a difference in platelet aggregation ability between ADP and betel leaf ethanol extract. The mean value of the betel leaf extract was higher than that of the ADP, indicating that the ethanol extract of betel leaf has the potential to be an alternative inductor agent, although the concentration added is not equivalent to that of the ADP reagent[11].

The response of the aggregating platelets to *Aloe vera* gel was evaluated at various time intervals, including 60 seconds, 120 seconds, 180 seconds, 240 seconds, and 300 seconds after the addition of aloe vera gel (Figure 3). The maximum percentage of platelet aggregation was seen 180 seconds after the addition of *Aloe vera* gel, reaching 85.7±2.3%. The percentage of platelet aggregation rose to about 1.36% after the addition of *Aloe vera* gel from 60 seconds to 120 seconds, reaching a maximum percentage at 180 seconds. There is a gradual decrease after reaching the maximum percentage at 240 seconds and 300 seconds. These results indicate that the optimal response of platelets to *Aloe vera* gel is 180 minutes after reacting it with citrate blood.

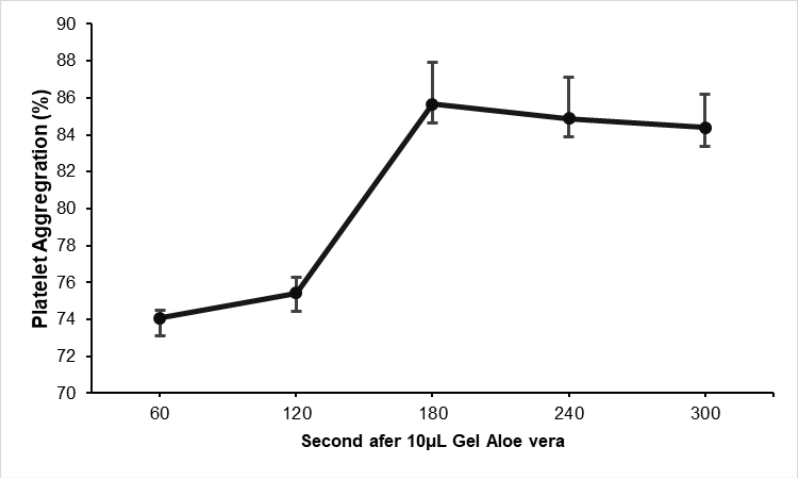

Figure 3. The percentage of platelet aggregation after the addition of 10µL *Aloe vera* gel at various time intervals (all result expressed as mean value ± 1SD)

A comparison of the percentage of platelet aggregation with the addition of different inductors reported using the Light transmission platelet aggregometry method with platelet-rich plasma samples. The results indicate the addition of different inductors to stimulate different platelet activation pathways. ADP (low dose) induces both primary and secondary waves of aggregation (biphasic curve). ADP < 1 µmol induces only a reversible form of platelet aggregation (primary aggregation), without thromboxane synthesis or intraplatelet release of ADP. However, increasing the ADP dosage may produce more significant irreversible aggregation (secondary aggregation)[24,25,26,27].

Arachidonic acid, collagen, and ristocetin induce one wave of aggregation (monophasic curve). Arachidonic acid will induce a change in platelet shape followed by maximum aggregation at 180 seconds then will be constant from 240 seconds to 300 seconds. These results are contrasted with the response of aggregating platelets to *Aloe vera* gel because *Aloe vera* gel used in the study still contains other substances. One of them is the flavonoid which can inhibit the

metabolism of arachidonic acid, reducing the level of aggregating platelets[28]. This is a limitation in this study, the pure aloe vera gel used includes several active components which can affect the performance of aloe vera gel as an inductor[29,30,31].

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All authors contributed equally to this work.

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Case Report

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Hyperuricemia, use of antituberculosis drugs, and liver injury: Case Report

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Abstract: Anti-tuberculous drug can cause idiosyncratic drug-induced liver injury (DILI). Considering the benefit risk, there will discontinuation therapy and rechallenge after symptom resolve. In addition to anti-tuberculosis drugs, liver injury can occur in patients with hyperuricemia. We report a 60-year-old male patient who had just used the initiation phase of OAT for 20 days experiencing hepatotoxic side effects characterized by complaints of nausea and vomiting for one week. Liver function examination results were normal with AST 23 u/L and ALT 9 u/L. OAT administration was temporarily stopped and started gradually with 150 mg rifampicin, 150 mg isoniazid and 500 mg ethambutol. The second day after using OAT again, given the full dose of 300 mg rifampicin, 300 mg isoniazid and 1000 mg ethambutol. The patient's condition improved after this modification of therapy so that therapy with three anti-TB drugs was continued until he was discharged from the hospital.

