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Original Research Article

The Impact of Butterfly Pea Flower (*Clitoria ternatea L.*) Extract on Atherosclerosis Biomarker Profiles in Obese White Rats (*Rattus norvegicus L.*)

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Abstract

Clitoria ternatea L., commonly known as butterfly pea flower, has gained attention as a potential agent in the prevention and treatment of atherosclerosis due to its rich content of bioactive compounds, such as anthocyanin, a type of flavonoid renowned for its potent antioxidant and anti-inflammatory properties, along with its ability to enhance the immune system.

Objective: This study aimed to elucidate the positive impact of butterfly pea flower extract on atherosclerosis biomarker profiles.

Methods: The approach framework used in this research was a true experimental laboratory with a Control Group Post-Test design. Obese male white rats were selected as the research subjects. Thirty-six obese white rats were randomly divided into six groups using the Completely Randomized Design (CRD) method. The data collected were VCAM and IL-6 levels from the blood serum of obese white rats tested using an ELISA photometer. The tunica intima thickness was measured using a microscope. Data were analyzed employing SPSS 18 software, utilizing one-way ANOVA statistical tests and post hoc Tukey tests.

Results: The research showed that with the increase in Butterfly Flower Extract (BPFE) dose, there was a consistently lower level of inflammatory biomarkers, such as VCAM-1 and IL-6, compared to positive controls and other variations, as well as tunica intima thickness was thinning than others. A dose of 600 mg/kg BW (P3 group) had VCAM-1 levels up to 30.40 ± 6.71 ng/mL, IL-6 levels up to 17.70 ± 8.29 ng/mL, and tunica intima thickness up to 3.18 ± 1.24 µm.

Conclusion: BPFE effectively lowers inflammatory biomarkers and thins the tunica intima thickness in obese white rats at 600 mg/kg BW dose. It may offer promising therapeutic potential in addressing atherosclerosis.

Keywords: *VCAM-1; IL-6; tunica intima thickness; butterfly pea flower extract.*
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INTRODUCTION

In the modern era, obesity has emerged as a complex multifactorial disease of increasing concern. It is characterized by excessive adipose tissue accumulation in the body, resulting from an imbalance between caloric intake and expenditure. This condition poses immediate physical risks and significantly elevates the risk of various severe diseases, with atherosclerosis often referred to as the 'silent killer' due to its asymptomatic nature until advanced stages.[1,2] Atherosclerosis, a highly hazardous arterial disease, is characterized by the progressive accumulation of atheromatous plaques on the arterial walls. This pathological process commences with the endothelial cell damage within the arteries, coupled with increased free radicals that oxidize low-density lipoproteins (LDL) into oxidized LDL (Ox-LDL). This initial step is a pivotal foundation for the subsequent stages of atherosclerosis.[3–5]

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Table 1. Sample Treatment Groups

Group	Sample Code	Treatment for 45 Days
Negative Control	NC	Given standard feed
Positive Control	PC	Given HFAD feed
Na-CMC	P0	Given HFAD feed with Na-CMC 1%
BPFE-200	P1	Given HFAD feed with Na-CMC 1% and BPFE at 200 mg/kg BW doses
BPFE-400	P2	Given HFAD feed with Na-CMC 1% and BPFE at 400 mg/kg BW doses
BPFE-600	P3	Given HFAD feed with Na-CMC 1% and BPFE at 600 mg/kg BW doses

Notes: BPFE = Butterfly Pea Flower Extract, HFAD = High-Fat Atherogenic Diet, Na-CMC = sodium carboxymethyl cellulose

Ox-LDL migrates to the sub-endothelial layer in conjunction with monocytes, transforming monocytes into macrophages, which subsequently phagocytize Ox-LDL, forming foam cells.[3,4] The accumulation of foam cells initiates the formation of fatty streaks within the arteries, consequently narrowing the arterial lumen and amplifying inflammation via the upregulation of pro-inflammatory cytokines, such as Vascular Cell Adhesion Molecule-1 (VCAM-1), Interleukin-6 (IL-6), and Tunica Intima (TI) thickening. This intricate cascade of events underscores the dangerous nature of atherosclerosis.[6]

Presently, the treatment of atherosclerosis predominantly relies on pharmacological interventions involving drugs such as simvastatin. Although practical, prolonged usage of simvastatin is associated with severe adverse effects, including myopathy and kidney failure, underscoring the imperative need for safer therapeutic alternatives. One promising alternative is traditional herbal medicine, specifically the extract derived from the butterfly pea flower (*Clitoria ternatea L.*). Butterfly pea flower extract is rich in bioactive compounds, primarily anthocyanins belonging to the flavonoid family, renowned for their potent antioxidant and anti-inflammatory properties. These compounds can fortify the immune system and mitigate inflammation within arterial walls.[7,8] Numerous prior investigations have lent credence to the therapeutic potential of butterfly pea flower extract in managing diverse ailments. For instance, Maneesai et al.[9] documented the efficacy of butterfly pea flower extract in ameliorating cardiovascular dysfunction and decreasing oxidative stress in nitric oxide-deficient hypertensive rats. Maulidy et al.[10] also observed that butterfly pea flower extract could reduce total cholesterol levels in high-fat diet-induced obese rats (*Rattus norvegicus L.*). Furthermore, Widowati et al.[11] research unveiled the promising utility of butterfly pea flower extract in treating diabetes mellitus and dyslipidemia.

The extraction process used the maceration method with ethanol as the solvent to obtain bioactive compounds from butterfly pea flowers. While maceration is effective, it has the drawback of being time-consuming and yielding low extraction efficiency. Therefore, a more efficient extraction approach, such as the sonication method, is needed. Sonication utilizes ultrasonic vibrations to facilitate the disruption of cell walls in the extracted sample, allowing the chemical components within to be removed more easily from the cells. Moreover, the sonication extraction process requires less time than conventional methods such as maceration and Soxhlet extraction.[12]

This study aimed to investigate the effect of butterfly pea flower extract (BPFE) administration on atherosclerosis biomarker analysis in obese rats, employing a more efficient extraction method to assist in identifying the therapeutic potential of butterfly pea flower extract in addressing atherosclerosis conditions in obese rats.

MATERIALS AND METHODS

Research Design

This study adopted a pure experimental design with a post-test-only control group approach to assess the impact of ethanol extract from butterfly pea flowers on obese male white rats. The research was conducted from March 2022 to February 2023 at the Organic Chemistry Laboratory and Pharmacology Laboratory, Faculty of Pharmacy, Institut Kesehatan Medistra Lubuk Pakam, Deli Serdang, Indonesia. This research has undergone ethical considerations and received approval from the Research Ethics Committee of Institut Kesehatan Medistra Lubuk Pakam (Ethical Clearance Number 027.D/KEP-MLP/III/2022).

Population and Samples

The research population was composed of obese male white rats (*Rattus norvegicus L.*). The sample consisted of 36 male white rats. Each sample had to meet precise inclusion criteria, which encompassed an initial body weight falling within the range of 180-200 grams, an age of 3-4 months at the study's commencement, a state of good health, and a Lee index > 300 as an indicator of obesity. Samples that did not survive during the study were excluded based on the predefined exclusion criteria. The research samples were allocated into six groups, each randomly comprising six white rats. Each group underwent specific treatment for 45 days. Further details regarding the treatments administered to each sample group can be found in Table 1.

Preparation of High-Fat Atherogenic Diet (HFAD)

The high-fat diet used to induce obesity in male white rats consisted of goat fat 10%, cholesterol 1%, quail egg yolk 5%, cooking oil 1%, and standard feed. The HFAD

was prepared by crushing the common feed using a grinder, sequentially adding goat fat 10%, cholesterol 1%, quail egg yolk 5%, and cooking oil 1% to the feed. The mixture was stirred until well-distributed, reshaped using a noodle-making machine, and baked in an oven until fully cooked.

Preparation of 1% Na-CMC Solution

Ten grams of sodium carboxymethyl cellulose (Na-CMC) were dissolved in 200 mL of distilled water, heated, and allowed to float on the surface of the distilled water for approximately 15 minutes while stirring until a transparent and homogeneous mass formed. Then, distilled water was added to the mass until 1000 mL was obtained while continuously stirring. Na-CMC served as a hanging or stabilizing agent to prepare BPFE suspension to administer to white rats, keeping BPFE dispersed in the liquid and not settling.

Preparation of Butterfly Pea Flower Extract (BPFE) Using the Sonication Method

The butterfly pea flowers (BPFE) of 2,000 g are thoroughly washed, dried under sunlight, placed in an oven at 105°C for 60 min to remove moisture, blended into a simplisia powder, and filtered through a 50-mesh sieve. The simplisia powder of 100 g was dissolved in 500 mL of ethanol 96% and introduced into an ultrasonic batch operating at 42 KHz for 60 min. Next, it was filtered, resulting in a liquid extract, which was subsequently concentrated using a rotary evaporator at a temperature of 40°C. It was then dried using a water bath to yield a butterfly pea flower extract.[13]

Preparation and Administration of Butterfly Pea Flower Extract Suspension

The BPFE suspension was prepared individually for each group at 200/400/600 mg/kg BW doses. The BPFE was calculated and mixed with a 1% Na-CMC solution to reach a volume of 42 mL, then stirred until homogeneous. This suspension was prepared for use over one week, with daily administration to white rats for seven days. The same preparation and administration of BPFE suspension were continued into the following week, lasting until day 45.

Phytochemical Screening of Butterfly Pea Flower Extract

The phytochemical screening of butterfly pea flower extract involved several methods. Firstly, for alkaloid analysis, 2 mL of the extract solution was evaporated until residue formation, followed by dissolution in 5 mL of hydrochloric acid 2N, which was then divided into two reaction tubes. Tube one received three drops of Dragendorff's reagent, and tube two received three drops of Mayer's reagent. Orange and yellow precipitates indicated the presence of alkaloids. For flavonoid analysis, 1 mL of the extract was dissolved in concentrated acetone with borax and oxalic acid, heated, and mixed with ether. Yellow fluorescence under 366 nm UV light indicated the presence of flavonoids. Saponin assessment involved vigorously mixing 10 mL of the extract and adding one drop of hydrochloric acid 2N, with persistent foam after 10 minutes indicating the presence of saponin. The examination for tannins was conducted by reacting the BPFE with a ferric chloride solution of 10%, resulting in the formation of a greenish-black color, which indicates the presence of tannins. Lastly, terpenoid analysis used the Lieberman-Burchard reaction: evaporating 2 mL of extract, dissolving the residue in 0.5 mL chloroform, adding 0.5 mL glacial acetic acid, and then 2 mL of concentrated sulfuric acid to observe a brown or purple ring at the interface, signifying the presence of terpenoids[13,14].

Measurement of Atherosclerosis Biomarker

This study measured atherosclerosis biomarkers, specifically the concentrations of VCAM-1 and IL-6 taken from blood serum and the tunica intima thickness taken from the aorta in obese white rats. VCAM-1 and IL-6 measurements were conducted using enzyme-linked immunosorbent assay (ELISA) kits. Final absorbance was assessed using an ELISA photometer from Bio-Rad (Model 680) and then converted to ng/mL. Tunica intima thickness was measured using a microscope equipped with an ocular micrometer.

Data Analysis

Data analysis was conducted using One-way ANOVA SPSS version 18. The significance level set was 95% ($\alpha = 0.05$). A *p*-value exceeding 0.05 signified the absence of a significant difference, while a *p*-value below 0.05 signified a considerable difference. The analysis results were continued with the Post Hoc Tukey test.

RESULTS

Phytochemical Screening Results of Butterfly Pea Flower Extract

The phytochemical screening results using various reagents demonstrate that the butterfly pea flower extract contains bioactive compounds such as flavonoids, saponins, tannins, and terpenoids. It is evident in Table 2.

Table 2. Phytochemical Screening Results of Butterfly Pea Flower Extract

No	Secondary metabolite compounds	Result
1	Alkaloids	-
2	Flavonoids	+
3	Saponins	+
4	Tannins	+
5	Terpenoids	+

Notes: (+) = contained in the extract

Atherosclerosis Biomarkers Analysis of VCAM-1 in Obese White Rats

Obesity occurs due to the accumulation of excess fat in adipose tissue, causing atherosclerosis. Atherosclerosis triggers an inflammatory response, causing an increase in VCAM-1 levels. The impact of BPFE on VCAM-1 levels is presented in Table 3.

Table 3. V-CAM Levels in Obese White Rats

Group	VCAM-1 Level (ng/mL)	*ρ*-value
NC	28.33 ± 6.31	0.009
PC	112.90 ± 51.44	
P0	108.30 ± 56.09	
P1	95.05 ± 14.58	
P2	73.00 ± 42.12	
P3	30.40 ± 6.71	

Notes: NC = Negative Control, PC = Positive Control, P0 = Na-CMC, P1 = Na-CMC + BPFE-200, P2 = Na-CMC-BPFE-400, and P3 = Na-CMC+BPFE-600

Table 3 reveals that the mean VCAM-1 levels in the NC group were 28.33 ± 6.31 ng/mL, PC group were 112.90 ± 51.44 ng/mL, and P0 group were 108.30 ± 56.09 ng/mL. This study demonstrated that the higher the BPFE dose, the more significant the reduction in VCAM-1 levels. The 600 mg/kg BW dose (P3 group) showed a lower VCAM-1 level of 30.40 ± 6.71 ng/mL. The analysis demonstrated a significant difference among the treatment groups (ρ-value of 0.009), indicating that BPFE affected VCAM-1 levels in obese rats.

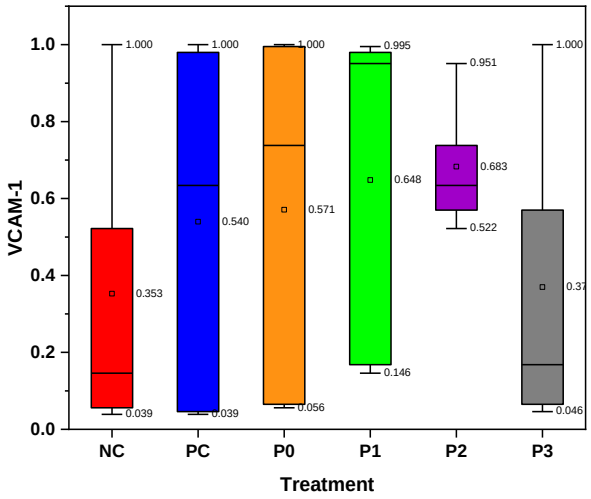

Figure 1. Tukey Box Plot of VCAM-1 in Obese White Rats

Tukey box plot (Figure 1) showed there were significant differences in the PC – P3 group (ρ = 0.046, ρ < 0.05) with an average difference in the VCAM-1 level of 82,500. Meanwhile, the other groups did not show significant differences in VCAM-1 levels. These results indicate that differences in VCAM-1 levels only occurred in certain groups, while other groups did not show significant differences.

Atherosclerosis Biomarkers Analysis of IL-6 in Obese White Rats

Obesity occurs because excess fat accumulation causes atherosclerosis, which, in turn, triggers an inflammatory response, resulting in increased IL-6 levels. The impact of BPFE administration on IL-6 levels in obese white rats is presented in Table 4.

Table 4. IL-6 Levels in Obese White Rats after BPFE Administration

Sample Code	IL-6 Level (ng/mL)	*ρ*-value
NC	22.90 ± 4.22	0.006
PC	95.65 ± 10.86	
P0	85.28 ± 65.69	
P1	55.75 ± 15.91	
P2	44.25 ± 20.63	
P3	17.70 ± 8.29	

Notes: NC = Negative Control, PC = Positive Control, P0 = Na-CMC, P1 = Na-CMC + BPFE-200, P2 = Na-CMC-BPFE-400, and P3 = Na-CMC+BPFE-600

Table 4 illustrates that the initial IL-6 levels were highest in the PC and P0 groups, while the NC group had the lowest. However, when the BPFE was given to obese rats, the IL-6 levels dropped. The most significant drop occurred in the P3 group, which got the highest extract dose. Statistical analysis confirmed that the BPFE significantly impacted IL-6 levels in obese rats, with IL-6 levels measuring 17.70 ± 8.29 ng/mL, which means there was a significant difference among the treatment groups (ρ-value of 0.006), indicating that BPFE affected IL-6 levels in obese rats.

In the Tukey box plot of IL-6 levels (Figure 2), there were significant differences in three groups, namely, the PC – P3 group (ρ = 0.016) with an average difference of 77,950, and group P0 – P3 (ρ = 0.044) with an average difference of 67.575. Meanwhile, the other groups did not show significant differences in IL-6 levels. These results indicated differences in IL-6 levels in certain groups, while other groups did not show significant differences.

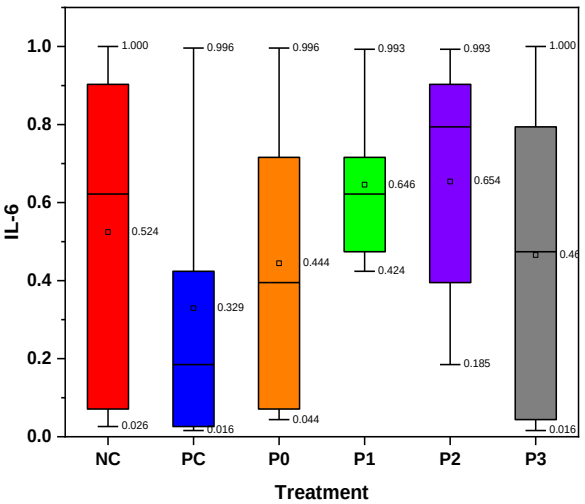

Figure 2. Tukey Box Plot of IL-6 in Obese White Rats

Atherosclerosis Biomarkers Analysis of Tunica Intima in Obese White Rats

Atherosclerosis, caused by fat accumulation due to obesity, can trigger an inflammatory response that increases tunica intima thickness. Histological results of the aortic tunica intima in obese white rats are presented in Figure 3.

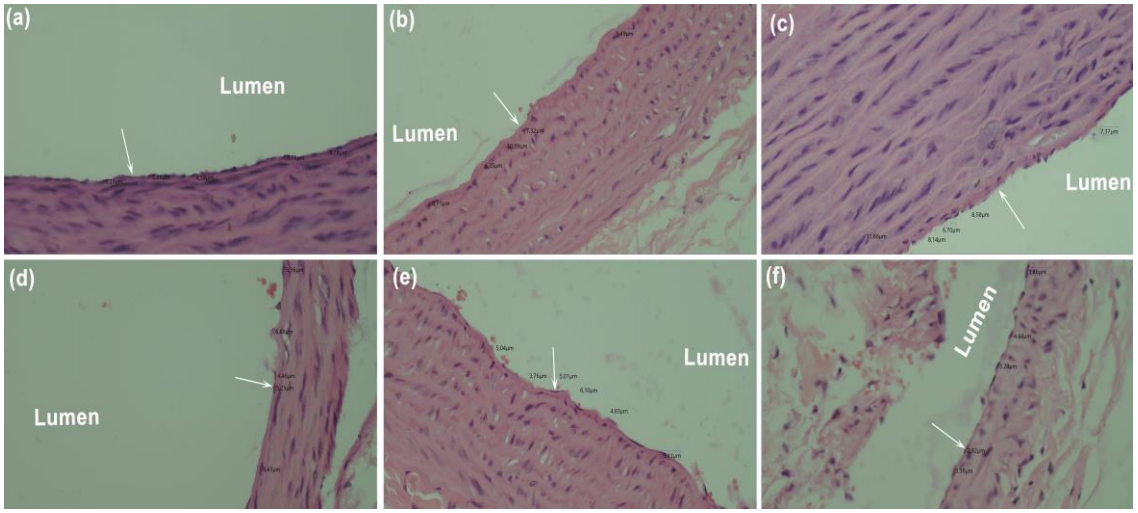

Figure 3. Histology of the Aortic Tunica Intima in the Obese White Rat of (a) NC, (b) PC, (c) P0, (d) P1, (e) P2, and (f) P3

Table 5. Tunica Intima Thickening in Obese White Rats after BPFE Administration

Group	Sample Code	Tunica Intima Thickness (µm)	*ρ*-value
Negative Control	NC	4.77 ± 2.00	0.001
Positive Control	PC	7.94 ± 0.61	
Na-CMC	P0	6.92 ± 1.93	
BPFE-200	P1	6.49 ± 1.05	
BPFE-400	P2	4.87 ± 0.73	
BPFE-600	P3	3.18 ± 1.24	

The NC group (Figure 3A) fed a standard diet showed aortic structures without atherosclerotic lesions. The endothelial and smooth muscle cell layers remained intact and organized with an intimal thickness of 4.77 ± 2.00 µm. The PC group (Figure 3B) was exposed to HFAD with apparent atherosclerotic lesions characterized by the accumulation of macrophages, inflammatory cells, foam cells, fat accumulation in smooth muscle cells, and endothelial damage[15]. The thickness of the tunica intima in the PC group was 7.94 ± 0.61 µm. The P0 group (Figure 3C) showed a slight improvement in the aortic structure compared with the PC group, but some damage remained. The P1 group (Figure 3D) showed further progress in the aortic structure. The P2 group (Figure 3E) showed that the aortic structure continued improving. The P3 group (Figure 3F) showed that the aortic structure was restored without atherosclerotic lesions, accumulation of macrophages, foam cells, or accumulation of intracellular fat in smooth muscle cells. The endothelial and smooth muscle cell layers showed organized characteristics with an intimal thickness of 3.18 ± 1.24 µm.

The effects of BPFE administration on tunica intima thickness in obese white rats are displayed in Table 5.

Table 5 shows that the NC group has an intima wall thickness of 4.75 ± 1.98 µm, while the PC group has a thickness of 7.92 ± 0.66 µm, and the P0 group has a thickness of 6.94 ± 1.99 µm. The PC group showed the thickest average intimal wall thickness, while the P3 group had the thinnest average tunica intima thickness. After administering BPFE to obese white rats, the thickness of the tunica intima became thinner, whereas the P3 group at a dose of 600 mg/kg BW had a tunica intima thickness of 3.18 ± 1.24 µm. Further statistical analysis showed significant differences between treatment groups ($\rho < 0.05$) with a ρ-value of 0.001, indicating that BPFE affected the thickness of the intima in obese white rats. Next, data pairs that differ significantly or not are presented in Figure 4.

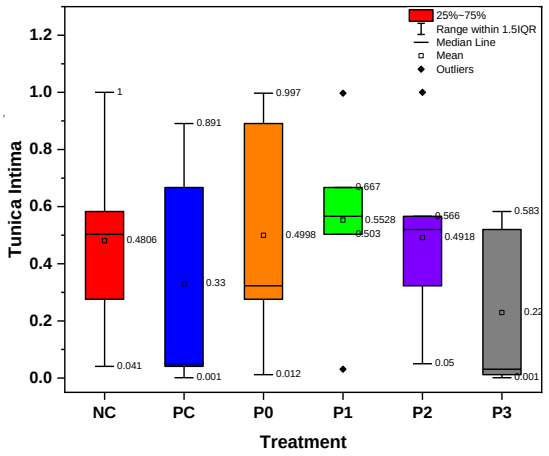

Figure 4. Tukey Box Plot of Tunica Intima in Obese White Rats

In the Tukey box plot of tunica intima thickness (Figure 4), there were some significant differences, namely between the PC – P3 group (p = 0.001) with an average difference in the tunica intima thickness of 4.765 µm, between the P0 – P3 (ρ = 0.012) with an average

difference in the tunica intima thickness of 3,740 μm, and between group P1 – P3 (ρ = 0.031) with an average difference of the tunica intima thickness 3.310 μm. Meanwhile, the other groups did not show significant differences in tunica intima thickness. The results of this study indicated significant differences in Tunica Intima thickness in certain groups, and these findings can be the basis for further research to understand the clinical implications. Additionally, the results of this study also noted differences in VCAM-1 and IL-6 levels between other groups, highlighting the complexity of the relationship in the context of this study.

DISCUSSION

Phytochemical Evaluation of Butterfly Pea Flower Extract

Phytochemical screening of butterfly pea flower extract (Table 2) identified key bioactive compounds: flavonoids, saponins, tannins, and terpenoids. These findings align with Jeyaraj et al.[16] research on butterfly pea flowers, which reported similar compounds. When exposed to the Lieberman-Burchard reagent, the extract exhibited intense yellow fluorescence under ultraviolet light, indicative of flavonoids, consistent with the Kumar & More[17] study. Furthermore, the extract's reaction with 2N hydrochloric acid produced a significant foaming response, confirming the presence of saponins. This observation echoes the findings of Manjula et al.[18], who reported on the polar glycoside and nonpolar steroid compounds of saponins. In addition, the extract's interaction with $FeCl_3$ reagent produced a dark green color, signifying tannin presence, correlating with Lijon et al.[19], that tannins' phenolic compounds are water-soluble and polar. Lastly, a brownish ring formed when the extract reacted with the Lieberman-Burchard reagent, indicative of terpenoid presence, in line with the findings of Ginting et al.[20]. Terpenoids, characterized by long hydrocarbon chains (C30), exhibit nonpolar characteristics and possess cyclic structures, such as alcohols, aldehydes, or carboxylic acids with -OH groups, rendering them semi-polar. These diverse bioactive compounds in butterfly pea flower extract hold promise for various potential health benefits and applications.

Impact of Butterfly Pea Flower Extract on VCAM-1 Levels in Obese Rats

The statistical analysis revealed a significant difference between the positive control and the administration of BPFE-200, BPFE-400, and BPFE-600, indicating that BPFE for 45 caused VCAM-1 to be lower than the positive control. The highest VCAM-1 levels were observed in the control group fed with HFAD or positive control groups. The administration of BPFE lowers the VCAM-1 level, where the higher the extract dose, the lower the VCAM-1 level. The results of the analysis showed that the BPFE-600 and the positive control showed significant differences in VCAM-1 levels. This finding aligns with research by Maneesai et al.[9], who reported that BPFE, which contains flavonoids, effectively improved cardiovascular dysfunction and oxidative stress due to a reduction in VCAM-1 levels in nitric oxide-deficient hypertensive rats. The lower VCAM-1 levels can be attributed to anthocyanin compounds in butterfly pea flower extract, a flavonoid known for its antioxidant and anti-inflammatory effects. Anthocyanins effectively inhibit enzymes involved in the inflammatory process, arachidonic acid metabolism, and LDL oxidation. This result is corroborated by the studies conducted by prior studies.[21,22]

Impact of Butterfly Pea Flower Extract on IL-6 Levels in Obese Rats

Our study showed that administration of BPFE-200, BPFE, 400, and BPFE-600 significantly lower IL-6 levels compared with positive controls. This finding aligns with research conducted by Widowati et al.[11], who reported that BPFE contains a bioactive flavonoid compound that functions as an antioxidant and anti-inflammatory so that it can lower IL-6 levels in experimental rats with diabetes mellitus and dyslipidemia. Muniroh et al.[23] also reported that BPFE has an anti-inflammatory function in leprosy, significantly reducing IL-6 levels. The reduction in IL-6 levels can be attributed to bioactive flavonoids in BPFE, which act as antioxidants and anti-inflammatory agents. Flavonoids can capture free radicals, inhibit LDL oxidation, and protect endothelial cell integrity. This finding aligns with research conducted by prior studies[24]. Maneesai et al.[9] also reported that the antioxidant properties of flavonoids prevent LDL oxidation and protect cell structures. Other studies by Rakha et al.[15] and Yamin et al.[25] also reported that flavonoids directly capture free radicals and stabilize reactive oxygen species (ROS) due to their hydroxyl groups, supporting the results of this study. The most significant results are observed at the BPFE-600 dose, as this dose possesses the optimal concentration to stimulate the desired biological response. According to Maneesai et al.[9], variability in this response may be influenced by factors such as dosage, administration time, rat health conditions, and the variability in BPFE composition. The BPFE holds promising therapeutic potential in addressing atherosclerosis. The section contains various bioactive compounds, primarily flavonoids such as anthocyanin, known for their antioxidant and anti-inflammatory properties. Flavonoids can protect arterial walls, inhibit LDL oxidation, and lower inflammation, all of which play roles in the prevention and treatment of atherosclerosis.

Impact of Butterfly Pea Flower Extract on Tunica Intima Thickness in the Obese Rats

The outcomes signify the significant impact of BPFE administration on aortic thickness, with the most effective dose for reducing tunica intima thickness being 600 mg/kg BW/day. This reduction can be attributed to flavonoids in BPFE, which function as antioxidants, inhibit LDL oxidation by macrophages, mitigate aortic thickening, and stimulate cellular immunity to prevent foam cell formation and smooth muscle cell proliferation within the tunica intima. These findings are consistent with studies by Ji et al.[26] and Ahmed,[27] which reported that flavonoids act as antioxidants and thins tunica intima thickness. Additionally, flavonoids exert anti-inflammatory effects by inhibiting prostaglandin synthesis through cyclooxygenase inhibition, preventing the conversion of arachidonic acid into prostaglandins.

One of the weaknesses of this study is the relatively short duration of intervention in the animal model, and the BPFE dosage difference was quite wide (i.e., 200 mg/kg) instead of a smaller dosage difference (for instance, 100mg/kg). There is a possibility that the dosage can be reduced is a smaller concentration difference or longer intervention duration is applied. Additionally, while the results suggest promising therapeutic potential, the long-term effects and safety of using BPFE in humans must be explored in clinical trials. The implication of this research provides valuable insights into the potential of BPFE as a natural remedy for atherosclerosis and related cardiovascular issues. The ability of BPFE to effectively lower inflammatory biomarkers and improve aortic health in obese rats suggests its therapeutic promise. This research sets the stage for further exploration of BPFE as a complementary approach to human cardiovascular health, offering a potentially safer and more natural alternative to traditional medications.

CONCLUSION

The butterfly pea flower (Clitoria ternatea L.) extract exhibits significant potential in reducing the levels of atherosclerosis biomarkers, including VCAM-1 and IL-6 concentration and intimal tunica thickness in the aorta of obese male white rats. The research findings indicate that administering butterfly pea flower extract for 45 days lowers VCAM-1 and IL-6 levels and thins the tunica intima thickness in obese white rats. The most effective dose in reducing intimal tunica thickness is 600 mg/kg body weight daily.

This butterfly pea flower extract effectively treats various diseases and can be a safer therapeutic alternative than promising pharmacological drugs such as simvastatin. Although not statistically significant, the reduction in levels of VCAM-1, IL-6, and tunica intima with the use of Na-CMC needs to be studied more deeply regarding the effect of Na-CMC on atherosclerosis biomarker analysis. It is necessary to validate these findings and identify more detailed mechanisms behind the positive impact of butterfly pea flower extract on atherosclerosis. Further research is needed to validate these findings and determine the more complex mechanisms behind the positive effects of butterfly pea flower extract on atherosclerosis.

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Original Research Article

Correlation Between Corrected TIMI Frame Count with the Extent of Myocardial Fibrosis on ST-Elevation Myocardial Infarction Patients Undergoing Primary Percutaneous Coronary Intervention

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Abstract

Background: Microvascular injury after primary percutaneous coronary intervention (PPCI) reperfusion contributes to necrosis propagation. Corrected TIMI Frame Count (CTFC) is a surrogate marker of microvascular dysfunction and can stratify in-hospital mortality risk in patients with final TIMI flow 3. The extent of myocardial fibrosis after STEMI is associated with a higher incidence of major cardiovascular events. This study was aimed to determine the relationship between CTFC in the infarct-related artery and myocardial fibrosis area based on cardiac magnetic resonance (CMR) in STEMI patients undergoing PPCI.

Methods: This retrospective cohort study included 31 STEMI patients who had undergone PPCI and CMR examination between days 60 and 75 after STEMI as the sample. CTFC was measured in the infarct-related artery from post-PPCI angiogram recordings. The myocardial fibrosis area was measured from late gadolinium enhancement CMR (LGE-CMR) imaging results.

Results: In this study, the mean age was 51.61±10.49 years, 90.3% were male, non-anterior infarction location was 58.1%, mean total ischemic time was 489.48±228.33 minutes, mean CTFC was 27.4±9.3 frames, and mean myocardial fibrosis was 18.33±7.87%. There was no significant correlation found between CTFC and myocardial fibrosis (p=0.530), however total ischemic time had a positive and significant correlation with myocardial fibrosis (p=0.025, r=0.403).

Conclusion: CTFC in the infarct-related artery is not correlated with myocardial fibrosis area in STEMI patients undergoing PCI.

Keywords: *CTFC; myocardial fibrosis; LGE-CMR; STEMI; PPCI*
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INTRODUCTION

In Indonesia, death from coronary heart disease is one of two leading causes of death after stroke.[1] Reperfusion therapy, either by thrombolysis or primary percutaneous coronary intervention (PPCI) in patients with ST-elevation myocardial infarction (STEMI) is the preferred treatment recommendation[2] and is aimed to save the myocardium and reduce mortality.[3] Patency of epicardial coronary artery flow on the infarct-related artery is a successful marker of reperfusion therapy, but despite good and effective blood flow in the epicardial vessels, myocardial tissue perfusion remains ineffective in the presence of coronary microvascular dysfunction, and it contributes significantly to the final severity of infarction and is an independent predictor of morbidity and mortality.[4]

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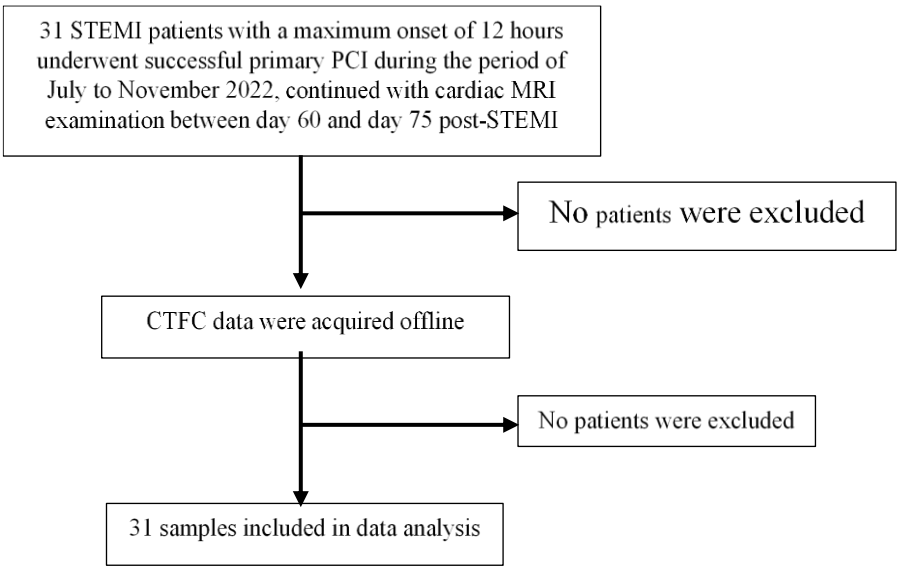

Figure 1. Research Flow Diagram

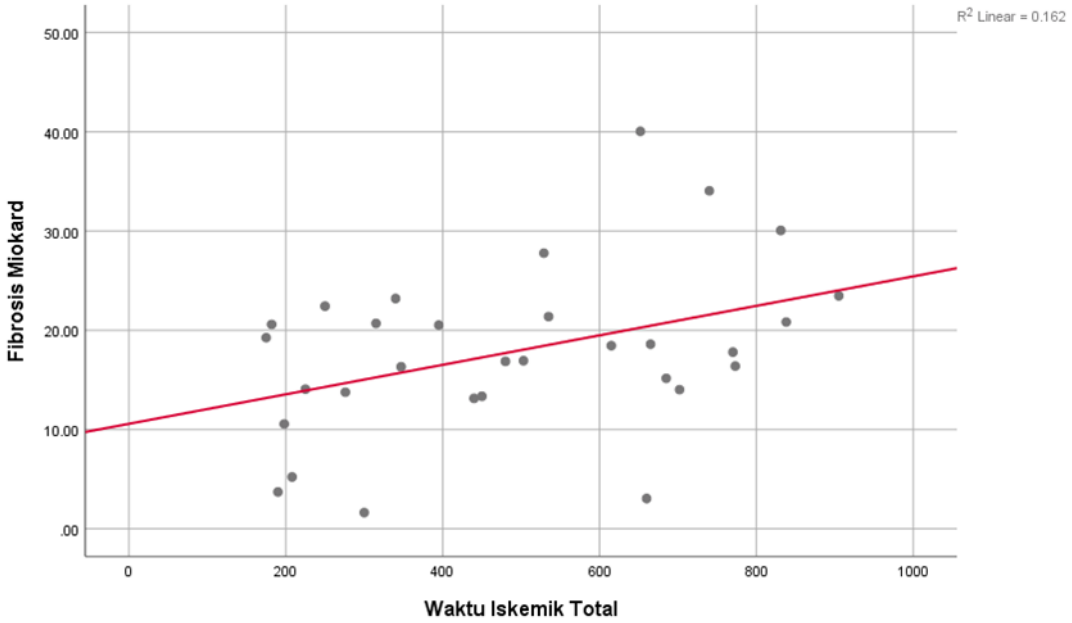

Figure 2. Pearson Correlation Test Between Total Ischemic Time and The Extent of Myocardial Fibrosis

Corrected TIMI frame count (CTFC) quantifies the extent of TIMI flow using the time required for contrast to fill the length of the epicardial artery and is one of the diagnostic methods for microvascular disorder in STEMI. This method is consistent with the results of SPECT and has been associated with various clinical outcomes such as improved mortality[5], better functional recovery and lower complication rates in patients undergoing PPCI.[6] Cardiac remodelling after acute myocardial infarction is characterized by the presence of fibrosis in myocardial tissue, which also has implications for long-term prognosis, causing contractile and rhythm dysfunction[7,8] Late gadolinium enhancement (LGE) imaging from cardiac magnetic resonance (CMR) is considered the gold standard for assessing infarct size and fibrosis. CMR-based infarct size is a strong independent predictor of all-cause mortality and heart failure hospitalization within 1 year.[9] 27 This study aimed to find a relationship between CTFC and myocardial fibrosis in STEMI patients undergoing PPCI.