Keywords: Adverse effect, Antituberculosis, Drug induced liver injury

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis.[1] It remains a significant cause of health decline and mortality worldwide, second only to the 2019 Coronavirus pandemic (COVID-19). According to the 2021 Global Tuberculosis Report, there were 9.9 million global TB infection cases, with TB incidence decreasing from previous years, but an increase in mortality cases estimated at 1.3 million. In 2020, two-thirds of global TB cases were contributed by India, China, and Indonesia. Indonesia reported an estimated 647 TB incidents per 100,000 population.[2]

Despite successful treatment in around 85% of TB cases, drug-related side effects, including hepatotoxicity, skin reactions, gastrointestinal, and neurological

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disorders, significantly contribute to morbidity and impact treatment efficacy.[3] Drug-induced liver injury (DILI) can occur at therapeutic doses or in cases of overdose; it can be a direct effect of the drug or idiosyncratic (Kumachev & Wu, 2021). The risk of DILI side effects is higher in patients on combination regimens compared to monotherapy.[4] About 28% of DILI cases are associated with combined OAT therapy (Devarbhavi et al., 2019) and Fixed-Dose Combination (FDC) therapy.[5] Isoniazid is the most common culprit for hepatotoxicity, followed by rifampicin and pyrazinamide.[3,6,7] Apart from drug use, liver injury can also be caused by hyperuricemia conditions.[8]

The occurrence of DILI side effects undoubtedly influences drug selection and treatment regimens significantly, potentially leading to OAT discontinuation.[8] Addressing these adverse events requires intervention, including treatment modification, to prevent worsening of hepatic function and potential hepatic failure.[9] Treatment adjustments are also necessary to support medication adherence and prevent treatment failure, relapse, and drug resistance.[8,10] This case report aims to highlight a DILI case based on the patient's clinical symptoms and the management guided by clinical recommendations.

CASE REPORT

A 60-year-old adult patient, weighing 45 kg and with a height of 158 cm, was admitted to the hospital with a diagnosis of pulmonary tuberculosis (TB), functional dyspepsia, and hyperuricemia. The patient underwent a six-day hospitalization in November 2022. The patient was already on Fixed-Dose Combination Therapy (FDC-TB) for the last twenty days prior to hospitalization. There was no history of allergies, and the patient had previously worked as an intercity bus driver. The patient reported experiencing nausea since the initiation of Anti-Tuberculosis Drugs (ATDs), which persisted for 5 days. The day before admission, the patient had vomited more than 5 times within a day. Respiratory rate increased to 28 breaths per minute. Laboratory investigations revealed a slight decline in hemoglobin and serum uric acid, with liver function tests showing AST 23 IU/L and ALT 9 IU/L. Radiological assessment demonstrated active pulmonary TB based on X-ray images.

The therapeutic approach involved switching from FDC-TB to a Single Dose Combination (SDC) regimen: rifampicin 150 mg, isoniazid 150 mg, and ethambutol 500 mg. After two days on this regimen, the dosages were doubled to rifampicin 300 mg, isoniazid 300 mg, and ethambutol 500 mg. Additionally, curcumin tablets were administered 3 times daily, along with intravenous methylprednisolone 2 times daily at a dose of 62.5 mg, L-cysteine tablets 3 times daily, and sucralfate 3 times daily. Upon discharge, the patient continued with the following regimen: rifampicin 300 mg, isoniazid 300 mg, L-cysteine 1000 mg 3 times daily, allopurinol 300 mg, sucralfate 3 times daily, and domperidone 3 times daily. This case report illustrates a comprehensive therapeutic approach to managing drug-induced hepatotoxicity in a pulmonary TB patient, with multiple interventions aimed at ensuring patient well-being and effective treatment outcomes.