METHODS

Study Design and Participants

This was a retrospective cohort study done in Kariadi Hospital, Semarang, Indonesia from July to November 2022. Infarct size was measured using cardiac magnetic resonance in patients who had undergone successful PPCI. The goal was to determine a relationship between CTFC and infarct size. Patients were enrolled at the Dr. Kariadi General Hospital, Semarang, Indonesia who met the following criteria; (1) had a first-ever ST-segment elevation myocardial infarction (STEMI) with onset within 12 hours; and (2) had successfully completed a

PPCI procedure (final TIMI flow III criteria and having residual stenosis of less than 30% in the culprit artery); (3) had underwent CMR examination in 60 – 75 days post myocardial infarction. Patients with a history of previous PCI, a history of previous coronary artery bypass graft (CABG) surgery, patients with non-ischemic heart valve disease greater than moderate degree, atrial fibrillation, pulmonary hypertension, hypertrophic cardiomyopathy, chronic kidney failure, a history of previous heart failure and patients with contraindications to MRI examination were excluded from study. The institutional Ethics Committee of Dr. Kariadi General Hospital had approved the study. (Ethical approval No. 1478/EC/KEPK-RSDK/2023) The research flow diagram can be seen in figure 1.

CTFC Acquisition

The assessment of CTFC was performed offline on the last angiographic image of the infarct related artery after the procedure had finished using RadiAnt DICOM software. The acquisition method of CTFC was as described by Gibson. CTFC were acquired by 2 observers blinded to subject data.

CMR Image Acquisition

The assessment of myocardial fibrosis was performed using LGE-CMR between day 60 and day 75 after STEMI, with an average examination time of 68±4 days. The GE Signa Voyager 1.5 Tesla MRI machine was used for the assessment of myocardial fibrosis. LGE imaging was performed 10-15 minutes after the intravenous bolus injection of 0.2 mmol/kg body weight of Gadobutrol contrast agent (Gadovist 1.0, Bayer Inc.). The MRI results were then analyzed using Philips Intellispace Portal 12 software by 2 observers blinded to subject data.

Table 1. Demographic Data

Variable	Description (n=31)
Age (y.o)*	51,61±10,49
Male**	28 (90,3%)
Systolic Blood Pressure (mmHg)*	126,41±34,29
Diastolic Blood Pressure (mmHg)*	81,25±20,3
Heart Rate (beats per minute)*	74,83±15,48
CAD Risk Factors**	
Dyslipidemia	23 (74,2%)
Smoker	21 (67,7%)
Hypertension	16 (51,6%)
Diabetes Melitus	12 (38,7%)
Medical Therapy**	
Aspirin	31(100%)
Ticagrelor	26 (83,9%)
clopidogrel	5 (16,1%)
ACE-Inhibitors	30 (96,8%)
Beta-blockers	24 (77,4%)
Statin	31 (100%)
Spironolactone	11 (35,5%)
Laboratorium	
Hemoglobin (g/dl)*	14,6±1,47
Random Blood Glucose (mg/dl)*	156±64
creatinin (mg/dl)*	1,17±0,24
Angiographic Parameters	
Total Ischemic Time (minutes)*	489,48±228,33
< 360 minutes	12 (38,7%)
≥ 360 minutes	19 (61,3%)
Infarct Location	
Anterior**	13 (41,9%)
Non-anterior	18 (58,1%)
Culprit Lesion**	
LAD	13 (41,9%)
LCX	3 (9,7%)
RCA	15 (48,4%)
CTFC*	27,4±9,3 frames
≤ 27 frames**	17 (54,8%)
> 27 frames**	14 (45,2%)
Multivessel Disease**	
Yes	16 (51,6%)
No	15 (48,4%)
Complete Revascularization**	17 (54,8%)
Echocardiographic Parameter*	
LVEF biplane (%)	52,82±8,96
GLS (%)	-11,49±3,15

Statistical Analysis

The mean, standard deviation, and percentage of the data were presented. The data's normality was examined using the Shapiro-Wilk test. Data CTFC and fibrosis area were examined using Pearson's correlation test. The IBM SPSS software version 23 was used to conduct all statistical analyses, and a P value of 0.05 was utilized to determine statistical significance.

RESULTS

Clinical and Demographic Data

A total of 31 subjects met the inclusion criteria. The baseline characteristics of patients are shown in Table 1. The majority of patients in this study were male, with a total of 28 patients (90%). Among the risk factors for coronary heart disease, dyslipidemia was the most commonly found risk factor, accounting for 74% of the cases. The mean body mass index (BMI) was indicative of obesity, with a value of 25.17±4.45 kg/m2. The most common location of the infarction was in the non-anterior region, with 18 patients (58.1%). High-intensity statins were given to all patients along with dual antiplatelet treatment. ACE inhibitors were given to a total of 30 patients (97%) and beta-blockers to 24 individuals (77%) in total. When patients had arrived at the hospital, the average STEMI symptom onset time was 6.16±3.43 hours, and the mean total ischemic time was 489.48±228.33 minutes. 17 patients (54.8%) underwent complete revascularization, while 16 patients (51.6%) had multivessel disease. There were 15 patients (48.4%) with the culprit vessel in the right coronary artery**.**

Correlation Between CTFC and the Extent of Fibrosis

The mean CTFC for the entire sample in this study was 27.4±9.17 frames. The mean infarct size measure by LGE-CMR were 18.33±7.87%. The analysis using Pearson's correlation showed no significant correlation between CTFC and infarct size by LGE-CMR (p=0.530, r=0.117).

Correlation Between Total Ischemic Time and the Extent of Fibrosis

In this study, we found that the mean total ischemic time was 489.48±228.33 minutes. The infarct size and total ischemic time were correlated using Pearson's correlation, and the analysis produced a p-value of 0.025, showing that the correlation was significant (Figure 2). A positive correlation with a moderate strength of correlation is indicated by the r = 0.403.

DISCUSSION

In this study, no significant correlation was found between CTFC in infarct related artery and the area of myocardial fibrosis assessed by CMR. De Luca et al[10] in their study had showed a significant linear relationship between CTFC and enzymatically assessed infarct size according to CKMB concentration. Creatine kinase (CK) is an intracellular enzyme that catalyzes the reversible conversion of creatine and ATP to creatine phosphate and ADP.[11] Disruption of cell membranes due to hypoxia or other damage results in the release of the CK enzyme through cell cytoplasm into the circulatory system[12], the CKMB isoenzyme is found specifically and predominantly in myocardial tissue and its release occurs only upon cardiomyocyte death.[13] However, due to its high molecular weight, it cannot detect small myocardial damage and its elevation may be caused by non-cardiovascular causes.[14] The study by Dai et al[15] showed a significant correlation between the incidence of perivascular fibrosis and CTFC in the LAD , describes the impairment of coronary blood flow in patients with non-ischemic heart failure. However, this study only included patients with non-ischemic heart failure.

Microvascular injury after PCI in STEMI patients is caused by a combination of external capillary compression due to interstitial edema and intramyocardial hemorrhage, as well as intraluminal obstruction processes. This microvascular injury will cause prolonged myocardial tissue hypoperfusion so that areas expected to improve after reperfusion still undergo prolonged ischemia which causes myocardial necrosis and infarcts, and ultimately will affect the final extent of myocardial fibrosis after STEMI. CTFC is a surrogate marker to detect microvascular injury. Based on the data above, the researchers assumed that microvascular injury after PCI as depicted by longer CTFC values would correlate with the final extent of myocardial fibrosis seen on CMR examination. However, the results of this study did not show a correlation between the two.

Confounding factors for CTFC had been accounted in the analysis of this study. These factors included heart rate, hemoglobin, random blood glucose, total ischemic time, and whether multivessel disease was present or not. All of these confounding factors had no correlation to CTFC. (p=0.647; p=0.203; p=0.313; p=0.774; p=0.552 respectively)

According to the study, it is believed that the duration of ischemia has a greater impact on the area of myocardial fibrosis. The increase in myocardial fibrosis area caused by continued microvascular injury after reperfusion does not seem to be as significant as compared to the damage that has already occurred before reperfusion, particularly before 360 minutes. The findings of Reimer and Jennings et al[16] who used canine models with total occlusion of the LCx artery are worth mention mentioning here. They discovered that irreversible ischemic myocyte death initially occurred in the subendocardial region and progressively spread like a wave moving towards the subepicardial region within 6 hours. Cell death was not formed immediately or simultaneously even in areas experiencing severe ischemia.[17] Occlusion of the LCx for 15 minutes was still completely reversible, in that reperfusion completely prevented necrosis, but sometimes small foci of necrosis were found after 20 minutes of occlusion. Reperfusion after 40 minutes of occlusion will always result in foci or confluences of subendocardial necrosis. Occlusion for 3 hours results in significantly more average necrosis, where usually there is confluence in the subendocardial myocardium with involvement of foci in the mid- or subepicardial myocardium. At 6 to 24 hours, the spread of necrosis is almost transmural. Viable myocardium at this point is mainly seen in subepicardial foci, which are often adjacent to blood vessels.[16]

Microvascular damage that occurs after or is caused by reperfusion will not add to the myocardial fibrosis area because a) reperfusion results in a smaller average infarct expansion within 3 hours and no difference after 6 hours compared to permanent infarcts, and b) the transmural progression of myocyte necrosis appears to precede the development of vascular damage.[16] The earliest cell death occurs in the subendocardial zone of the severely ischemic area and about half of the myocardium experiencing ischemia and becoming necrotic at 24 hours has died at 40 minutes of total occlusion. However, cell death usually occurs more slowly in the mid- and subepicardial areas, where on average about a third of the ischemic and at-risk myocardium can still be salvaged at 3 hours. By 6 hours, only a little myocardium can be salvaged.[16] Therefore, although microvascular injury is detected as indicated by prolonged CTFC after reperfusion, the myocardial fibrosis area will not increase too much if reperfusion is performed after 6 hours, because the most substantial myocardial cell necrosis occurs within 3 hours of total occlusion, and its progression will be relatively small after 6 hours of occlusion.

No correlation was found between CTFC and myocardial fibrosis area mainly affected by total ischemic time exceeding 360 minutes, where even if microvascular injury was detected by the prolonged CTFC, the progression of myocardial fibrosis was relatively slow because the damage had already been extensive and there's only a small percentage of salvageable myocardium left. If the reperfusion was completed with total ischemic time less than 360 minutes, the microvascular injury, that was indicated by prolonged CTFC, may still add to the expansion of myocardial fibrosis area because at time between 180 to 360 minutes of occlusion there's more percentage of salvageable myocardium left that may become fibrotic due to post reperfusion microvascular injury. Our study was limited by the retrospective design, small total sample number of patients, total ischemic time that exceeded 360 minutes, and the majority of non-anterior infarct location. However, this is only a pilot study, further study with prospective design may be needed to confirm our findings.

CONCLUSION

From this study we conclude that CTFC was not correlated with the extent of fibrosis based on LGE-CMR on STEMI patients who underwent PPCI. Further prospective studies with larger total sample number may be needed to confirm our findings which including sample of anterior infarct location (LAD and LCX infarct related artery) only, and total ischemic time less than 360 minutes so that there's still bigger percentage of salvageable myocardium to see the progression of fibrosis and the sample is more homogenous.

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Original Research Article

Association between Neutrophil to Lymphocyte Ratio and Post Operative Atrial Fibrillation after Coronary Artery Bypass Graft operation

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Abstract

Background: Post operative atrial fibrillation (POAF) is associated with cardiovascular outcomes such as stroke, heart failure, and mortality. Neutrophil to lymphocyte ratio (NLR) is one of biomarker of inflammation. The use of cor pulmonary bypass (CPB), surgical trauma and reperfusion injury during cardiac surgery causes inflammation. NLR is suspected to be a biomarker that represents the inflammatory response as a modulator of POAF, but data are still lacking. This study analyzed the association between NLR and POAF after coronary artery bypass graft (CABG) operation.

Methods: This was an Observational analytic study, involving patients after the CABG procedure in Kariadi Hospital between June 2022 to September 2023. NLR was assessed in the first three hours after operation. ECG Holter was attained for 96 hours post-CABG procedure.

Results: The total subject was 62 patients, consisting of 57 men and 5 women with a mean age of 58,7 ± 6.39 years. Mean postoperative NLR 4.95 ± 2.17 with a median of 4.56 (1.52-11.00). There was a significant association between post-operative NLR with POAF after CABG (p=0.006, OR 4.64, 95%CI 1.50-14.35). High inotropic dose and *β* blocker initiation time less than 45 hours correlate significantly with POAF after CABG (p<0.001, OR 6.94 dan p=0.001, OR 0.17).

Conclusions: Postoperative NLR is associated significantly with POAF after CABG.

Keywords: *Neutrophil to Lymphocyte Ratio; Post Operative Atrial Fibrillation, coronary artery bypass graft; ECG Holter*

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INTRODUCTION

Post Operative Atrial Fibrillation (POAF) is defined as new onset atrial fibrillation which occurs after surgery. POAF could develop in cardiac surgery and noncardiac surgery. Prevalence of POAF after Coronary Artery Bypass Graft (CABG) was around 20%.[1] Hence, the prevalence of POAF after CABG in 2021 at Kariadi Hospital Semarang was 55%.

POAF was considered to be a benign and self-limiting complication. Nowadays, it is known to be associated with cardiovascular events, such as stroke, heart failure, and mortality. POAF is also associated with prolonged hospital and intensive care stays. [1,2]

Pathophysiology of POAF after CABG is caused by several factors. Fibrotic substrates which develop chronically added with acute factors that are caused by surgery, propagate atrial fibrillation. Factors that make ideal conditions for AF to occur are defined as the modulator. Inflammation, oxidative stress, and sympathetic nerve activation serve as a modulator of POAF. Inflammation that occurs in cardiac surgery is caused by cardiopulmonary bypass and injury reperfusion.[1,3,4]

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Table 1. Baseline Characteristics

Variable	Description
Age (years)	58.7± 6.39
Sex	
Men	57 (89.1%)
Woman	5 (10.9%)
Body Mass Index (kg/m^2)	25.30 ± 3.41 24.60 (16.76-34.81)
GFR (mL min-1 1.73 m^2)	61.03 ±18.71
ACEI or ARB	
yes	52 (83.8%)
no	10 (16.2%)
β-blocker	
yes	59 (92.2%)
no	3 (7.8%)
Calcium channel blocker	
yes	19 (31.7%)
no	43 (69.3%)
Mineral receptor antagonis	
Yes	26 (41.9%)
No	36 (58.1%)
Statin	
Yes	61 (98.3%)
No	1 (1.7%)
LV *ejection fraction* (%)	50.46 ± 10.42
LA diameter (mm)	36.46 ± 5.39
Amount of SVG *graft*	
2 graft	22 (25.2%)
3 graft	33 (53.2%)
4 graft	7 (11.6%)
IABP	
Yes	11 (17.7%)
No	51 (82.3%)
Reoperation	
Yes	3(7.8%)
No	59 (92.2%)
Aortic cross clamp time (minutes)	55.29 ± 10.67
Cardiopulmonary Bypass time (minutes)	67.86 ± 11.75
Post operative Atrial Fibrillation	
Yes	28 (45.2%)
No	34 (54.8%)
Total Hospital stay (days)	
Mean ± SD	12.08 ± 4.99
Median (min-max)	11 (6-31)
Total ICCU stay (days)	
Mean ± SD	5.42 ± 2.53
Median (min-max)	5 (2-14)
Total ventilator time (hours)	
Mean ± SD	51.29 ± 52.49
Median (min-max)	24.5 (12-192)
Mortality in hospital	
Yes	9 (14.5%)
No	53 (85.5%)

GFR: Glomerular Filtration Rate; ACEi: Angiotensin Converting Enzyme inhibitor; ARB: Angiotensin Receptor Blocker; LV : Left ventricle; IABP : Intra Aortic Ballon Pump, ICCU : Intensive Cardiac Care Unit

The neutrophil-to-lymphocyte ratio is a popular inflammation marker associated with cardiovascular disease. NLR is suspected to be a biomarker that represents the inflammatory response as a modulator of POAF, but data are still lacking. In 2019, Study by Weedle showed high preoperative and postoperative NLR associated with POAF after cardiac surgery (CABG and valvular surgery) ($p=0.011$ and $p<0.001$).[5] These studies aimed to assess the association between postoperative NLR with POAF after CABG.

METHODS

Study Design and Participants

This was an observational analytic study with a cross-sectional design done in Kariadi Hospital. Patients were enrolled at the Dr. Kariadi General Hospital, Semarang,

Indonesia who were (1) ≥ 18 tahun years of age and (2) underwent elective on pump CPB procedures. Informed consents were obtained before cardiac surgery, patient given detailed information about study and asked for permission. Patient recruitment occurred between June 2022 and September 2023. Exclusion criteria included a history of atrial fibrillation, diagnosed with moderate-severe valvular disease and scheduled for valve replacement, consumed anti-arrhythmic drug class I or III, history of chronic kidney disease, ongoing chemotherapy treatment, history of leukemia, diagnosed with HIV and didn't have complete blood count assessment 0-3 hours after surgery. POAF was evaluated with a Holter ECG 96 hours after surgery.

Table 2. Correlation analysis of Postoperative NLR with POAF after CABG

Variable	POAF after CABG	
	Coefficient Correlation (r)	p-value
Post operative NLR	r=0.32	p=0.01[b*]

Statistical analysis using Spearman test

Statistical Analysis

Data were displayed in mean, standard deviation, and percentage. The Kolmogorov-Smirnov test was used to check the normality of the data. Either Pearson or Spearman test was used to analyzed the correlation within the variable. The cut-off value was defined with receiver operating characteristic (ROC) analysis. The numeric variable was converted to a nominal variable and underwent bivariate analyses. The chi-square test was used to analyzed the association between post-operative NLR and POAF after CABG. All statistical analyses were performed using the IBM SPSS software version 26 and a P value of <0.05 was used to ascribe statistical significance.

RESULTS

A total of 62 subjects met the inclusion criteria. The baseline characteristic of patients is shown in **Table 1**. 57 patients (89.1%) were male. Mean age was 58.7± 6.39 years. Majority of patients had 3 vessel disease (89.1%). Mean left ventricle ejection fraction was 50.46±10.42%. Mean aortic cross-clamp time was 55.29±10.67 minutes and cardiopulmonary bypass time was 67.86±11.75 minutes.

Table 2 shows the correlation between postoperative NLR with POAF after CABG. There was a positive correlation with weak correlation power between post-operative NLR with POAF after CABG with p=0.01 r=0.32.

Table 3 shows the cut-off value of post-operative NLR which divides high risk and low risk of developing POAF after CABG. The cut-off value was 4.14 with a sensitivity of 78% and specificity 56%.

Table 4 shows the association between post-operative NLR and POAF after CABG. There is a significant association between post-operative NLR and POAF after CABG with p=0.006 OR 4.64 95%CI 1.50-14.35.

Table 5 shows the association between confounding factors and POAF after CABG. Confounding factors analyzed in this study such as Age > 60 years, Hypertension, DM, COPD, LVEF > 40%, LA diameter > 38,5mm, High inotropic dose, and Initiation of β blocker < 45 hours. High inotropic dose and Initiation of β blocker < 45 hours were significantly associated with POAF after CABG with p=0.000 OR 6.94 95%CI 2.27-21.27 and p=0.001 OR 0.17 95%CI 0.05-0.50.