DISCUSSION

Hepatotoxicity is a side effect that most frequently reported on patients undergoing therapy with anti-tuberculosis drugs such as isoniazid, rifampin and pyrazinamide.[11] Drug-Induced Liver Injury (DILI) remains a critical concern during tuberculosis (TB) treatment, with varying definitions and management approaches across guidelines.[12] The World Health Organization's Adverse Drug Reaction Terminology (WHO-ART) defines DILI grades based on alanine aminotransferase (ALT) levels: grade 1 (mild) <2.5 times the upper limit of normal (ULN), grade 2 (mild) 2.5–5 times ULN, grade 3 (moderate) 5–10 times ULN, and grade 4 (severe)

>10 times ULN.[13] Additionally, American Thoracic Societies (ATS) and British Thoracic Societies (BTS) guidelines define DILI due to anti-tuberculosis drugs as ALT elevation of 3-5 times ULN with or without symptoms.[14] DILI because of OAT occurred within 2 months after administration and the highest incidence occurred in the first 2 weeks of therapy.[15]

Several studies reveal that DILI symptoms include nausea, vomiting, and jaundice.[3,5,16] TB patients with hepatotoxicity often report symptoms such as nausea and vomiting, fatigue, and jaundice, occurring 8-56 days after the intensive phase of TB therapy.[17] Notably, liver damage due to anti-tuberculosis drugs occurs within the initial 2 weeks of treatment.[18,19]

Isoniazid plays a role in metabolism pathway via N-acetyltransferase 2 (NAT2) and microsomal enzyme cytochrome P4502E1 (CYP2E1) which will convert isoniazid to acetyl diazine which is a toxic metabolite. Next, acetyl diazine will be broken down to activate acetyl onium ion, acetyl radical and ketene which will bond covalently with hepatic macromolecule triggering liver injury. Beside that, isoniazid inhibits glutathione peroxidase which is an antioxidant against free radicals. There are other mechanisms regarding the hepatotoxicity of isoniazid, the presence of immune mediated idiosyncrasy as a response mechanism liver adaptation to isoniazid and hepatotoxicity.[20,21]

Rifampicin activates CYP3A4 increase in metabolism Isoniazid produces toxic metabolites and cause hepatotoxicity. Rifampicin also induces isoniazid hydrolase, which causes thereby increasing hydrazine production increase toxicity when combined with isoniazid. Additionally, rifampicin able to inhibit bile salt exporters pump (BSEP) thereby causing conjugate hyperbilirubinemia.[20,21] Pyrazinamide inhibits activity CYP450 and disrupt levels nicotinamide – acetyl dehydrogenase (NAD) thus producing free radicals mediates hepatotoxicity.[20]

To manage DILI, a patient's treatment regimen might be modified or alternative therapies introduced. International Union against Tuberculosis and Lung Disease and WHO recommend temporarily halting treatment until liver function returns to normal, followed by reinitiating therapy.[17] ATS and BTS guidelines suggest gradual reintroduction starting with the safest drug, rifampicin, and progressively adding others like isoniazid.[17,22,23] National Institute for Health and Care Excellence (NICE) guidelines propose discontinuing treatment until ALT is below twice ULN and then reintroducing full-dose therapy, beginning with ethambutol or isoniazid.[24]

Despite the approaches mentioned, there is no evidence-based treatment to support the comparison of full-dose versus gradual reintroduction of anti-tuberculosis drugs.[14,25] A retrospective study employing gradual dose escalation reported high regimen completion rates (75.2%).[14] Mechanisms underlying pirazinamid – induced hepatotoxicity are complex, involving dose-dependent effects and metabolic pathways.[26–28]

In conclusion, managing DILI during TB treatment requires vigilant monitoring, dose adjustments, and careful drug selection. An integrated approach considering various guidelines, patient characteristics, and evolving evidence can effectively address DILI, ensuring optimal TB treatment outcomes and patient safety.

CONCLUSION

DILI side effects due to the use of OAT can occur at the beginning of treatment. Management of OAT-DILI is temporarily stopping the use of OAT until normal liver function and reusing the treatment regimen. A two-drug hepatotoxic regimen with gradually increasing doses was adopted in the management of this case. The use of hepatoprotectors such as curcuma can be done.

AUTHORS' CONTRIBUTIONS

Vina Yuwantari took research data and wrote this journal. Nur Oktafiyani, Nurmelinda Hadi Ningrum, M. Hari Pristantiningtyas, Herya Putra Dharma and Muhammad Muchlis chose cases in the hospital that could be used as case reports, as well as guiding the writing of this journal. Jainuri Erik Pratama, Fauna Herawati, Adji Prayitno Setiadi and Marisca Evalina Gondokesumo reviewed and supervised the journal. All authors have read and approved the final journal.

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Case Report

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Hepatoprotector in cases of Dengue Hemorrhagic Fever as a prevention of hepatic damage: A case report

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Abstract: Dengue Hemorrhagic Fever (DHF) is classified as an arbovirus disease which is a public health problem in the world. The causative agent of DHF is the dengue virus, namely the RNA virus of the genus Flavivirus and the family Flaviviridae. Hepatic impairment is often observed in DHF cases with asymptomatic or asymptomatic elevations in serum transaminases up to severe manifestations in the form of acute liver failure. It was reported that DHF patients had mild hepatic impairment with clinical manifestations of mild hepatomegaly, hypoalbumin, normal SGOT/SGPT values, and clinical conditions of nausea – vomiting. To prevent worsening liver damage, combination therapy with Ursodeoxycholic Acid (UDCA) and curcumin was given as a hepatoprotector. The use of curcumin can reduce serum levels of transaminase, Malondialdehyde (MDA) or markers of oxidative stress and increase hepatic glutathione concentrations which work in free radical detoxication, while the role of UDCA is as a hepatoprotector by reducing the level of oxidative stress in liver cells.

Keywords: DHF, Liver damage, Hepatoprotector, Curcumin, UDCA

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) falls within the spectrum of arboviral diseases and stands as a significant global public health challenge. Annually, a staggering 390 million cases of dengue virus infections are documented worldwide, with approximately 96 million cases displaying pronounced symptoms[1].The highest DHF incidence is observed among urban populations in tropical and subtropical regions, particularly within Southeast Asia[2]. Indonesia, an extensive endemic region for dengue, reported 73,518 DHF cases and 705 fatalities in 2021. The national tabulated Incidence Rate for DHF cases is 27 individuals per 100,000 population[3].

The etiological agent of DHF is the dengue virus, an RNA virus belonging to the Flavivirus genus within the Flaviviridae family[1]. Classified as an arbovirus

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(arthropod-borne virus), the dengue virus comprises four serotypes: dengue virus 1, 2, 3, and 4 (DENV-1, DENV-2, DENV-3, and DENV-4)[4]. All these serotypes have the potential to induce severe and fatal dengue infections, though DENV-2 and DENV-3 are more frequently associated with severe cases[5,6,7]. The primary transmission of the dengue virus is facilitated by Aedes mosquitoes, particularly Aedes aegypti and Aedes albopictus[8,9]. Additionally, non-vector transmission routes have been documented, including through blood transfusions, organ transplants, intrapartum, and breastfeeding[10]. The incubation period for dengue virus infection ranges from 4 to 10 days.

Clinical manifestations of dengue infection encompass asymptomatic cases, as well as mild flu-like syndromes termed dengue fever (DF)[11]. Severe and life-threatening forms include DHF and dengue shock syndrome[12]. Acute dengue infections manifest as fever lasting 2-7 days, accompanied by bleeding, thrombocytopenia, heightened hematocrit, ascites, pleural effusion, and hypoalbuminemia due to plasma leakage arising from hemoconcentration[13]. Although early fever phase symptoms resemble those of DF, DHF cases exhibit increased vascular permeability or plasma leakage, potentially leading to reduced intravascular volume and shock[14].

The high virulence of the dengue virus can result in multi-organ complications, including the liver[15]. Hepatic impairments are frequently observed in DHF cases, varying from asymptomatic to symptomatic elevation of serum transaminases, and even acute hepatic failure[16]. Liver dysfunction manifestations often range from mild to severe elevations in serum transaminases, presenting symptoms such as abdominal pain, nausea, vomiting, anorexia, and hepatomegaly[17,18]. Currently, no established therapeutic management exists for addressing hepatic dysfunction in DHF cases.

We present a case report of an adult patient exhibiting elevated serum transaminases and hepatosplenomegaly during inpatient care. This case report aims to explore the application of hepatoprotective agents for hepatic impairment in DHF patients.

CASE REPORT

A 62-year-old female patient was admitted to the hospital with complaints of fever fluctuating for approximately one week, occasionally accompanied by nausea. The patient reported no history of allergies. Upon examination at the Emergency Department, the patient's psychological status was cooperative, extremities were warm to touch, and no pallor was observed. Vital signs indicated a temperature of 38.4°C, blood pressure of 113/65 mmHg, heart rate of 126 beats per minute, respiratory rate of 20 breaths per minute, oxygen saturation of 96%, and random blood glucose level of 119 mg/dL, with capillary refill time < 2 seconds. The patient complained of abdominal pain with a pain intensity scale of 3 and described the pain as stabbing.