Table 4. Association of postooperative NLR with POAF after CABG

Association	POAF after CABG		
	OR	P value	95% CI
Post operative NLR	4.64	p=0.006[*]	1.50-14.35

DISCUSSION

Atrial fibrillation is multifactorial arrythmia that share classical cardiovascular risk factor.[6] Risk factor of atrial fibrillation which chronically overdue in patient, creating fibrotic substrate that maintain atrial fibrillation once triggered. In CABG patients, another risk factors due to surgical procedure and medical management after surgery, such as inflamation, stress oxydative and symphatetic nervous activation, acts as modulator of atrial fibrillation which increase susceptibility of FAPO.[1,3,4]

Confounding preoperative factors that led to the formation of fibrotic substrate were analysed and assessed. These factors included ageing, hypertension, diabetes mellitus, chronic pulmonary obstructive disease, left ventricle ejection fraction, and LA diameter.[7–11] Because the population in this study was younger (mean age 58 years) than in previous studies (mean age 65 years), there was no significant correlation found between ageing and POAF[12]. Furthermore, this study found no evidence of a significant correlation between FAPO and LA diameter. In terms of demographics, the population in this study had a mean LA diameter of 36.46±5.39 mm, which was smaller than the mean LA diameter of 40.09±3.47 mm in another study that demonstrated a significant correlation.[13] There were no significant association between each confounding pre operative factors with POAF after CABG in this study, due to relatively younger and less structural LA remodelling compared to other significant prior studies.[12–15]

Table 3. Cut off Post Operative NLR

Variable	cutoff	AUC	p-value	Sensitivity	Specitifity
Post operative NLR	4.14	0.684	0.012	78%	56%

Table 5. Association of confounding factor with POAF after CABG

Confounding factor	Description	POAF after CABG		
		OR	p value	95% IK
Age		1.15	0.82	(0.41-3.06)
≥ 60 years	30 (51.6%)			
< 60 years	32 (48.4%)			
Hypertension		3.37	0.14	(0.64-17.75)
Yes	53 (82.8%)			
No	9 (17.2%)			
DM		0.56	0.26	(0.20-1.55)
Yes	27 (42.2%)			
No	35 (57.8%)			
COPD		1.22	0.89	(0.07-20.47)
Yes	2 (3.1%)			
No	60 (96.9%)			
LVEF > 40%		0.95	0.94	(0.28-3.24)
≥40%	49 (79.7%)			
<40%	13 (20.3%)			
LA Diameter >38.5mm		1.59	0.38	(0.57-4.42)
≥ 38.5mm	25 (42.2%)			
< 38.5mm	37 (57.8%)			
High dose inotropic use		6.94	0.000*	(2.27-21.27)
Yes	33 (51.6%)			
No	29 (40.4%)			
Initiation of B-blocker <45 hour		0.17	0.001*	(0.05-0.50)
<45hour	32 (48.4%)			
>45hour	30 (51.6)			

CABG procedure triggered inflammation response due to tissue injury, use of *Cardio Pulmonary Bypass* (CPB) and reperfusion injury.[16]. The use of inotropic after surgery and initiation time of β blocker affected sympathetic nerve activation. Both mechanism, inflammation and sympathethic nerve activation act as modulator of POAF which enchanced susceptibility of POAF.[2] There were significant association between high dose inotropic use and also β blocker initiation time with POAF after CABG (p<0.001 and p=0.001).This study mainly focused to assesed association between postoperative NLR which stands as a marker of inflammation with FAPO after CABG.

NLR is an inflammation biomarker arranged into a ratio of absolute neutrophil count divided by absolute lymphocyte count taken from vein blood. It could describe two kinds of immunity systems, innate immune response and adaptive immune response.[17,18] This study assessed post-operative NLR taken 0-3 hours after surgery.

The mean postoperative NLR was 4.95 ± 2.17 with a median of 4.56 (1.52-11.00). The correlation test of postoperative NLR and POAF after CABG concluded a positive correlation with weak correlation power (p=0.012; r= 0.317). Cut-off value assessed to divide subjects with a high risk of POAF based on postoperative NLR. The ROC analysis concluded 4.14 as a cut-off value, with sensitivity of 78% and specificity of 56%.

Mean postoperative absolute neutrophil count was 9.54 ± 3.95 10^3/mL with a median of 8.72 (4.08-22.35) 10^3/mL. Correlative analysis showed no significant correlation between postoperative neutrophil and POAF after CABG with p=0.09. Despite no significant correlation, it can be seen that the trend of neutrophil increase has occurred 0-3 hours after surgery. The study by Kawahito et al, showed the trend of neutrophil increase started right after the end of CPB, with a peak 12 hours after surgery and decreased gradually after.[19]

Mean post operative absolute lymphocyte count was 2.19 ± 1.05 10^3/mL with median 2.01(0.50-5.68) 10^3/mL. Correlative analysis showed no significant correlation between postoperative lymphocyte and POAF after CABG with p=0.55.

The chi-square test used to assess the association between postoperative NLR and POAF after CABG, concluded a significant association with p=0.006, OR 4.64 dan 95% CI 1.5-14.35. Similar findings were also obtained from a prospective study by Gibson et al, which showed a significant association of postoperative NLR with POAF after CABG with p< 0.001.[12] Weedle et al, also showed a significant correlation of post-operative NLR with POAF after CABG with p=0.03 dan OR 1.04.[5] Postoperative NLR value showed immune response happened when blood was sampled. Postoperative stress was the cause of high immunity response. Production of reactive oxygen species, ongoing hypoperfusion, contact activation with CPB, and tissue injury were causing innate immunity response and increased neutrophil count.[20] The dynamic of adaptive immunity started with transient lymphocytopenia early after surgery. The study by Jankovicova et al, showed nadir point of decreasing lymphocytes happened on the first day after surgery.[21]

The limitation of this study is that there is no evaluation of the activation sympathetic nervous system using actual biomarkers. Also, there is no data about inotropic and

vasoactive drug doses used intraoperatively. The fibrotic substrate also wasn't assessed directly with imaging modality. Lastly, the history of AF only asses by preoperative ECG.

CONCLUSION

The results of this study demonstrated that the neutrophil-to-lymphocyte ratio is associated with the incidence of POAF after CABG. If it confirmed by further studies, it might be beneficial if we monitor the NLR after CABG to predict POAF event.

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Original Research Article

Novel Endophytic Bacteria Isolates from Andaliman (*Zancthoxylum acanthopodium* DC.) which Potentially Inhibit *Escherichia coli* and *Staphylococcus aureus*

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Abstract

Background: Irrational use of antibiotics has various side effects to resistance. Utilization of secondary metabolites of Andaliman plant endophytic bacteria (*Z. acanthopodium* DC) is an alternative known to act as an antibacterial to overcome this problem.

Objective: This study aimed to determine the antimicrobial activity of the crude extract of Andaliman endophytic bacteria against *Escherichia coli* and *Staphylococcus aureus*.

Methods: This research is an experimental laboratory with qualitative data collection methods. The initial stages will be the isolation of endophytic bacteria from Andaliman, the antagonist test of Andaliman endophyte isolates against *E. coli* and *S. aureus*, and the extraction of endophytic bacterial isolates using ethyl acetate as solvent. An antagonistic test of bacterial isolate extract was performed using the disc diffusion method against pathogenic bacteria *E. coli* and *S. aureus* and observed by an inhibition zone. The final stage is the minimum inhibition test of endophytic isolates by dilution method on three potential endophytic isolates.

Results: The results of the extraction of isolates EAA22, EAA28, EAB5, EAB6, and EAB7 Andaliman endophytic bacteria have inhibitory activity against *S.aureus*, and the results of the extraction of isolates EAB5, EAB6, EAA22 have inhibitory activity against *E. coli*.

Conclusion: Endophyte bacteria isolates from Andaliman produce antibacterial compounds against *E. coli* and *S. aureus*.

Keywords:
Endophytic bacteria; extraction; antibacterial compound; Andaliman; minimum inhibitory concentration
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INTRODUCTION

Infectious diseases occur when interactions with microbes cause damage to the host body; this damage causes various clinical symptoms and signs, one of the causes of which is pathogenic bacteria.[1] Giving antibiotics is still the primary choice for treating bacterial infections. Various studies have found that around 40–62% of antibiotics are misused.[2] The Global Antimicrobial Surveillance System (GLASS) 2018 revealed widespread antibiotic resistance among 500,000 people with suspected bacterial infections in 22 countries.[3] In 2019, antimicrobial resistance indicators were included in the monitoring framework of the Sustainable Development Goals (SDGs). This indicator monitors the frequency of bloodstream infections due to two specific drug-resistant pathogens: *Staphylococcus aureus* and *Escherichia coli*.[2]

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S.aureus is a microbiome in the skin, nose and throat. Diseases caused by *S. aureus* bacteria in Indonesia are 28%.[4] *S. aureus* can cause serious infections such as septicemia, pneumonia, endocarditis, osteomyelitis, gastroenteritis and abscesses.[5] Meanwhile, *E. coli* is the most common commensal in the human digestive tract. Diarrhea caused by *E. coli* has the highest prevalence in Indonesia, almost reaching 10%.[6] Other clinical infections, such as urinary tract infections, bacteremia, and septicemia, can also be caused by *E. coli.*[7]

In 2015, as many as 13,300 patients died due to resistant bacterial infections.[8] The discovery of new antibiotics has not offset the increase in cases of bacterial resistance.[9] Efforts that have been made to reduce the incidence of antibiotic resistance include controlling the use of antibiotics, developing research to understand genetic resistance mechanisms better, and discovering new drugs, both synthetic and of natural origin from natural ingredients, it is still being used, one of which is as an antibiotic.[2]

Nature provides many alternative plants with antimicrobial potential against pathogenic microbes, including the Andaliman. Removing bioactive compounds from a plant can be done by extracting parts of the plant. This method is undoubtedly ineffective because if the plant is continuously taken to extract its bioactive compounds, the availability of the plant in the environment will decrease. One way to obtain bioactive plant compounds is to utilize the plant's endophytic bacteria, so there is no need to cut down the original plant to take simplicia, which will most likely take a long time to harvest.[10]

Endophytic bacteria can produce unique natural compounds that are sometimes the same as the compounds produced by their host plants, one of which is the ability to synthesize the same antibacterial compounds as their host plants.[10] The bioactive compounds synthesized by endophytic bacteria are several secondary metabolites such as alkaloids, steroids, terpenoids, peptides, flavonoids and phenols, which are essential in therapeutic applications, one of which is antimicrobial.[11] These bioactive compounds can be further explored for medical and pharmaceutical use, which is very important for further exploration.

Extraction of secondary metabolites of endophytic bacteria from *Anredera cordifolia* leaves shows the ability to produce effective antimicrobials against several pathogenic bacteria such as *S. aureus, E. coli* and *B. cereus.*[12] The results of the antibacterial activity test showed that the BH2 isolate extract from the endophytic bacteria of the Nyawai plant (*Ficus variegate* Blume) could inhibit the bacteria *Bacillus subtilis, S. aureus, E. coli, and Pseudomonas aeruginosa.*[13] Research by Tendani in 2020 reported that crude extracts of endophytic bacteria from *Crinum macowanii* Baker leaves showed inhibitory activity against gram-positive and gram-negative bacterial species.

Antimicrobial activity can be seen in the MIC (minimum inhibitory concentration), the lowest concentration capable of inhibiting the growth and development of bacteria. An increase in the MIC value describes the initial stage of resistance. Antibiotic resistance by bacteria that cause infections requires discovering and developing new antibacterial drugs derived from nature, one of which is by utilizing endophytic bacterial extracts from medicine plants.

Seeing the importance of the function of endophytic bacteria for plants and the antibacterial potential of Andaliman, it is crucial to carry out this research to obtain isolates of endophytic bacteria from Andaliman that have activity as producers of antibacterial compounds that can inhibit pathogenic bacteria activity. Therefore, it is hoped that future results of this research can be used and developed to overcome health problems, mainly due to pathogenic bacterial infections.

MATERIALS AND METHODS

Isolation of Endophyte Bacteria

The parts of the Andaliman plant used are leaves, stems and roots. Fresh leaves, stems, and roots were cleaned with running water, cut into 2-5 cm long pieces, and separated according to plant parts. The sample pieces were then surface sterilized by immersing them in 70% alcohol for 1 minute, 5.25% sodium hypochlorite (NaOCl) solution for 5 minutes, and 70% alcohol for 2 minutes. The sterilized sample pieces were cut again and then planted in King's B media. The media containing the sample was incubated at room temperature in the dark and observed every day until there was colony growth.

Antagonist Test

Pure isolates of *S. aureus* and *E. coli* (IPBCC) were rejuvenated into Tryptic Soy Broth (TSB) liquid media and incubated at room temperature for 24 hours. Then, the turbidity was measured using spectrophotometry (OD = 0.3, concentration 10^6 - 10^7 cells/ml). Bacterial antagonist test for *E. coli* and *S. aureus* used NA media. The bacterial antagonist test of endophytic isolates against the growth of target bacteria was carried out using a two-layer agar media technique consisting of semi-solid and solid media. The target bacteria whose turbidity has been measured using spectrophotometry (OD = 0.3, concentration 10^6 - 10^7 cells/ml) in the broth medium are mixed into the semi-solid medium and then poured over the solid medium that has been previously frozen in a cup. After freezing, the endophytic bacterial isolate was streaked onto it. The culture was incubated for 24 hours at room temperature.

Observations were made after the bacterial sensitivity test culture had been incubated for 24 hours. A bacterial isolate is said to be positive for potential inhibitory power if the isolate produces an inhibition zone in the bacterial antagonist test. The diameter of the inhibition zone measured endophytic bacterial isolates that were positive for producing potential inhibitory activity, then inoculated into NA media and incubated at room temperature for 24 hours.[14]

Table 1. Inhibition Category

Diameter	Category
≥ 20 mm	Very Strong
10-20 mm	Strong
5-10 mm	Moderate
≤ 5 mm	Weak

Table 2. Andaliman Endophyte Bacterial Antagonist Test Results against E. Coli

No	Isolate Code	Isolate diameter (mm)	Isolate + Clear zone Diameter (mm)	Inhibition zone diameter (mm)	Category
1	EAA28	4,2	4,9	0,7	Weak
2	EAB06	3,4	4,5	1,1	Weak

Table 3. Andaliman Endophyte Bacterial Antagonist Test Results against S. aureus

No	Isolate Code	Isolate diameter (mm)	Isolate + Clear zone Diameter (mm)	Inhibition zone diameter (mm)	Category
1	EAA03	5,6	6,7	1,1	Weak
2	EAA16	8,2	12,8	4,6	Weak
3	EAA22	5,9	8,5	2,6	Weak
4	EAA26	5,7	7,6	1,9	Weak
5	EAB03	5,6	6,4	0,8	Weak
6	EAB05	6,6	15,0	8,4	Moderate
7	EAB06	5,2	9,6	4,4	Weak
8	EAB07	5,3	10,4	5,1	Moderate
9	EAB10	5,8	7,2	1,4	Weak
10	EAB11	5,9	7,0	1,1	Weak
11	EAB16	5,6	6,8	1,2	Weak
12	EAB18	5,4	6,7	1,3	Weak
13	EAB21	5,1	6,3	1,2	Weak
14	EAD07	6,3	6,8	0,5	Weak
15	EAD10	6,4	7,2	0,8	Weak
16	EAD13	5,3	5,9	0,6	Weak
17	EAD14	5,6	6,7	1,1	Weak

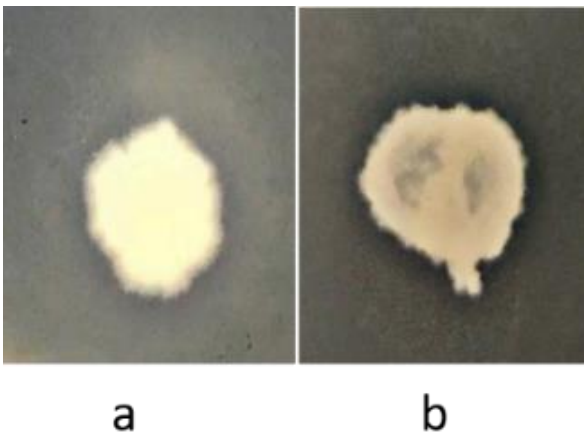

Figure 1. Inhibition zone formation in Andaliman Endophyte Bacterial Antagonist Test against *E. Coli*. a. EAA28, b. EAB06

Minimum Inhibitory Concentration (MIC) Test

Minimum Inhibitory Concentration (MIC) is the lowest antimicrobial concentration that can still inhibit the growth of certain organisms. The MIC test in this study was carried out using the dilution method. This method is mainly used in determining the minimum inhibitory concentration (MIC). Generally, determining the MIC using the dilution method involves inoculating the target microbe at various concentrations of antimicrobial compounds and incubating for 18 to 24 hours. The MIC test was conducted because a standard concentration of endophytic bacteria isolates from the Andaliman plant had not been found to test its effectiveness as an antimicrobial on *E. coli* and *S. aureus*.

The Andaliman endophytic bacterial isolate was cultured in an NB medium and then incubated for three days at room temperature. Escherichia coli and Staphylococcus aureus were rejuvenated in NB (Nutrient Broth) media and incubated for 24 hours at room temperature. The antimicrobial-producing Andaliman endophytic bacterial isolate that had been incubated was centrifuged at 6000 rpm for 1 hour. The supernatant was taken and then filtered using a 0.22 µm syringe filter and put into 5 test tubes with concentrations of 6.25%, 12.5%, 25%, 50%, and 100%, respectively. Apart from these five concentrations, a positive control was also made by adding an antibiotic in the form of amoxicillin with a concentration of 50 µg/mL, a negative control by not adding antibiotics or endophytic bacterial supernatant, and a media control containing only NB media. After that, each tube was filled with 1 ml of target bacteria, except for the media control.

The MIC test culture was then incubated for 24 hours. Then, the turbidity of each culture was observed in the test tube and measured using spectrophotometry at a wavelength of 620 nm. MIC is determined based on the turbidity of the culture compared to the control.[14] The MIC value is determined by observing the minor concentration at which the test tube appears clear, compared to the control, and confirmed by measuring the OD (Optical Density) of the solution in the test tube using spectrophotometry.

Table 4. MIC test results for endophytic bacterial isolates against S.aureus and E.coli

Staphylococcus aureus					
Concentration	*Optical Density*				
	EAB5	EAB6	EAB7	EAA22	EAA28
100%	0,143	0,080	0,138	0,080	0,094
50%	0,213	0,169	0,213	0,082	0,141
25%	0,240	0,186	0,240	0,156	0,208
12,5%	0,241	0,227	0,241	0,290	0,232
6,25%	0,243	0,229	0,243	0,295	0,234
K(-)	0,271				
K(+)	0,149				

Escherichia coli			
Concentration	*Optical Density*		
	EAB5	EAB6	EAA22
100%	0,089	0,143	0,112
50%	0,130	0,182	0,230
25%	0,214	0,253	0,235
12,5%	0,222	0,261	0,246
6,25%	0,281	0,288	0,258
K(-)	0,282		
K(+)	0,090		

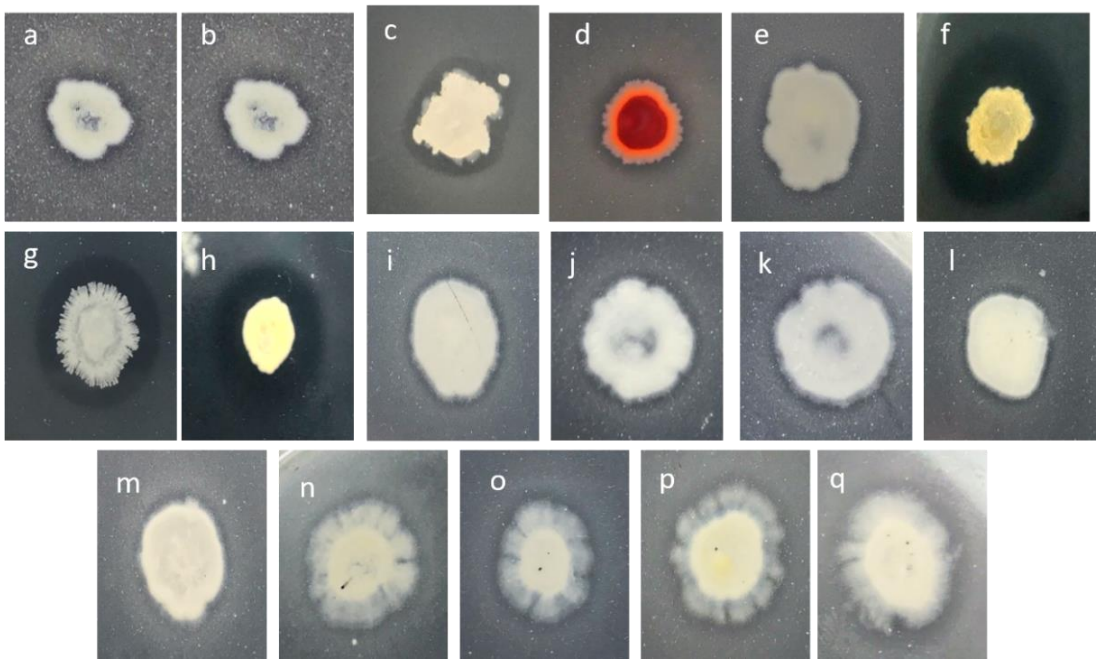

Figure 2. Inhibition zone formation in Andaliman Endophyte Bacterial Antagonist Test against *S. aureus*. a. EAA03; b. EAA16; c. EAA22; d. EAA26; e. EAB03; f. EAB05; g. EAB06; h. EAB07; i. EAB10; j. EAB11; k. EAB16, l. EAB18; m. EAB21; n. EAD07; o. EAD10; p. EAD13; q. EAD14.