Laboratory results revealed a decreased hemoglobin level of 10.4 g/dL, normal leukocyte count of 5,770/mm3, decreased platelet count of 58,000/mm3, decreased red blood cell count of 3.42 x 1012/L, normal hematocrit level of 31.2%, normal SGOT/SGPT levels at 29 and 25 U/L, and a triglyceride level of 183 mg/dL. Positive Dengue immunoglobulin G (IgG) results indicated secondary infection. A chest X-ray impression suggested non-specific bronchitis, with no infiltrates in either lung. The radiologist suggested irritation due to acute respiratory infection. A diagnosis of pancytopenia, characterized by decreased levels of three types of blood cells resulting in anemia, leukopenia, and thrombocytopenia, was made[19]. The patient was hospitalized for seven days.

The patient received treatment that included Intravenous Fluid Drain (IVFD) Bfluid 20 drops per minute at the Emergency Department, Futrolit 20 drops per minute, one ampoule of Lansoprazole intravenously daily, one intravenous dose of Ondansetron 8 mg, three tablespoons of Sucralfate syrup daily, two capsules of

cough medication containing Paracetamol 500 mg, Acetylcysteine 200 mg, Mebhydrolin 50 mg, and Codeine 10 mg twice daily, and one 300 mg Gemfibrozil capsule at night.

On the second day, the patient still complained of fluctuating fever and nausea. The patient's general condition was weak, extremities warm, blood pressure 100/60 mmHg, hemoglobin decreased to 9.3 g/dL, platelet count decreased to 43,000/mm3, red blood cell count was normal at 3.17 x 1012/L, and hematocrit was decreased to 28.8%. The patient received a transfusion of four Thrombocyte Concentrate (TC) units (1 unit = 500 mL, 4 units = 2 L). Cough medication was not administered again due to patient refusal.

On the third day, the patient continued to experience fluctuating fever, but the nausea had decreased. The patient remained weak, extremities warm, blood pressure 111/68 mmHg, hemoglobin 8.9 g/dL, leukocyte count 3,100/mm3, platelet count 47,000/mm3, red blood cell count 3.01 x 1012/L, and albumin had decreased to 2.7 g/dL. The patient received a transfusion of four TC units and two Packed Red Cells (PRC) units.

On the fourth day, the patient was afebrile but complained of nausea and difficulty in defecation. Blood pressure was 146/80 mmHg, hemoglobin had increased to 12.2 g/dL, platelet count was still low at 44,000/mm3, and hematocrit was normal at 37.5%. The patient was diagnosed with hypertension, thrombocytopenia, non-specific bronchitis, dengue fever, and dyslipidemia. The patient received additional treatment including one daily fleet enema, three tablespoons of Lactulax thrice daily, three Inbumin tablets daily, a transfusion of four TC units, and continued other therapies.

On the fifth day, the patient complained of fatigue and ongoing fluctuating fever. Blood pressure was 140/86 mmHg, hemoglobin was 11 g/dL, and platelet count was 38,000/mm3. Abdominal ultrasound indicated mild hepatomegaly of unknown cause. No abnormalities were found in the pancreas, gallbladder, both kidneys, internal genitalia, or bladder on the abdominal ultrasound. No signs of ascites or pleural effusion were observed. The patient received a transfusion of four TC units and the doctor added Methylprednisolone 125 mg intravenously thrice daily and Trolit sachets thrice daily.

On the sixth day, the patient complained of ongoing fever, fatigue, and bloating. Blood pressure was 156/75 mmHg, hemoglobin was 11.7 g/dL, and platelet count was 47,000/mm3. The patient was diagnosed with pancytopenia, hypertension, dengue fever, non-specific bronchitis, hepatosplenomegaly, and dyslipidemia. The patient received a transfusion of four TC units and additional treatment including two Disflatyl tablets to be chewed twice daily, three Ursodeoxycholic Acid (UDCA) 500 mg tablets thrice daily, and three Curcuma tablets thrice daily.

On the seventh day, the patient reported weakness with blood pressure of 147/80 mmHg and platelet count of 30,000/mm3. The patient received a transfusion of four TC units and continued with other therapies. The patient requested discharge. Medications provided upon discharge included two daily Disflatyl tablets to be chewed, three UDCA 500 mg tablets thrice daily, three Curcuma tablets thrice daily, two daily Dexamethasone 0.5 mg tablets, one nightly Gemfibrozil 300 mg capsule, three tablespoons of cough medication thrice daily, and two daily Cefixime 100 mg tablets (for five days).