Extraction of Andaliman endophytic bacterial isolates

The six best andaliman endophytic bacterial isolates that had been isolated from the previous step and had been subcultured were each re-cultured into 500 ml of Nutrient Broth (NB) media and incubated on a shaker (170 RPM /Revolutions Per Minute, 30 ºC) for 72 hours so that the bacteria grow uniformly. After that, the culture was centrifuged at 3600 rpm for 10 minutes to separate the supernatant and cells. The supernatant that had been taken was added to 150 mL of ethyl acetate and incubated. Then, it was shaken for 20 minutes and transferred to a separating funnel. After leaving it for 10 minutes, the solvent (transparent top layer) and medium (yellow bottom layer) were separated. The top layer was put into a glass container and evaporated using a vacuum rotary evaporator at a temperature of 40ºC with a speed of 90 rpm.[12] The resulting crude extract of endophytic bacterial isolates in liquid form was stored at four ºC for further use.[15]

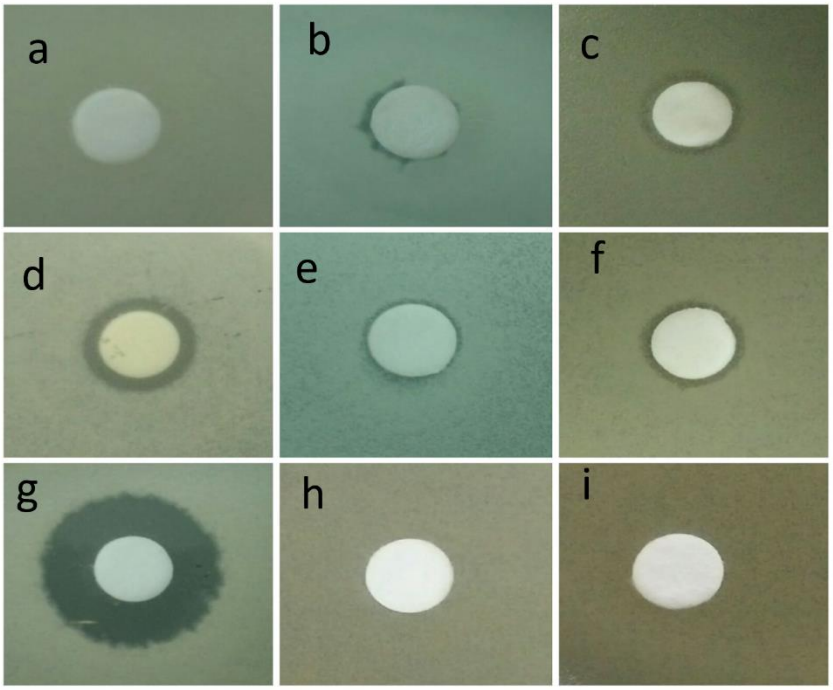

Figure 3. Inhibition zone formed from crude extract of isolates of andaliman endophytic bacteria against S. aureus; a = EAA16; b = EAA22; c = EAA28; d= EAB5; e= EAB6; f=EAB7;g=Amoxicilin; h= Ethyl Acetate; i= Aquadest

Table 5. Inhibition Test of Crude Extract of Andaliman Endophyte Bacterial Isolate against Staphylococcus aureus and Escherichia coli

Isolate Code	Isolate diameter (mm)	Isolate + Clear zone Diameter (mm)	Inhibition zone diameter (mm)	Category
Inhibition to *Staphylococcus aureus*				
EAA16	6	6	-	Negative
EAA22	6	6,3	0,3	Weak
EAA28	6	6	7	Weak
EAB5	6	9,2	3,4	Weak
EAB6	6	6,6	0,6	Weak
EAB7	6	7,6	1,6	Weak
Amoxicillin	6	12,7	6,7	Moderate
Ethyl Acetate	6	6	-	Negative
Aquades	6	6	-	Negative
Inhibition *Escherichia coli*				
EAA16	6	6	-	Negative
EAA22	6	6,6	0,6	Weak
EAA28	6	6	-	Negative
EAB5	6	6,35	0,35	Weak
EAB6	6	6,30	0,30	Weak
EAB7	6	6	-	Negative
Amoxicillin	6	14	8	Moderate
Ethyl Acetate	6	6	-	Negative
Aquades	6	6	-	Negative

Inhibition Test of Crude Extract of Andaliman Endophytic Bacterial Isolate

Stocks of pathogenic bacteria were taken and then grown in 10 mL of sterile liquid NB media—next, they were shaken in an incubator at 37°C for 24 hours. Then, the turbidity was measured using spectrophotometry (OD = 0.3 concentration 10^6-10^7 cells/ml). A 2.5 mL pathogenic bacterial culture originating from liquid NB media was put into 50 mL of semi-solid NB media, stirred or stirred, poured into a petri dish of ±10 mL, and cooled. The test was carried out using the diffusion method; crude extracts of endophytic bacterial isolate compounds dissolved in ethyl acetate were dropped onto paper discs 100 µl each. Next, the paper disc on which the extract has been dripped is waited to dry to eliminate water access, then placed on the surface of NB semi-solid media containing indicator microbes and incubated for 24 hours. The formation of a clear zone indicates positive

test results.[15] The diameter of the inhibition zone around the test disc is measured using a ruler or a calliper.

RESULTS

Isolation of Endophyte Bacteria

The first stage of this research was isolating the Andaliman plant using two parts of each of the roots, stems, and leaves from three different plants. From the isolation using the cutting-planting method, 252 colonies were produced from all the parts taken. The details of the number of colonies that grew on King's B media were that on the roots, the results were 150 colonies; on the stems, the results were 81; on the leaves, the results were 21.

Antagonist Test

After observing the microscopic characteristics of the Gram stain, a bacterial sensitivity test using the spot technique on Double Layer Agar media was followed. Of the eighty-five isolates tested against pathogenic bacteria, 18 isolates were obtained which showed inhibitory activity against the pathogenic bacteria *E. coli* and *S. aureus*, with details on the roots of 5 isolates from 28 isolates (17.8%), on the stems nine isolates from 40 isolates (22.5%) and four isolates from 17 isolates (23.5%) on leaves. Table 2 and Table 3 display the results of the Antagonist Test for Andaliman endophytic bacteria against the pathogenic bacteria *E. coli* and *S. aureus.* Two isolates have inhibition activity against *E. coli* (Figure 1) and 17 isolates have inhibition activity against *S. aureus* (Figure 2).

Minimum Inhibitory Concentration (MIC) Test

Based on measuring the absorbance value using spectrophotometry, the MIC (minimum inhibitory concentration) value was obtained for *S. aureus* bacteria at a concentration of 6.25% for isolates EAB7, EAB5, EAB6, EAA28 and a concentration of 25% for isolate EAA22 (Table 4). Meanwhile, the MIC value for *E. coli* bacteria was at a concentration of 6.25% for isolate EAA22 and 12.5% for isolate EAB5 and EAB6. The MIC value is based on the isolate concentration's optical density value, which is lower than the negative control.

Extraction and Inhibition Test of Andaliman Endophytic Bacterial Isolates

The extraction results for each isolate EAB5, EAA16, EAA28, EAB5, EAB6, and EAB7 resulted in less than 5 mL of extraction stored in a tube bottle. Antagonism test of the six extract isolates tested, it was found that five isolates, EAA22, EAA28, EAB5, EAB6, and EAB7, had weak inhibitory activity against the pathogenic bacteria *S.aureus*, and three isolates, namely EAB5, EAB6 and EAA22, had weak activity against *E. coli.*

DISCUSSION

Endophytic bacteria live in host plant tissues without causing disease symptoms.[16] The mechanism of endophytic bacterial invasion into tissue can be carried out in several ways, namely through roots, stomata, lenticels, natural wounds, and damaged leaves. Bacteria in plant tissue then colonize at the point where they enter or spread to all parts of the plant through the xylem.[17] This study found endophytic bacterial isolates in roots, stems and leaves. Based on the isolation results, it was found that there were more endophyte isolates from the roots than stems and leaves, following research conducted by Balosi et al.[18] that the population of endophytic bacteria is more abundant in roots and decreases in stems and leaves. The number of endophytic bacteria in plants cannot be determined with certainty, but these bacteria can be isolated using agar media.[19] In this study, the agar medium used to isolate endophytic bacteria was King's B medium. This medium is rich in

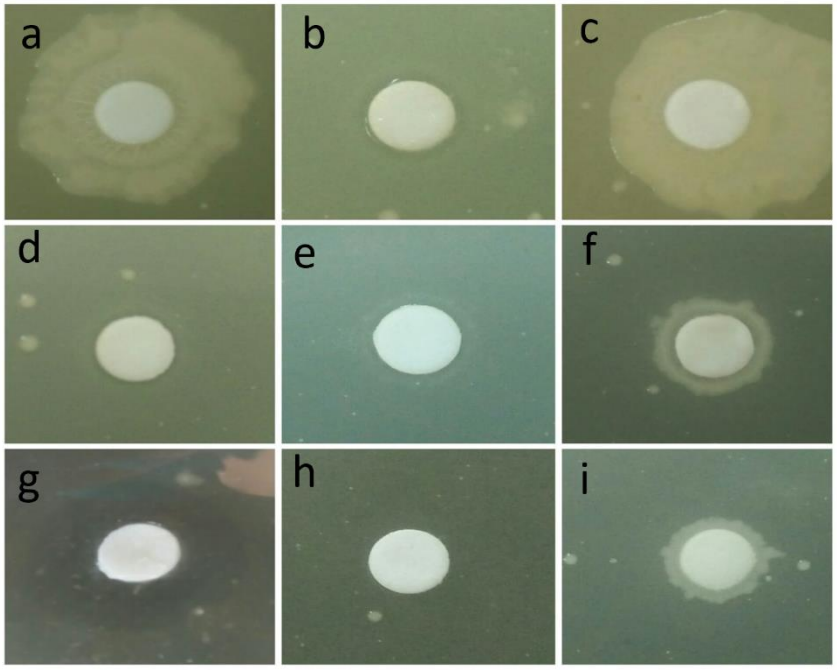

Figure 4. Inhibition zone formed from crude extract of isolates of andaliman endophytic bacteria against E. coli; a = EAA16; b = EAA22; c = EAA28; d= EAB5; e= EAB6; f=EAB7;g=Amoxicilin; h= Ethyl Acetate; i= Aquadest

peptone, glycerol, magnesium sulfate, dipotassium phosphate, and agar, which helps bacterial growth. Endophytic bacteria can live on this media due to its complex nature, which allows it to have a composition similar to conditions in plants and is a suitable medium for the non-selective isolation of bacteria.[20]

Bacterial sensitivity testing was carried out to determine the activity of Andaliman endophytic bacterial isolates in inhibiting the growth of *E. coli* and *S. aureus*. Before a sensitivity test, the target bacteria will be rejuvenated into agar media. The media used is *Tryptic Soy Broth* (TSB) agar media because this media helps support the growth and development of various microorganisms, predominantly anaerobic and facultative aerobic bacteria. This sensitivity test was carried out by growing endophytic and pathogenic bacteria in petri dishes using a two-layer agar technique.[14] This technique is helpful so that the results of the inhibition zone obtained are more clearly visible and the nutrition obtained by the target and endophytic bacteria is sufficient. Following research by Oktavia et al.[21] in a sensitivity test, the concentration of target bacteria can influence the inhibition zone formed, the concentration of endophytic bacteria being tested, temperature and incubation time, and other factors such as the type of medium used.

The pathogenic bacteria used in this study were *E. coli* and *S. aureus*. *Escherichia coli* was chosen to represent Gram-negative pathogenic bacteria, and *S. aureus* was chosen to represent Gram-positive pathogenic bacteria.[22] The inhibitory activity of endophytic bacteria against pathogenic bacteria is characterized by the appearance of a clear zone around the area of endophytic bacteria that is spotted on media that already contains pathogenic bacteria. The inhibition zone that appears indicates that there is secondary metabolite activity at work. The inhibitory mechanism of antibacterial compounds will generally work by damaging cell wall synthesis, disrupting membrane function, protein synthesis, nucleic acid synthesis, and antimetabolites.[23]

Based on the sensitivity test carried out in this study, it was found that of the 18 isolates that gave rise to the inhibition zone, 17 isolates could inhibit the growth of *S. aureus* bacteria, and two isolates could inhibit *E. coli*. Isolate EAB6 could inhibit both pathogenic bacteria. It is supported by several studies that their research show the potential inhibitory power of the Andaliman plant against *S. aureus*. Several studies report that isolates isolated from endophytic bacteria of a plant will have higher inhibitory zone activity against Gram-positive pathogenic bacteria such as *S. aureus*.[19,24] It can happen because Gram-negative pathogenic bacteria have more robust defence capabilities when attacked by inhibitory compounds than Gram-positive pathogenic bacteria. Gram-negative bacteria have good defence because they have different cell wall components. The cell walls of Gram-positive bacteria are relatively thinner because most of them are only composed of peptidoglycan. At the same time, Gram-negative bacteria are not only composed of peptidoglycan. However, they are also composed of other components such as lipoproteins, outer membranes, and lipopolysaccharides, making inhibitory compounds from Andaliman endophytic bacterial isolates not to enter Gram-negative bacterial cells.[22]

The dilution method determines the MIC because it is easier and more efficient, does not require agar media, and only uses liquid media in a tube.[25] The MIC test results showed that various isolated supernatants had different minimum inhibitory concentrations. For Staphylococcus aureus bacteria, the optical density value of isolates EAB7, EAB5, EAB6, and EAA28 at a concentration of 6.25%, and isolate EAA22 at a concentration of 25% had a value that was smaller than the optical density value of 0.271 which was a negative control. In Escherichia coli bacteria, the optical density of isolates EAB5 EAA22 at a concentration of 6.25% and isolate EAB6 at 12.5% was 0.282 smaller than that of a negative control. The MIC value is determined based on the isolate concentration's optical density value, which is lower than the negative control. Spectrophotometry is used because of the tool's detailed capabilities regarding the absorption of chemical energy by chemical species, thus allowing greater accuracy in qualitative details and measurements.[23]

The incubation process produces a suspension in NB media in the form of bacterial secondary metabolites in the stationary phase. During the stationary phase, namely when the number of bacterial cells is constant, meaning the number of bacteria that die is the same as the number of bacteria that grow, when the nutrients for the bacteria in the medium begin to run out, competition between bacteria occurs and produces secondary metabolites to defend themselves which are also beneficial for the host plant.[26] The supernatant taken from each liquid culture of endophytic fungi from the Javanese Ginseng Plant (*Talinum paniculatum* Jag.) contains several types of compounds that are antimicrobial, namely flavonoids, alkaloids, terpenoids, and tannins. These antimicrobial compounds have the potential to cause damage to the test bacterial cells, thereby inhibiting their growth.[27]

Endophytic bacterial isolates grown in Nutrient Broth (NB) medium were shaken for three days (72 hours) with the aim of aeration, and it is hoped that they will obtain optimal cell biomass. Based on research conducted by Kuntari et al.[26] on root endophytic bacteria of the *Moringa oleifera* L, the three-day incubation process produced a suspension in NB media in the form of bacterial secondary metabolites in the stationary phase. The shaker with a speed of 170 rpm is also intended so that the bacteria can secrete the antibacterial compounds they produce into the culture medium optimally.[25]

The supernatant was extracted using ethyl acetate, the process of extracting metabolite compounds with the aim of the metabolite compounds diffusing into the solvent. Ethyl acetate is an excellent solvent for extraction because it can be quickly evaporated, is not hygroscopic, has low toxicity, and is semi-polar, so it is expected to attract metabolites of both polar and non-polar compounds.[28] Ethyl acetate macerate was separated from the culture medium using a separating funnel and evaporated using a vacuum rotary evaporator to obtain an isolate extract of endophytic bacteria.

The inhibition test results of Andaliman endophytic bacterial isolate extract against *S. aureus* showed that five endophytic bacterial isolates could inhibit the growth of *S. aureus* bacteria. Meanwhile, the results of the

antagonist test of endophytic bacterial isolates against *E. coli* showed that three endophytic bacterial isolates could inhibit the growth of *E. coli* bacteria. Andaliman plant extract contains alkaloids, flavonoids, glycosides, saponins, tannins, triterpenes/steroids, and glycosides, which have antimicrobial activity in inhibiting the growth of *S. aureus.*[29]

The flavonoid content in andaliman extract effectively inhibits the growth of *S. aureus*. Flavonoids are polar, so they more easily penetrate the peptidoglycan layer of microbes, which are also polar than the non-polar lipid layer, thereby disrupting the permeability of cell membranes.[30] Saponin compounds reduce the surface tension of bacterial cell walls and damage membrane permeability. Lysis of cell membranes can disrupt the survival of bacteria so that saponins can be used as antibacterials.[31]

Secondary metabolites from the endophytic bacterial isolate of *Anredera cordifolia* CIX1 leaves were extracted, and the antibacterial potential of the extract was evaluated against selected bacteria. The extract showed activity against Gram-positive bacterial strains. Gram-negative bacteria and a thin peptidoglycan layer (2 to 7 nm) have about 7 to 8 nm of outer membrane. This outer membrane consists of an additional protective lipopolysaccharide layer that exhibits toxicity and antigenicity to antibacterial agents. *E. coli*, which are Gram-negative bacteria, have a more complex cell wall structure than *S. aureus.*[12]

The difference between the initial screening results of Andaliman endophytic bacterial isolates and crude extracts was caused by several things. The diffusion speed of different substances and differences in the response of microbes to the substance being tested cause the resulting inhibitory diameter.[32] The differences in bacterial growth that occur at each fermentation time are caused by the different abilities of the bacteria to reproduce. The specific growth rate for each bacteria is different because the enzyme content in each bacteria is different, which influences the bacterial metabolic process in producing secondary metabolites.[33]

CONCLUSION

Based on the isolation of Andaliman endophytic bacteria, 252 colonies and 85 isolates with different characteristics were obtained. The bacterial antagonist test showed that 18 isolates of Andaliman endophytic bacteria had the potential to inhibit the growth of *E. coli* and *S. aureus* bacteria. The test results of Andaliman endophytic bacterial isolate extracts observed five bacterial isolate extracts that could inhibit *S. aureus* bacteria in the weak inhibitory category with the highest inhibitory diameter value in the EAB5 isolate. Three isolate extracts could inhibit *E. coli* in the weak inhibitory category with the highest inhibitory diameter value for isolate EAA22. In the MIC test, the MIC value of isolates EAB7, EAB5, EAB6, and EAA28 at a concentration of 6.25% and isolate EAA22 at a concentration of 25% inhibited *S. aureus.* For *E. coli* bacteria, the MIC value of isolates EAB5 and EAA22 was at a concentration of 6.25% and isolate EAB6 was 12.5%.