DISCUSSION

Dengue fever is classified as an acute infection caused by the dengue virus transmitted through Aedes aegypti mosquitoes. Indonesia, a tropical and subtropical country, is an endemic location for dengue infections. The classification of dengue infections and the severity levels of Dengue Hemorrhagic Fever (DHF) according to WHO are divided into Dengue Fever (DF) and DHF Grades I, II, III,

and IV. In this case, the patient was classified as having DHF Grade I, diagnosed based on fever complaints, bleeding manifestations (positive tourniquet test), evidence of plasma leakage indicated by hypoalbuminemia, and positive dengue IgG test results. Treatment for the patient was based on the Clinical Management Guidelines for Dengue by WHO (2012) and the National Guidelines for Medical Services in Adult Dengue Infections (2020), involving fluid therapy and symptomatic treatment. Additionally, a mild increase in serum transaminase levels was observed[20].

Dengue virus infection can lead to liver cell damage, characterized by an elevation in Serum Glutamic-Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT) levels[21,22]. The detailed pathophysiology of liver dysfunction in DHF cases remains not fully understood. Among various theories, three mechanisms associated with liver dysfunction are direct cellular apoptosis, ischemic and hypoxic hepatopathy due to decreased perfusion to the liver, and immune-mediated end-organ damage[21,23]. Recent research indicates that disruptions in hepatic microcirculation and reduced portal blood flow significantly contribute to the pathogenesis of severe liver dysfunction[24]. The administered hepatoprotective therapy involved a combination of curcumin and Ursodeoxycholic Acid (UDCA).

The use of curcumin as a hepatoprotective agent in cases of DHF with liver dysfunction has been explored previously. Firmansyah (2020) reported a case involving the administration of a combination of curcumin and silymarin three times a day in a patient with DHF and acute fulminant hepatitis. After three days of treatment, SGOT improved by 12.7% and SGPT by 19.1%[25]. Moreover, curcumin has been reported to have a protective effect by preventing DNA fragmentation and preserving mitochondrial functionality[26]. Curcumin, an active component of curcumin tablets, exhibits anti-inflammatory activity used in hepatitis cases. The use of curcumin can decrease serum transaminase levels, Malondialdehyde (MDA) levels (an oxidative stress marker in the liver), and increase hepatic glutathione concentration, which contributes to free radical detoxification[27,28].

Currently, no research exists on the effectiveness of UDCA in patients with DHF-related liver dysfunction. UDCA is recommended for treating patients with primary biliary cholangitis (PBC)[29]. Furthermore, UDCA can activate anti-apoptotic pathways, providing protection to cellular structures like plasma membranes and mitochondria, thus preventing hepatocyte damage. UDCA also inhibits reactive oxygen species (ROS) formation in Kupffer cells, reducing oxidative stress levels in liver cells[30,31]. Administration of UDCA for four weeks in patients with liver dysfunction resulted in a 40% decrease in ALT enzyme levels, 34% decrease in AST levels, and 23% decrease in GGT levels[32].

CONCLUSION

The combination of UDCA and curcuma can be used as a prophylaxis for liver damage in DHF patients, but further monitoring is needed to assess the effectiveness of the therapy.

AUTHORS' CONTRIBUTIONS

Emilia Gan and Yenry Sumarlim took research data and wrote this journal. Desantika Wuryana, M. Hari Pristantiningtyas, Herya Putra Dharma and Muhammad Muchlis chose cases in the hospital that could be used as case reports, as well as guiding the writing of this journal. Jainuri Erik Pratama, Adji Prayitno Setiadi and Marisca Evalina Gondokesumo reviewed and supervised the journal. All authors have read and approved the final journal.