ACKNOWLEDGMENTS

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Case Report

Squamous Cell Carcinoma Cervical Uterine Metastasis in Abdominal Wall: A Rare Case Report

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Abstract

Background: Squamous cell carcinoma of the cervix accounts for greater than 80 percent of cervical malignancies. After radical surgery for squamous cervical carcinoma, abdominal metastasis is a rare occurrence.

Case Presentation: A 33-year-old female was treated for cervical cancer with radical hysterectomy, bilateral salpingo-oophorectomy, and bilateral pelvic lymphadenectomy. The histopathological examination revealed moderately differentiated non-keratinizing squamous cell carcinoma. Three months after surgery, a 6 cm mass, suggestive of metastasis, emerged from the abdominal region. For the treatment of the metastasis mass, abdominal wall reconstruction surgery was performed. Due to the rarity of abdominal metastasis in cervical cancer, the optimal treatment for this condition remains undetermined. Individualized treatment based on the severity of the disease and the presence of metastases.

Conclusion: The optimal treatment approach for cervical cancer remains uncertain, primarily due to the infrequency of abdominal metastases. Individualized treatment plans are formulated based on the patient's condition and the presence of metastatic disease.

Keywords: *Squamous Cell Carcinoma; Hysterectomy; Cervical Malignancies*
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INTRODUCTION

Due to its high prevalence worldwide, cervical cancer is a significant public health concern. Cervical cancer is the fourth most prevalent malignancy in women worldwide.[1,2] It is the second most prevalent malignancy among Indonesian women, with an incidence rate of 348,809 cases in 2018. It is the fourth leading cause of cancer-related mortality, with more than 270 thousand women dying of cervical cancer each year worldwide. Women in developing nations have a higher mortality risk from cervical cancer. Variations in screening availability and the prevalence of human papillomavirus (HPV) infection account for the substantial geographic variation in cervical cancer rates. It is known that the oncogenic subtype of HPV, specifically subtypes 16 and 18, causes cervical cancer.[3] History of smoking, parity, oral contraceptive use, early age of onset of coitus, higher number of sexual partners, history of sexually transmitted disease, certain autoimmune disease, and chronic immunosuppression are epidemiologic risk factors for cervical cancer. Approximately 80% of cervical malignancies are caused by squamous cell carcinoma, while 20% are caused by adenocarcinoma.[4]

According to the 2020 National Comprehensive Cancer Network Guidelines, the treatment of cervical cancer is stage-dependent. Planification of treatment should involve general practitioners, general gynecologists, gynecologic oncologists, radiologists, radiation oncologists, pathologists, and surgeons. Management of cervical cancer could include surgery, chemotherapy, radiotherapy, or a combination of the aforementioned modalities.[5]

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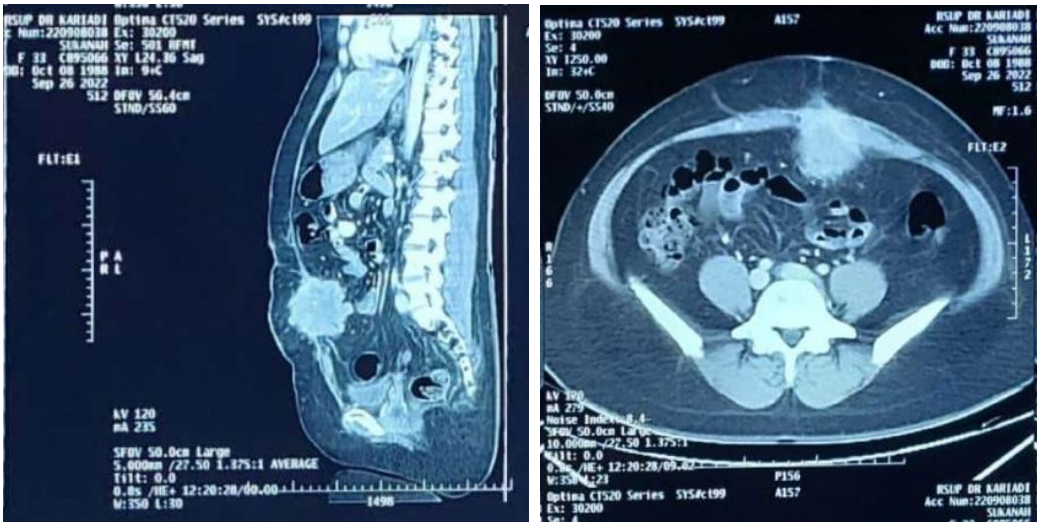

Figure 1. CT scan showing a suggestive metastatic mass at the infraumbilical region.

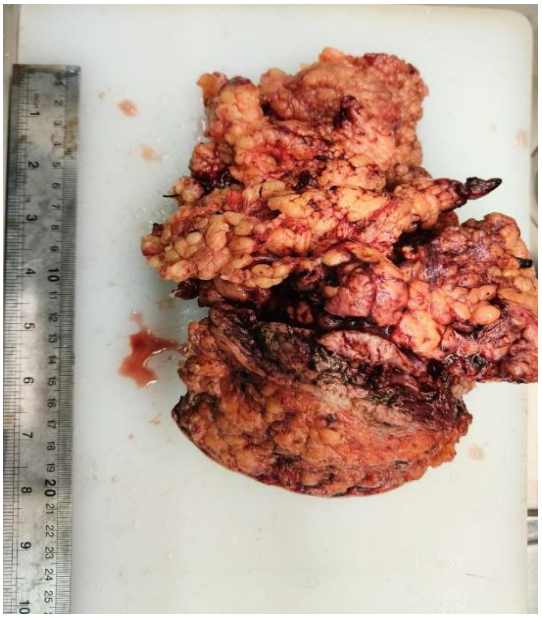

Figure 2. Abdominal Wall Metastasis Mass.

The National Comprehensive Cancer Network's 2020 guidelines state that the best course of action for treating cervical cancer is condition-specific. The whole healthcare team, from family doctors to radiologists to radiation oncologists to pathologists to surgeons, should be included in the treatment planning process. Numerous modalities could be used for cervical cancer management such as surgery, chemotherapy, radiotherapy, or a combination of the three above.[1,4]
Despite advances in cervical cancer treatment, the majority of recurrent cases occur within two years of initial treatment. Recurrent cervical cancer is the regrowth or redevelopment of tumor in lymph nodes or distal metastases following regression of the primary lesion. The most prevalent locations of recurrence are the parametrium, pelvic lymph nodes, and vagina. Typically, lung, bone, and liver are sites of distant metastasis. After radical surgery for squamous cervical carcinoma, abdominal metastasis is a rare occurrence. To date, there are very few articles reported the case of abdominal wall metastases of cervical cancer.[6–12] The treatment of recurrent cases is dependent on previous therapeutic approaches, the location, and the severity of the recurrence.[1,13,14]

We present the case of a 33-year-old multipara woman with abdominal metastases in recurrence of cervical carcinoma following cisplatin-based neoadjuvant chemotherapy, radical hysterectomy, bilateral salpingo-oophorectomy, and bilateral pelvic lymphadenectomy, followed by external radiotherapy and brachytherapy. This was a challenging instance because the patient required complex surgery. With cytoreductive surgery, adhesiolysis, omentectomy, and abdominal wall reconstruction, the patient was successfully treated.

CASE REPORTS

A 33-year-old woman, a mother of two children and no previous history of abortion, was currently in her second marriage for five years, and of Javanese ethnicity, was referred from a secondary health facility to the Obstetric and Gynecological clinic at Kariadi Central Hospital due to cervical tumor, suspicious for malignancy. The patient presents with a chief complaint of vaginal bleeding, without any accompanying or supplementary grievances. Vaginal bleeding became more profuse since three months before the patient consulted the health facility. The patient had no difficulties in micturition and defecation. The history of using an injection contraceptive was reported. There was no history of smoking or cervical cancer in her family.

Physical Examination

The patient's health was excellent overall. All of the vitals were within the typical range. She was in grade 1 obesity according to Asia Pacific BMI criteria. Neither conjunctival pallor nor scleral icterus was found. No abnormalities were detected within the thorax and abdomen examinations. In the pelvic examination with a speculum, blood clots and infiltrate in the vagina were shown. A friable mass was observed at the uterine portion of the cervix with the size of 5x4x5 cm. In the bimanual pelvic examination, the uterus was normal in size. An infiltrate in the right parametrium was palpable.

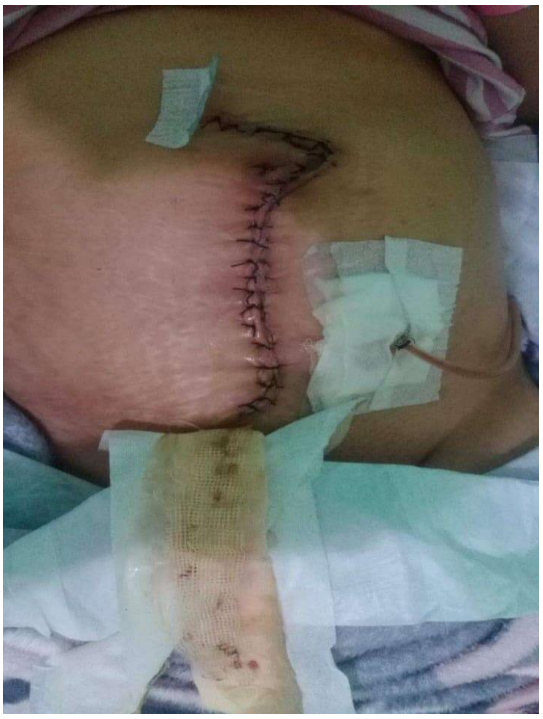

Figure 3. Operation wound at the end of surgery.

Supporting Examination

A blood test was performed on the patient. The hematocrit was 35.5% and the hemoglobin level was 10.2 g/dL. There were 13,300 total leukocytes and 367,000 platelets. Her glucose level was measured at 137 mg/dL. The results of the liver and renal tests were normal. Both electrolyte levels (Sodium, Kalium, Chloride) and coagulation study were also within normal limits. Based on an abdominal ultrasound examination, an inhomogeneous solid mass was revealed at the cervix uteri, 5.72 x 5.55 cm in size without infiltration to the urinary bladder. No abnormalities were detected in the other intraabdominal organs. The histopathological examination resulted in non-keratinizing squamous cell carcinoma moderately differentiated.

After obtaining informed agreement, a radical hysterectomy was done along with bilateral salpingo-oophorectomy and pelvic lymph node dissection. The patient received a cisplatin-based neoadjuvant chemotherapy. The procedure was performed under general anesthesia. The fixed-rigid vesico-uterine ligament was revealed intra-operatively. It did not reach the radicality procedure.

The histopathological examination within the cervix revealed a stratified squamous epithelial lining possessing features of hyperplasia with oval-shaped nucleus, moderate-to-high degree of pleomorphism, hyperchromatic with coarse chromatin and prominent nucleoli, abnormal mitotic activity with some exhibiting visible cleavage furrow, as well as koilocytosis with solid and nested arrangement, infiltrating the fibrous stroma, hyperemic, and permeated by inflammatory cells, namely lymphocytes and PMN, with eosinophils and histiocytes as the dominating types. Lymphangioinvasion was also found. Therefore, the suspicion for malignancy was confirmed, as the result concluded a moderately-differentiated, Cancer of the cervix composed of non-keratinizing squamous cells with lymphangioinvasion. Other parts, such as the endometrium, left and right uterine adnexa, left and right parametrium, anterior incision margin, and left and right pelvic lymph nodes provided normal images, with no signs of tumor. However, the myometrium exhibited characteristics of leiomyoma, while infiltration of malignant cells with hyperemic fibromuscular tissue, with hemorrhagic foci and mild permeation of lymphocytes and histiocytes was found within the posterior incision margin.

Following the operative management, the patient underwent adjuvant 25 x 2 Gy external radiotherapy and internal radiotherapy, then. A follow-up was conducted after the therapy had been completed.

Three months later in a regular follow-up, the patient complained for a painless abdominal lump since a month prior. A mass of duck egg-size located at the middle of umbilical and symphysis was revealed in an abdominal palpation. An ultrasound of the abdomen revealed a 6x6 cm hypoechoic mass in the infraumbilical region but no free intraabdominal fluid. Contrast computed tomography (CT) revealed a solid mass in the infraumbilical region on the patient's left side (AP 7.1 x LL 5.2 x CC 6.1 cm) that was attached to and difficult to separate from the left rectus abdominis muscles, as well as having surrounding fat stranding and internal calcification (Figure 1). It was presumed to be likely as a metastasis. Multiple lymphadenopathies were found in the paraaortic and interaortocaval region (largest size ± 1.7 x 0.8 cm, in the paraaorta) and no visible nodules in the liver, spleen, or lung were visualized on the CT scan. The mass was removed by cytoreductive surgery, adhesiolysis, and omentectomy. In addition, an abdominal wall reconstruction was performed. A unit of packed red blood cells was given to the patient during the operation.

On the subsequent histopathological evaluation, within the deep dermal and subcuticular fat layers of the recurrent abdominal-wall, exhibited proliferation of malignant cells with oval-shaped nuclei, mild-to-moderate degree of pleomorphism, filled with vesicles, prominent nucleoli, large eosinophilic cytoplasm, unclear intercellular margin, visible mitotic activity, and were structured in nested arrangement. The result also showed infiltration of inflammatory cells, namely PMN, lymphocytes, histiocytes, and multinucleated giant cells. Conclusions: Non-keratinizing squamous cell carcinoma with modest differentiation, which originated from the uterine cervix and metastasized to the abdominal wall and omentum. Concurrent comedonecrosis and lymphangioinvasion were also present. Two of the lateral margins and the base of incision, along with the omentum were revealed to be infiltrated by malignant cells, with characteristics similar to those found within the abdominal-wall mass.

Following the operation, an intraabdominal drain was placed and a urinary catheter was inserted. In order to prevent patients from postoperative infection, broad-spectrum antibiotic of Ampicillin-Sulbactam (1.5 gram/6 hours) was administered for 7 days. The dressing was changed once in three days and the urine output was monitored daily. Two days postoperatively, the patient complained of nausea. She vomited twice a day and had

not yet flatulated nor defecated. A nasogastric tube was inserted and the patient was consulted to the clinical nutrition specialist. Greenish gastric fluid had flowed through the nasogastric tube. The patient and her family was given educated about the sham feeding. The tube was finally removed three days later. The urinary catheter was removed on day 4, while the drain was removed on day 7 postoperatively. On day 8, the patient was discharged after being educated about wound care when she would had returned home (Figure 3).

Informed Consent

The patient has given explicit authorization for the publication of their documented medical case, encompassing medical records, physical examination findings, and imaging studies, in the form of a case report. The patient's personal information will be meticulously safeguarded to ensure strict confidentiality, and any sensitive data have undergone a stringent screening process to maintain the security of confidential information. Furthermore, a written informed-consent form has been duly acquired from the patient.

DISCUSSION

Seventy-five percent to eighty-five percent of invasive cervical cancers are squamous cell carcinomas. Squamous cell carcinoma is classified as an epithelial neoplasm by histopathologists, which in the case of cervical cancer, affects the flat-shaped cells that cover the cervix, with the most common form being malignant. Non-keratinizing squamous cell carcinoma of the cervix possesses these histopathological features: proliferation of epithelial cells; stromal infiltration of lymphocytes; nuclear enlargement; increased nuclear-cytoplasmic ratio; coarse chromatin; and no keratin mass.[15,16]

Definitive platinum-based chemoradiotherapy and brachytherapy are the preferred treatment. Every single chemotherapy treatment plan relied heavily on cisplatin. For locally advanced cervical cancer, the systematic review compared neoadjuvant chemotherapy followed by surgery to radical radiation alone. When data from all studies were pooled, they showed that neoadjuvant chemotherapy significantly reduced mortality.[4]

The recurrence of symptoms is not always present. Vaginal bleeding, back discomfort, leg oedema, haematuria, weight loss, and abdominal tumours are frequent complaints when patients seek medical attention. After reviewing the relevant literature, we determined that the average interval between recurrences was 14 months (range, 1.5–45 months). To establish the primary cause of the metastasis, a biopsy is required. Immunohistochemical markers, such as CD31 positivity, may also be useful in determining the extent to which a metastasis has spread from its initial site.[1,5] The patient did not have an immunohistochemistry analysis because to financial constraints.

Metastasis involves a number of intricate biological processes, such as cell detachment, control of cell motility and invasion, survival, proliferation, and immune system evasion. It's important to remember that cells may travel via the body's circulatory and lymphatic systems. Potential risk factors for such metastases may be broken down into four categories: in the first, you have patient-specific variables such local immune reactivity, wound hypoxia, and acidosis that might trigger angiogenesis and hematogenous dissemination around the umbilicus. Disease progression, adenocarcinoma cell type, peritoneal carcinomatosis, and lymph node disease make up the second category. Parameters of the laparoscopic environment, such as pneumoperitoneum and notably carbon dioxide usage, are discussed in the fourth group, while those of the surgical approach are discussed in the third group (mechanical port irrigation, not using endobags, trocar size direct installation by tools or gloves). Cervical cancer often spreads to other organs outside of the pelvis, including the lymph nodes, lungs, liver, and bone. Metastatic recurrence of squamous cervical cancer was sometimes seen in the abdomen wall (0.1-1.3%).[17]

To better understand how to present and care for such outliers, a multicenter investigation is recommended. Since significant surgery is difficult and requires a multidisciplinary approach, radiation therapy and chemotherapy are the usual methods of treatment. Abdominal lesion resection for palliation was followed by repair using different muscle flaps in two previously described instances of abdominal wall metastases. The prognosis of such a recurrence is murky since individuals with such advanced illness may not survive. In most situations, the outlook is grim.[17,18]

Due to its rarity of abdominal wall cancer recurrence, the optimal management of this condition remains unclear. Treatment plan of each individual are depended on what extent of the disease and the presence of metastasis.[19,20] Current patient had the unique location of recurrence site of abdominal wall, which was successfully treated with wide local excision without adjuvant chemotherapy. Kanao et al., reported that patients who underwent locally extended endopelvic resection with negative marhin for recurrent cervical carcinoma showed 62% of 5-year-survival rate.[21] While, abdominal wall reconstruction was needed in this case. Some parameters were found to be predictor of metastasis recurrence in patient’s condition, such as cut margin negativity and absence of other organ metastasis. After being discharged from hospital, through tight follow-up schedule, Oncology Gynecology team will consider the role of adjuvant radiotherapy for further management regarding patient’s condition.

CONCLUSION

In conclusion, cervical cancer metastasis to the abdominal wall is uncommon. The optimal treatment for this condition remains unknown. Individualized treatment is dependent on the extent of the disease and the presence of metastasis. With the advancement of plastic surgery, extensive resection with defect closure appears to be a reasonable option in appropriately selected cases in an effort to improve quality of life and, hopefully, extend survival. Moreover, a thorough follow-up after the initial surgery could have led to the earlier detection of the abdominal wall metastasis with a significantly smaller mass, a less aggressive procedure, a shorter recovery period postoperatively, and, naturally, a shorter delay of the chemoradiation.

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Review Article

The Influence of *CYP2C19* Gene Variants on Selective Serotonin Reuptake Inhibitors in Patients with Major Depressive Disorder: A Pharmacogenetic Prospecting Approach

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Abstract

Major Depressive Disorder (MDD) is a chronic disorder characterized by at least a two-week-long major depressive episode. MDD presents a significant global health burden, often treated with Selective Serotonin Reuptake Inhibitors (SSRIs). However, variable responses to SSRIs, including ineffectiveness and adverse effects, may be attributed to genetic factors influencing drug metabolism, particularly by the CYP2C19 enzyme. This review aimed to assess the impact of *CYP2C19* gene variants on the effectiveness and safety of SSRIs, specifically citalopram, escitalopram, and sertraline, in patients with MDD. A systematic search of databases including PubMed, Google Scholar, and ScienceDirect was conducted using relevant keywords. Articles published between 2013 and 2023 were included. Nine relevant studies from various countries were identified and analyzed. Findings indicated that *CYP2C19* gene variants, notably loss-of-function *(*2, *3)* and gain-of-function *(*17)*, influence the metabolism of SSRIs. Variability in enzyme activity among individuals carrying these variants can lead to significant alterations in drug efficacy and safety profiles. The review underscored the importance of considering genetic factors, particularly *CYP2C19* variants, in tailoring SSRI treatment for MDD.

Keywords: *Major Depressive Disorder; CYP2C19; Gene Variant; Selective Serotonin Reuptake Inhibitors; Drug Metabolism.*
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INTRODUCTION

Major Depressive Disorder (MDD) is a severe condition marked by at least one major depressive episode that endures for a minimum of two weeks. Distinguished shifts in mood and interest, significant changes in thinking patterns, and conspicuous autonomic symptom alterations accompany it.[1] According to information retrieved from the Institute of Health Metrics and Evaluation in 2023, global depression affects around 280 million individuals. Approximately 3.8% of the global population suffers from depression, with rates varying among different demographics, such as 5% among adults, split into 4% for men and 6% for women, and 5.7% among adults aged 60 years and older.[2] The statistics are influenced by a range of factors including variations in hormone levels among women, the impact of childbirth, varying psychosocial stressors encountered by both men and women, as well as the frequently experienced sense of helplessness among women.[3]

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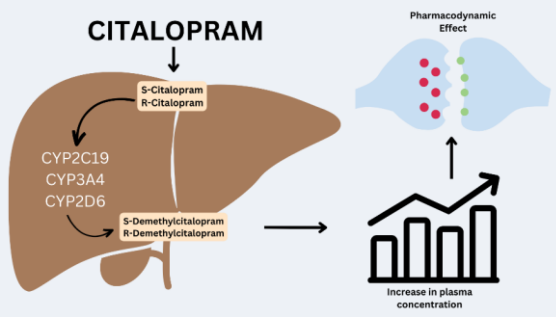

Figure 1. Metabolism of citalopram and escitalopram

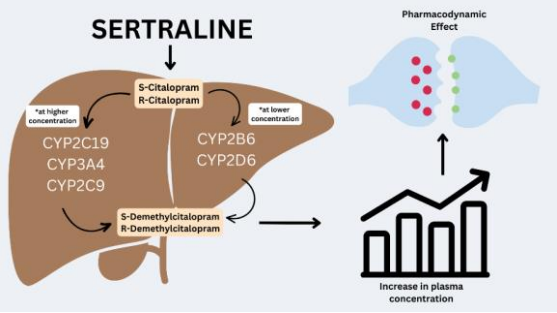

Figure 2. Metabolism of sertraline

MDD is frequently associated with chronic conditions such as diabetes, stroke, and other cardiovascular diseases, thus increasing concerns about this illness. Furthermore, MDD is known to lead to suicide among its sufferers.[4]

Although antidepressants remain crucial in addressing moderate to severe MDD, their effectiveness varies. A significant portion of individuals do not achieve optimal response or even discontinue treatment due to early side effects. Research shows that around 60-70% of individuals with depression do not attain remission, and approximately 30-40% either do not show a notable improvement or encounter medication-related adverse effects leading to discontinuation of treatment, non-adherence, and the emergence of chronic conditions. One significant factor contributing to variations in drug response is the varying activity levels of drug-metabolizing enzymes among individuals, leading to differences in drug exposure.[5,6]

Selective Serotonin Reuptake Inhibitors (SSRIs) constitute a commonly prescribed class of medications primarily utilized for treating depression. Their mode of action involves the inhibition of the serotonin transporter (SERT) at the presynaptic axon terminal. This inhibition results in a prolonged presence of serotonin (also known as 5-hydroxytryptamine or 5-HT) within the synaptic cleft, thereby extending stimulation of postsynaptic receptors. Due to their favorable safety profile, effectiveness, and tolerability, these drugs are recommended as the initial pharmacotherapeutic approach for depression and other mental disorders. Unlike other antidepressant categories, SSRIs have limited impacts on neurotransmitters such as dopamine or norepinephrine. Additionally, SSRIs tend to induce fewer side effects in comparison to Tricyclic Antidepressants (TCAs) and Monoamine Oxidase Inhibitors (MAOIs), mainly owing to their reduced influence on adrenergic, cholinergic, and histaminergic receptors.[7] Nevertheless, despite their extensive utilization, research conducted in the early 2000s indicated that one out of every three patients did not exhibit a positive response to SSRI therapy.[8] Subsequently, this observation garnered validation from other studies, highlighting that SSRIs, predominantly metabolized by the CYP2C19 isoenzyme, displayed susceptibility to variations attributable to many genetic variants.[9,10] SSRIs primarily metabolized by CYP2C19 include escitalopram, citalopram, and sertraline.[11,12] This study will review the influence of *CYP2C19* gene variant on the metabolism of SSRIs (escitalopram, citalopram, and sertraline).

MATERIALS & METHOD

The review process involved searching for relevant articles in Google Scholar, PubMed, and Science Direct databases. The search was conducted using keywords such as "CYP2C19 gene variants," "SSRIs," "major depressive disorder," "pharmacogenetics," "polymorphism," and "variants." Articles within the range of 2013-2023 were included in the review, resulting in the identification of 9 articles that matched the title.

METABOLISM OF CITALOPRAM AND ESCITALOPRAM

Citalopram (CIT) and escitalopram (ESC), both classified as selective serotonin reuptake inhibitors (SSRIs), are frequently recommended for addressing depression. Citalopram and its N-demethylated metabolite comprise a racemic combination encompassing S-enantiomer and R-enantiomer forms. In contrast, escitalopram is the S-enantiomer derived from the racemic citalopram compound.[13] Both enantiomers of citalopram undergo metabolism in the liver through the cytochrome P450 system. As depicted in Figure 1, the formation of R/S-demethylcitalopram is primarily facilitated by the isoenzymes CYP2C19, CYP3A4, and CYP2D6. In vitro and in vivo investigations consistently showed that the effects of citalopram and N-demethylcitalopram are primarily associated with their S-enantiomers, S-citalopram, and S-demethylcitalopram. These S-enantiomers exhibit significantly higher potency in inhibiting serotonin reuptake compared to their respective R-enantiomers, with S-citalopram being about 167 times more potent and S-demethylcitalopram being 6.6 times more potent. Furthermore, the conversion of citalopram to R/S-didesmethylcitalopram involves the action of CYP2D6. In vitro studies on human liver microsomes demonstrated that CYP2C19, CYP3A4, and CYP2D6 were responsible for the transformation of the biologically active S-enantiomer. Due to the racemic nature of citalopram, its administration results in separate steady-state concentrations of R- and S-stereoisomers. Additionally, processes involving N-oxidation and deamination, mediated by CYP2D6, create R/S-citalopram N-oxide and citalopram propionic acid metabolites.[14]

METABOLISM OF SERTRALINE

Sertraline is an effective and widely used antidepressant. Studies have found that sertraline metabolism occurs in the liver, primarily through N-demethylation. Moreover, research suggested that among the CYP450 enzyme family, CYP2C19 plays a

significant role in the sertraline metabolism process.[15,16] Majority of studies on sertraline metabolism asserted that the key metabolic pathway involves demethylation, converting sertraline into desmethylsertraline, which is the sole active metabolite of sertraline. In vitro studies suggested that when sertraline concentrations were higher, the primary contributors to its metabolism were CYP2C9, CYP3A4, and CYP2C19, with CYP2D6 and CYP2B6 making minor contributions (see Figure 2). However, when sertraline concentrations are lower, CYP2D6 and CYP2B6 assume a more prominent role in the formation of desmethylsertraline, while the impact of CYP3A4 on this process diminishes.[17]

CYP2C19 GENOTYPE

CYP2C19, a vital component of the cytochrome P450 superfamily (CYP450), accounts for approximately 16% of the total liver enzyme composition. This particular enzyme holds a pivotal role in the hepatic metabolism of diverse medications, such as antimalarials (proguanil), antiplatelets (clopidogrel), and antidepressants (amitriptyline, clomipramine). The existance of genetic variants within *CYP2C19* significantly impacts the metabolism of these substrates, thus shaping individual responses to these drugs [18]. Díaz-Ordóñez et al. (2021) successfully identified at least 40 *CYP2C19* variants in their study.[19]

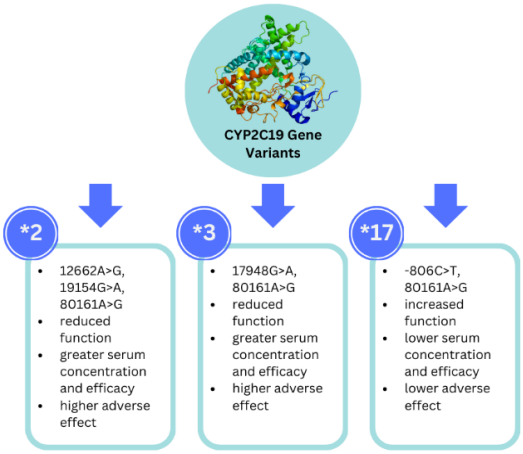

Figure 3. Characteristics of *CYP2C19* gene variant

Table 1. *CYP2C19* Variants diplotype and its enzyme activity[22].

Phenotype	Genotype	Enzyme activity
Ultra-rapid metabolizer	**1/*17* **17/*17*	Increased
Extensive metabolizer	**1/*1*	Normal
Intermediate metabolizer	**1/*2* **1/*3* **2/*17* **3/*17*	Intermediate
Poor metabolizer	**2/*2* **3/*3* **2/*3*	Reduced or none

The *CYP2C19* diplotype and its corresponding enzyme activity are outlined in Table 1. Loss-of-function variant alleles, specifically *CYP2C19*2* and **3*, are grouped as *CYP2C19Null*, whereas *CYP2C19*17* is the sole gain-of-function variant allele recognized. Patients with the Poor Metabolizer (PM) genotype (*CYP2C19Null*/*Null*) lack functional *CYP2C19* enzyme entirely. Intermediate Metabolizers (IM) (*CYP2C19**1/*Null* and *CYP2C19*17*/*Null*) possess one non-functional allele. Patients carrying the *CYP2C19Null*/**17* genotype are categorized as IM because the influence of the *CYP2C19Null* allele on the phenotype is more pronounced than that of the functional *CYP2C19*17* allele. Extensive Metabolizer (EM) or Normal Metabolizer (NM) individuals with the diplotype *CYP2C19*1/*1* have two copies of the wild-type *CYP2C19**1 allele, representing the reference genotype. Ultrarapid metabolizer (UM) denotes individuals who carry either *CYP2C19*1/*17* or *CYP2C19*17*/**17* [20,21]. Figure 3 provides an overview of the characteristics of the three most extensively studied variants, **2*, **3*, and **17*.[22]

The distribution of *CYP2C19* alleles displays a noteworthy trend. The most prevalent non-functional allele, *CYP2C19*2* (c.681G>A; rs4244285), exhibits an occurrence rate of 18% in African and European populations but surpasses 30% in Asian populations. In contrast, other alleles (**3*-**8) are present in limited numbers and lack functionality. *CYP2C19*3* (c.636G>A; rs4986893) possesses a minor allele frequency (MAF) of roughly 7% among East Asian populations. On the other hand, the increased-function allele *CYP2C19*17* (c.-806C>T; rs12248560) has a prevalence of around 23% among Europeans and Africans, with slightly lower occurrence in mixed American and South Asian populations (12%-14%) and nearly absent in East Asians. Consequently, the *CYP2C19* phenotype spans a wide range, from poor metabolism (PM) to ultra-rapid metabolism (UM), with the diversity of these alleles contributing to observed phenotypic variations in different populations.

CYP2C19 Variant and SSRI Metabolism

Several studies that have established a connection between *CYP2C19* variant and the effectiveness of SSRI drugs are presented in Table 2. Jukic et al. (2018) used a retrospective study method on 2087 patients in Norway and found the indications that patients with *CYP2C19* PM and IM genetic profiles would exhibit higher serum concentrations of escitalopram compared to EM.[20] Conversely, patients with UM metabolism status would show lower serum concentrations of escitalopram. The results implied that patients with this metabolism status might face treatment failure. Furthermore, the data suggested that patients with *CYP2C19* UM and PM metabolism conditions are more inclined to switch to other classes of antidepressants in comparison to those with EM metabolic conditions.

Table 2. Research conclusion related to *CYP2C19* variants and their effects on SSRI metabolism.

SSRI	Research type	Genes	Origin of Study	Conclusion	Research
ESC	Retrospective Study	**2, *3, *4, *17*	Norway (n=2087)	PM indicates a tendency for patients to switch medications, while UM shows serum drug concentrations below the therapeutic window.	20
ESC	Clinical Trial	**1, *2, *3*	Japan (n=142)	A significant correlation between *CYP2C19* Variant and ESC concentration can only be found at adjusted doses.	23
ESC	Clinical Trial	**1, *2, *3*	China (n=90)	PM has the highest AUC0-t and AUC0-∞, and the longest half-life. Different Cmax levels were found among the three phenotypic types, but they were insignificant.	21
ESC	Clinical Trial	**2, *3, *17*	Brazil (n=31)	Patients with UM metabolism status show the highest HDRS scale, signifying treatment failure.	5
CIT	Clinical Trial	**2, *17*	Turkey (n=2019)	*CYP2C19*2* variant contributes to inter-individual variability in CIT metabolism in vivo.	24
CIT	Clinical Trial	**2*	Russia (n=130)	Patients with GA genotype status (IM) have lower blood drug concentrations.	25
SET	Clinical Trial	**1, *2, *3, *17*	Scandinavia (n=1202)	*CYP2C19*17* does not significantly impact therapeutic response, whereas increased serum concentrations in PM and IM can elevate the risk of overexposure and adverse drug reactions.	34
SET	Clinical Trial	**2, *17*	Turkey (n=50)	There are no significant differences in plasma concentrations among *CYP2C19* subgroups.	27
ESC, CIT, dan SET	Retrospective Study	**2, *3, *17*	Australia (n=9168)	Participants with RM status show the best tolerability; IM faces a higher risk of side effects, while PM exhibits better efficacy.	6

Huang et al. (2021) studied 90 clinical trial participants in China that investigated the influence of CYP2C19 metabolism on patient pharmacokinetic profiles using the area under the curve (AUC), half-life ($t_{1/2}$), and maximum concentration (C_{max}) data.[21] The data indicated that PM patients had the highest AUC_{0-t} and $AUC0_{-\infty}$, and the most prolonged half-life compared to EM and IM. These AUC results can be interpreted as PM patients potentially having higher therapeutic exposure than EM and IM, which relates to treatment efficacy and safety. While there were differences in C_{max} values among patient profiles, they were not highly significant. UM metabolism status was not included in this study due to a lack of candidates.

In contrast, Tsuchimine et al. (2018) yielded opposing results in Japan.[23] No notable association was observed between *CYP2C19* variant and steady-state escitalopram concentrations when administered at typical doses. This lack of correlation is believed to result from the substantial variability in plasma concentration of escitalopram at a steady state. However, when adjusted for escitalopram doses, a significant correlation was

found. Furthermore, the authors raised the potential involvement of CYP3A4 in the N-demethylation of escitalopram, which could introduce complexity when measuring steady-state plasma escitalopram concentrations.

Different from previously discussed articles, a study by Bernini de Brito & Ghedini (2020) in Brazil utilized the HDRS (Hamilton Rating Scale for Depression) scale to measure indications of depression in patients through a questionnaire. The results showed that patients with *CYP2C19* UM metabolism conditions had higher HDRS scale scores, indicating more severe levels of depression compared to other metabolism statuses.[5]

Furthermore, investigations into the impact of *CYP2C19* variant on the effectiveness of citalopram were carried out by Uckun et al. in 2015 and Zastrozhin et al. in 2021.[24,25] The study by Uckun et al. (2015) indicated a significant role of *CYP2C19* variant in the CIT metabolism process. *CYP2C19*2* variant was crucial in inter-individual variations in in vivo CIT metabolism at therapeutic doses commonly used in clinical practice. Thus, the *CYP2C19*2* variant was predicted to yield better treatment outcomes and a higher risk of adverse drug effects. In contrast, this study found no significant differences in CIT and DCIT plasma concentrations between patients with the *CYP2C19*17* allele and those with *CYP2C19*1* homozygotes.[24]

Zastrozhin et al. (2021) specifically investigated the impact of *CYP2C19*2* variant on the effectiveness of citalopram. The variations were statistically significant in the equilibrium concentrations of citalopram among patients with different *CYP2C19*2* (681G>A) genotypes. Patients carrying the A allele exhibited lower drug equilibrium concentration levels than those with the G allele. This reduction seems to arise from decreased biotransformation processes and a slower elimination rate of citalopram in individuals with the A allele, leading to drug accumulation in the bloodstream. Consequently, there is a potential for an elevated risk of adverse drug reactions and the possibility of developing pharmacoresistance. These findings suggest that carriers of this polymorphic variant may face an increased likelihood of experiencing adverse drug reactions associated with citalopram, likely due to reduced CYP2C19 activity, impaired drug biotransformation processes, decreased elimination rates, and subsequent drug buildup in the bloodstream.[25]

The third SSRI drug primarily metabolized by CYP2C19 is sertraline. A study conducted by Bråten et al. (2020) found that individuals with CYP2C19 PM and IM variants exhibited notably higher serum concentrations of sertraline in comparison to those categorized as normal metabolizers (NM). This increase is attributed to the compromised metabolism of sertraline, a process typically facilitated by the CYP2C19 enzyme in individuals with PM and IM phenotypes. Interestingly, the study results indicated that the *CYP2C19*17* variant did not affect the therapeutic response to sertraline. However, the heightened serum concentrations observed in individuals with CYP2C19 PM and IM phenotypes pose a potential risk of drug overexposure, potentially leading to adverse drug reactions.[26] Based on these findings and using NM individuals as the reference group within a large patient population, it is estimated that the initial sertraline dose could be reduced by 60% for individuals with the CYP2C19 PM phenotype and by 25% for individuals with the CYP2C19 IM phenotype. These estimates are based on the relative differences between the IM and PM phenotypes compared to NM. Nevertheless, the slight variations in the ratio of sertraline to N-desmethylsertraline among different *CYP2C19* phenotype groups suggest that other enzymes may have a more substantial role in the N-demethylation process of sertraline. The result highlights the broader complexity of sertraline metabolism that must be considered when effectively adjusting doses and monitoring drug therapy.