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DATA AVAILABILITY STATEMENT

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

DISCLOSURE STATEMENT

The views and opinions expressed in this journal are those of the authors after reviewing various literatures and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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Original Research

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Antibacterial Blood Sample on the Stability of Hematological Examination

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Abstract: Transportation accounts for quite large errors up to 12% of the total errors. The error factor in this transportation activity comes from sending specimens that are too long and storage temperatures that are not right. Hematological examination is an examination that is often performed in various health services. The purpose of this study was to determine the stability of the hematological examination of blood samples transported for 1 hour and 2 hours at room temperature and cold temperatures. This study is a laboratory experimental study. Observations before treatment were controls, and treatments were carried out after 1 hour and 2 hours of transportation at room temperature and cold temperatures. This research was conducted at an average ambient temperature of 33.4ºC and humidity of 45%, room temperature of 27.8ºC and humidity of 43%, cold temperature of 4.5ºC and humidity of 52%, average shock of 54.9. The results showed that the parameters of Hgb, PLT, WBC, NEUT were stable for up to 2 hours in both room temperature and cold conditions. Meanwhile, the stability of LYMPH parameters is only capable at room temperature for 2 hours. Parameters Hct, RBC, MCV, MCHC and MXD there are differences in the results of the examination at cold temperatures after 1 hour and 2 hours. However, the parameters of RBC, MCV and MXD were stable for up to 1 hour at room temperature. We conclude that the hematological examination is stable for up to 1 hour with the recommended storage at room temperature. Only Hgb, PLT, WBC, NEUT tests are stable for up to 2 hours in both room temperature and cold conditions.

Keywords: Hematology, stability, transportation, time, temperature.

INTRODUCTION

Transportation accounts for quite large errors up to 12% of the total errors. The error factor in this transportation activity comes from sending specimens that are too long and the storage temperature is not right.[1,2] Hematology examination is an examination that is often carried out in various health services.[1,3,4] Regulation of the Minister of Health of the Republic of Indonesia Number 43 of 2013 stipulates the length of time for delaying hematological examinations to reach 2 hours except for Hct, which is 6 hours at room temperature.[4] Meanwhile, the International Council for Standardization in Haematology (ICSH) recommends a delay at room temperature not exceeding 4 hours and for diffcout not exceeding 1 hour, a delay at 4ºC reaching 24 hours to 72 hours and for diffcout reaching 8 hours.[5] The problem is that there are no reports on the stability of hematological examinations that are influenced by temperature and time on specimen transportation.

Clinical laboratories have a role in making doctor's decisions towards patients, about 60% to 70% of laboratory results can underlie the doctor's decisions.[6] These decisions include establishing a diagnosis by determining the

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cause of the disease, supporting an early warning system, monitoring treatment, maintaining health, and preventing the onset of disease.[4] Therefore, if this does not receive special attention, it can have an impact on the accuracy of laboratory examinations and can subsequently lead to misinterpretation by doctors so that it can have an impact on inappropriate treatment periods and even death.

Transport of specimens must meet the requirements for long delivery times that do not exceed the stability of the examination, are not exposed to sunlight and stored at a certain temperature.[7] Storage at cold temperatures (±4ºC) aims to inhibit blood cell metabolism which can prolong the life span and maintain cell morphology, so that the stability of the examination becomes longer. However, frozen blood specimens (±-4ºC), resulted in lysis of the specimen. In addition, mechanical factors such as shocks can also cause specimens to easily lyse.[8–10] Based on this, this study aims to determine the stability of the hematological examination of blood samples transported for 1 hour and 2 hours at room temperature and cold temperatures.

MATERIAL AND METHOD

This research is a laboratory experimental study with a pre-experimental research design through a onegroup pretest-posttest design approach. The study used human blood specimens which were then divided into two groups, namely room temperature and cold spoons. Observations before treatment were controls, and treatments were carried out after 1 hour and 2 hours of transportation. This research has been approved by the research ethics commission of Universitas Nahdlatul Ulama Surabaya with the number 091/EC/KEPK/UNUSA/2021. A total of 15 people participated in this study voluntarily.

Venous blood samples were taken from the fossa antecubital area using a K2-EDTA tube (BD Vacutainer, Becton Dickinson, USA). Each respondent was taken as many as 2 tubes with each volume of 3 cc. The samples obtained were immediately analyzed as research controls. Then the 1st tube is put in a transport container without ice (room temperature) and the 2nd tube is inserted into a transport container filled with ice (cold temperature). The containers were carried by motorized vehicles around the road, after 1 hour and 2 hours the analysis was carried out again.

Hematology analysis was performed using the hematology analyzer Sysmex XP-300 (Sysmex Corp., Japan) by taking the parameters of hemoglobin (Hgb), hematocrit (Hct), erythrocyte count (red blood cell, RBC), platelet count (platelet, PLT), leukocyte count (white blood cell, WBC), count the type of leukocyte (diffcount), MCV (meancorpuscular volume), MCH (mean corpuscular hemoglobin) and MCHC (mean corpuscular hemoglobin concentration). In addition, temperature and humidity measurements were carried out in the transport container and the environment using a digital thermometer and hygrometer (Therm Scan, China), shock measurements during transportation using a lutronvibrasimeter VB 8200 (Lutron electronic, Taiwan).

Inferential data analysis begins with a normality test using the Shapiro-Wilk test, the data is normally distributed if the p value > with a significance level of 5% or at = 0.05. Hypothesis testing was carried out to determine the difference in the results of the treatment with the control using Anova parametric analysis with the same subject. Ratio data that were not normally distributed were analyzed by nonparametric Anova Friedeman. The test results stated that there was a significant difference if the p-value < with a two-tailed significance and a significance value of 5% or = 0.05. Analyzes were performed using SPSS version 21 (IBM, United States).

RESULTS AND DISCUSSION

Maintaining examination stability is very important in laboratory services in order to maintain the quality of examination results, including hematology. This condition is generally found in laboratories that have a phlebotomy room far from the laboratory, sampling services in the field (homecare) or conducting examination referrals.[11]

During the study, the measured average ambient temperature was 33.4°C with an average humidity of 45%. The average temperature of the container without ice measured was 27.8°C with 43% humidity. The average temperature of the container with ice is 4.5°C with an average humidity of 52%. The average shock recorded in this study was 54.9. The results of this study are shown in Table 1.

Parameters of Hgb, PLT, WBC, NEUT are stable for up to 2 hours in both room temperature and cold conditions. While the stability of LYMPH parameters was only able at room temperature for a long time of 2 hours. Parameters Hct, RBC, MCV, MCHC and MXD there were differences in the results of the examination at cold temperatures after 1 hour and 2 hours. However, the parameters of RBC, MCV and MXD were stable for up to 1 hour at room temperature. While at room temperature parameters Hct and MCHC found differences in the results of the examination at 1 hour and no difference at 2 hours. The results of the unstable examination in this study, both at room temperature and cold temperatures, were the MCH parameter (Table 3).

Various studies related to examination stability have been published with various types of hematology analyzers. Publication of the stability of hematological examinations transported for 1 and 2 hours is still very limited, however, a study conducted by previous authors in the publication "Stability of routine hematological examinations on blood samples that were kept at room temperature using Cell-Dyn Ruby" showed that the parameters Hgb, Hct, RBC , MCV, MCH, MCHC, PLT, WBC and LYMPH were able to be stable at room temperature for up to 6 hours.[12]

When compared with various studies, the stability of hematological examination parameters with various hematology analyzers can give different results.[13–17] These differences can be influenced by various factors including the method used.[18] Uniquely, our study reported that the hematological examination was more stable at room temperature when transported.

Blood cells are cells that are easy to change if they are outside the blood circulation for a long time. Erythrocytes tend to change shape into spherocytes and crenation cells. Leukocytes generally undergo changes in the nucleus, granules, cytoplasm and vacuole formation. Meanwhile, the platelets changed shape to become spherical.[5,12,19–21] The stability of the examination in this study could be caused by these factors, generally it could be inhibited by a decrease in the storage temperature of the examination material. However, in this study, room temperature showed excellent examination stability. The shock factor that can affect the stability of specimens at cold temperatures cannot be explained because there are few publications regarding the stability of hematological examinations in transportation.

However, there are reports in other studies that unstable RBC parameters are caused by acid accumulation from cell metabolism and reduced ATP which causes RBC to change shape. Lack of ATP will also affect the RBC ionic pump so that it affects hemostasis which has an impact on the release of hemoglobin due to RBC lysis.[22] It could be that this condition causes the parameters of RBC and its derivatives to be unstable while HGB remains stable because the hemoglobin protein is still contained in the plasma. It was also reported in WBC, WBC morphological changes were associated with a decrease in ATP in cells and its decrease was associated with granulocyte degeneration.[22] WBC and NEUT are more stable than other leukocyte parameters, but it is still unclear because the number of studies is still small. However, we suspect that LYMPH and MDX morphological changes are faster than NEUT.

CONCLUSION

Our results show that the hematological examination is stable for up to 1 hour with the recommended storage at room temperature. Only Hgb, PLT, WBC, NEUT tests are stable for up to 2 hours in both room temperature and cold conditions.

AUTHORS' CONTRIBUTIONS

All authors contributed to the process of preparing this article.

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DATA AVAILABILITY STATEMENT

The utilized data in this investigation 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.

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