Another study that elucidates the impact of *CYP2C19* variant on sertraline efficacy is the research by Yuce-Artun et al., 2016.[27] The findings from this study support the 2020 study by Bråten et al. that the genetic variant *CYP2C19*17* does not significantly influence sertraline metabolism. Concurrently, this study investigated the relationship between *CYP2B6* variant and sertraline efficacy. However, it was discovered that the *CYP2B6*6* variant has a more pronounced role and appears to contribute to inter-individual variations in SERT metabolism under real-world conditions at therapeutic doses used in clinical practice.

Among the studied articles, Campos et al. (2022) is the only one that discusses all three drugs together, namely escitalopram, citalopram, and sertraline. This research was conducted retrospectively on 9500 participants in Australia. This study indicated that individuals with PM status experience significantly higher antidepressant efficacy than those with normal or rapid metabolism. Furthermore, it was discovered that individuals with Rapid Metabolizer (RM) status have higher tolerability levels. They tend to have a lower risk of discontinuing drug use than individuals with NM status due to side effects. However, individuals with intermediate metabolism, who fall between rapid and slow metabolizers, may have a higher risk of reporting side effects during treatment. These findings indicate that drug tolerability can vary depending on the *CYP2C19* metabolizer status, with RM status showing the best tolerability, IM facing a higher risk of side effects, and PM showing better efficacy.[6]

DISCUSSION

Pharmacogenetics and pharmacogenomics are two fields that have gained significant attention recently, alongside the rise of studies in personalized medicine. Despite appearing similar, these two fields have distinctions. Pharmacogenetics is a research field studying how genetic variations in individuals can affect drug responses. Its focus is on specific genes or particular genetic variations' roles in influencing drug metabolism, transportation, or mechanisms of action. On the other hand, pharmacogenomics utilizes a broader approach, examining the relationship between the overall genetic profile and drug responses.

Genetic profiling tests are in high demand due to the evidence of varying treatment outcomes in individuals based on their genetic conditions. One of the most prominent studies involves the *CYP2D6* gene's metabolism in relation to anticancer drug substrates. *CYP2D6* is a gene with numerous variants that often lead

to different therapeutic outcomes in cancer patients. In 2013, Westbrook & Stearns stated that remarkable progress has been made in breast cancer treatment by introducing targeted therapies like aromatase inhibitors (AI) and biological therapies like trastuzumab. Targeted therapy introductions have proven to aid in understanding factors contributing to individual variability in response to various breast cancer treatments, such as the impact of genetic variations on drug metabolism.[28,29] Pharmacogenomic studies are also considered to have great potential in altering asthma treatment regimens in other diseases, such as asthma. Data and findings from pharmacogenomic studies suggest that standard treatment guidelines cannot be uniformly applied to the entire asthma patient population. Understanding the influence of genetic variations on therapy responses can reduce side effects and enhance patient outcomes.[30]

Major depressive disorder is a proven hereditary illness. Individuals with a family history of MDD have a threefold higher risk of experiencing depression. Broadly, the two main options for treating MDD are psychotherapy and pharmacotherapy. Both approaches have demonstrated efficacy in reducing depressive symptoms and overall well-being. A recent study indicated that combining psychotherapy and pharmacotherapy as an initial treatment strategy produced more favorable outcomes than utilizing either treatment in isolation.[4]

The application of genetic profiling to tailor depression therapy has been extensively studied. A meta-analysis conducted in 2018 stated that depressed patients treated with pharmacogenetic guidance had a 1.71 times higher chance of symptom remission compared to patients who did not undergo pharmacogenetic testing.[31] Another systematic study in 2021 also declared that treatment guided by pharmacogenetic testing would positively affect symptom remission and better treatment response.[26]

In Indonesia, the initial investigation into the prevalence of the *CYP2C19* gene was carried out by Ikawati et al. in 2014, focusing on *CYP2C19* variants within the Bugis ethnic group.[32] This research was subsequently expanded upon by Miftahussurur et al. in 2021, encompassing a broader examination of variant distribution in Indonesia and comparing various ethnic groups. The findings revealed that the frequency of recessive genes responsible for diminished CYP2C19 activity (**2* and **3* alleles) stood at 40.7% (135 out of 332). In summary, the prevalence rates for rapid, intermediate, and poor metabolisms in Indonesia were 38.5%, 41.6%, and 19.9%, respectively. This data indicates that more than half (61.5%) of participants exhibited reduced CYP2C19 enzyme activity.[33]

Despite its clear significance, pharmacogenetic and pharmacogenomic studies in Indonesia are still relatively rare. Some private health laboratories offer genetic profiling services, which are considered exclusive. In the future, pharmacogenomic and pharmacogenetic studies are expected to gain more attention, considering Indonesia's diverse demographic, ethnicities, and genetic background. This review, especially, has the potential to shed light on how genetic variations (*CYP2C19*) within the population can influence drug responses (CIT, ESC, and SERT) uniquely. The findings from such research can directly impact clinical practice, helping healthcare providers make informed decisions about medication choices and dosages, ultimately improving treatment outcomes and reducing adverse drug reactions.

CONCLUSION

Variants in *CYP2C19* have been demonstrated to influence the efficacy of SSRIs in treating patients with Major Depressive Disorder symptoms. Patients with UM metabolism status typically exhibit lower plasma serum concentrations, indicating a risk of treatment failure. Conversely, patients with IM or PM metabolism status tend to display higher plasma serum concentrations and an increased risk of adverse drug reactions (ADRs). Hence, it would be beneficial for patients prescribed SSRIs to undergo pharmacogenetic testing prior to treatment initiation, enabling an assessment of their pharmacogenetic profile and the formulation of an appropriate treatment plan. While such studies are still relatively scarce in Indonesia, future challenges may encompass financial constraints, the necessity for specialized infrastructure and expertise, and the integration of pharmacogenetic testing into routine clinical practice. However, considering the importance of this research, it will be necessary to gather all information about human genetic profiles to inform drug choices in Indonesia. This approach can increase the likelihood of treatment success and mitigate the occurrence of adverse reactions.

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Original Research Article

Effect of Myristica fragrans on PGC1α and *Synaptophysin* Expression in Male Wistar Rats Hippocampus

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Abstract

Background: Nutmeg is an indigenous plant from Indonesia that has been used extensively in herbal treatment. Nutmeg seed extract (NuSE) contains the active compound macelignan, which exhibits pharmacological activities. A previous study stated that NuSE is PPARγ or peroxisome proliferator-activated receptor gamma agonist that potentially enhances synaptic signal modulation. PPARγ activation can activate PGC1α or peroxisome proliferator-activated receptor gamma coactivator-1 alpha as the primary regulator of mitochondrial biogenesis. Mitochondria are involved in synaptic transmission. Increased modulation of signals at synapses can increase neuroplasticity, potentially improving the brain's cognitive function, as seen by the amount of *Synaptophysin* in the synaptic vesicle membrane for evaluating synaptogenesis.

Objective: This research demonstrates how nutmeg seed extract (NuSE) affects PGC1α and *synaptophysin* expression compared with DHA or docosahexaenoic acid, which has been evidenced to promote neurite growth.

Methods: Twenty-four Wistar male rats aged eight weeks were divided into four groups (control, PGA group, NuSE group, and DHA group). The treatment group was administered for 12 weeks using a gavage. After that, the rats were sacrificed, and the hippocampus neurons were collected. The PGC1α and *Synaptophysin* mRNA expression was measured using semiquantitative reversed PCR, visualized with electrophoresis, and then quantified with ImageJ. The analysis used in this study was a one-way ANOVA test to measure differences between groups using SPSS 26.0. If the test leads to significant results, a post hoc test is used to to confirm the differences between groups statistically.

Results: This study showed that NuSE increased *synaptophysin* and PGC1α mRNA expressions compared to the control group with significance statistic ($p=0.017$, $p<0,05$) in *synaptophysin* expression but did not increase PGC1α expression significantly ($p=0.364$, $p>0,05$).

Conclusion: In conclusion, nutmeg seed extract (NuSE) impacts synaptogenesis in *synaptophysin* expression to modulate synaptic transmission.

Keywords: *Hippocampus; PGC-1a; NuSE; Synaptophysin*
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INTRODUCTION

Neuroplasticity is a process of structural and functional adaptive changes.[1] Plasticity in neuronal morphology and electrical responsiveness occurs with regular changes in the nervous system.[2] Other factors that affect neuroplasticity are changes in the strength and number of synapses and the reorganization of neural circuits.

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An increase or decrease in synaptic activity results in a continual increase or decrease in synaptic strength so that experience (environment) directly affects the brain's physical and functional properties. Neuroplasticity is essential for learning and memory processes because it is the central nervous system rearranging neural networks to respond to environmental stimuli. Structurally, most brain areas are related to the function of processing memory, namely the hippocampus, fornix, temporal lobe, and cerebellum, which are interconnected to and between other brain regions.[3] The process of neuroplasticity as brain development is influenced by various factors, one of which is PPARγ or peroxisome proliferator-activated receptor gamma. PPARγ is an activated ligand for transcription factors bound to the nuclear membrane.[4] Studies suggest that PPARγ has the potential to promote neuron development.[5] PPARγ activation can activate PGC1α or peroxisome proliferator-activated receptor gamma coactivator-1 alpha as the primary regulator of mitochondrial biogenesis. Mitochondrial biogenesis is a complex biological process that aims to maintain cell homeostasis by forming new mitochondria and destroying damaged mitochondria.[6,7] Substances that can increase the activity of PPARγ from natural substances, one of which is using docosahexaenoic acid (DHA), which is a group of omega-3 or omega-3 polyunsaturated fatty acids (n-3 PUFAs) for consumption by pregnant and lactating women.[8] Apart from DHA, the use of other natural substances that have the potential to increase PPARγ activity is nutmeg seed extract.

Nutmeg, or *Myristica fragrans*, is a spice plant native to Indonesia with high economic value. This plant has long been used in various foods and has medical benefits.[9] Based on the previous research, NuSE contains an active compound commonly known as macelignan, which has been extensively characterized for its antioxidant and anti-inflammatory properties through the activation of PPAR-γ.[4] Previous research stated that the content of nutmeg seed extract (NuSE) has the potential as a PPARγ agonist that could potentially increase signal modulation at synapses. PPARγ, as a receptor in the nucleus, can increase the process of mitochondrial biogenesis, which causes mitochondrial function also to increase. Mitochondria are involved in synaptic transmission in long-term potentiation that is essential in the learning and memory process.[10] Increased modulation of signals at synapses can increase neuroplasticity, potentially improving the brain's cognitive function, as seen by the amount of *synaptophysin* mRNA expression. *Synaptophysin* is the main integral glycoprotein forming the synaptic vesicle membrane, which delivers neurotransmitters into the synaptic cleft and transmits signals between neurons after fusion with the plasma membrane.[11]

The content of PPARγ agonists has the potential for neuroplasticity. However, until now, there has been no research about the potential neurostimulant impact of the PPAR-γ agonist from NuSE on the gene expression of PGC1α and synaptophysin in the hippocampus. This study explores the use of NuSE on PGC-1α's role as a regulator in the biogenesis of mitochondria and synaptophysin, aiming to elucidate synaptophysin's role as a marker for synaptic plasticity using rat hippocampal tissue.

MATERIALS AND METHODS

This study was conducted in according to the guidelines set by the Animal Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. The treatment procedures for the animals were approved by the Research Ethics Committee of Universitas Padjadjaran. According to our previous protocol, the NuSE in this study was extracted from the Glucopala caplet. Glucopala is a natural patent product obtained from the Faculty of Pharmacy, Universitas Padjadjaran (batch number FP08.A1604.001). The NuSE was dissolved in distilled water containing pulvis gum arabicum immediately before administration.[12]

Male Wistar rats, aged eight weeks, were obtained from Biofarma Laboratories and allowed to acclimate to the research facility for at least seven days before any experimental procedures. The rats were housed in groups of four per cage, maintained under a 12:12-hour light-dark cycle, and provided with a low-stress environment with a temperature of 22°C, 50% humidity, and minimal noise. Food and water were provided ad libitum. The rats were randomly assigned to four groups that consisted of 6 rats each: control group and treatment groups divided into three groups. The treatment groups in this study consisted of the PGA (pulvis gummi arabicum) group, NuSE (nutmeg seed extract) group, and DHA (docosahexanoenoic acid) group. According to a previous journal, PGA or pulvis gum arabicum was used as a control in this study because it is found in distilled water as a solvent for Glucopala caplet.[12] NuSE group receiving 0,945mg/day of NuSE while the DHA group receiving 0,835mg/day via gavage. The treatment period lasted for 12 weeks.

Following a 12-week treatment period, all rats underwent anesthesia using isoflurane at a flow rate sufficient to achieve a 5% or higher concentration. They were then euthanized through cervical translocation. Whole brains were carefully excised, washed with ice-cold phosphate-buffered saline (PBS), and stored at -80ºC until further analysis. The extracted brain samples used in this study are from the hippocampus area. RNA extraction from the hippocampus tissue was performed using 200 µl of TRIzol Reagent (Qiagen). The Transcriptor First Strand cDNA Synthesis kit (Takara Bio) was employed to synthesize cDNA from 500 ng of total RNA, using oligo dT and random primers. For the semi-quantitative Conventional RT-PCR, a reaction mixture was prepared containing 2.5 µl of reverse-transcribed cDNA, 0.5 µM of both sense and antisense primers, 200 µM of dNTPs, and 0.125 µl of Taq polymerase (Roche), resulting in a final volume of 25 µl. The optimal number of PCR cycles was determined to ensure amplification within the linear range. The reverse transcription step lasted 30 minutes at 50°C, followed by an initial activation step of 15 minutes at 95°C. Subsequently, denaturation was performed at 94°C for 40 seconds, followed by an annealing/extension step and repeated cycles specific to the mRNA targets (PGC1α and *synaptophysin*) as indicated in Table 1.[12]

Table 1. Table Primer PCR and Cycles

Name of Primer	Primer Sequence	Product Size Anneling (bp)	Anneling (℃)	Cycle
β-actin	F 5'-TGG AGA AGA TTT GGC ACC A-3' R 5'-CCA GAG GCA TAC AGG GAC AA-3'	193	60	37
Synap-tophysin	F 5'-GTG TAC TTT GAT GCA CCC TC-3' R 5'-TCT GCA GGA AGA TGT AGG TG-3'	177	55	37
PGC-1 alpha	F CGC ACA ACT CAG CAA GTC CTC R CCT TGC TGG CCT CCA AAG TCT C	263	62	37

To normalize the PCR results, the mRNA levels of β-actin were measured as an internal control. The experiments were repeated three times to validate the consistency of the results. All experimental parameters and conditions remained constant throughout the study. The resulting images were saved in TIFF format and subjected to digital image analysis using ImageJ software version 1.4.3u. The relative quantities of RNA from PGC1α and *synaptophysin* were determined by comparing the kinetic amplification of β-actin, which served as an endogenous control.

The collected data were quantified using ImageJ, illustrated in Figure 1A and Figure 2A. After quantification, the expression level of protein expression is then divided towards β-actin level as internal control. The data was analyzed using SPSS V.13. Data analysis was first tested for normality using the Shapiro-Wilk and homogeneity tests using the Levene Statistics tests. Differences in gene expression between these groups were analyzed with a One-Way Analysis of Variance (ANOVA) test showing the mean ± minimum standard error (SEM). A significance level of less than 0.05 ($p < 0.05$) was utilized to determine the statistical significance of the findings in this study.

RESULTS

After giving different treatments, we used semiquantitative PCR to analyze PGC1α and synaptophysin mRNA expression. The unit of protein expression data used in this study is arbitrary. The PGC1α mRNA expression is illustrated in Figure 1A. Compared to the control (1.055 ± 0.088 arbitrary unit), the NuSE group (0.968 ± 0.057) tended to increase PGC1α expression to the control group; however, this difference was not statistically significant, with a p-value of 0.364 ($p>0.05$). Other treatment groups, the PGA group (0.932 ± 0.035) and the DHA group (1.099 ± 0.091) did not increase the PGC1α expression if compared to the control group.

The results for synaptophysin mRNA expression are presented in Figure 1B. Compared to the control (1.050 ± 0.037), the NuSE group (1.238 ± 0.024) tended to increase synaptophysin expression and was statistically significant with a p-value of 0.017 ($p<0.05$) (Figure 2B). The DHA group (1.050 ± 0.037) also showed an increase significantly compared to the control group. Compared to the control, PGA (1.192 ± 0.065) did not increase the expression of PGC1α expression if compared to the control group.

DISCUSSION

Nutmeg is an indigenous plant originating from Indonesia with high economic value. It has been used extensively as a culinary and medicinal ingredient throughout history.[9] Prior investigations have indicated that nutmeg contains compounds acting as agonists for the PPARγ receptor, potentially enhancing synaptic signal modulation.[4,13] PPARγ within the cell nucleus promotes mitochondrial biogenesis, increasing mitochondrial functionality.[6] Mitochondria are involved in synaptic transmission during long-term potentiation, a vital process underlying learning and memory.[7] This study explored the effect of NuSE on PGC-1a, a marker regulator of mitochondrial biogenesis, compared with docosahexaenoic acid.

Based on the previous research, macelignan as an active compound in NuSE that has a natural ligand of PPAR γ agonist assumed contribution in increasing PGC-1α in the biogenesis of mitochondria and synaptophysin as a marker for synaptic plasticity.[4] Macelignan exhibits pharmacological activities such as antibacterial, anti-inflammatory, anticancer, and neuroprotective effects.[12] This study yielded no significant differences between the control and treatment groups administered PGA, NuSE, and DHA. The extract used in this research excluded myristicin and safrole from nutmeg seeds due to their hallucinogenic and hepatotoxic properties, respectively.[13] Phytochemical analysis revealed the presence of various secondary metabolites, including polyphenols, flavonoids, tannins, quinones, monoterpenoids, and sesquiterpenoids.[14] Other secondary metabolites may influence the outcomes of this study in the treatment group. Other research studies have reported that tannins can downregulate the mRNA expression of transcription factors in PPARγ.[15,16] Another study utilizing plants with tea leaves' phenols, polyphenols, and tannins demonstrated the ability to reduce PGC1α mRNA levels.[17] The presence of other compounds apart from macelignan in this research could potentially affect the study results.

Macelignan not only impacts the increase in PPARγ activity due to its dual agonist nature for PPARα and PPARγ.[13] Another study suggests that macelignan demonstrated a higher level of effectiveness as an agonist for PPARα when compared to PPARγ. PPARα plays a critical role in regulating the synthesis of neuroprotective proteins and controlling synaptic signaling.[18] In addition to PPARα, another study indicates that nutmeg significantly reduces acetylcholinesterase activity, which affects the cholinergic pathways that play a prominent role in learning and memory processes.[19]

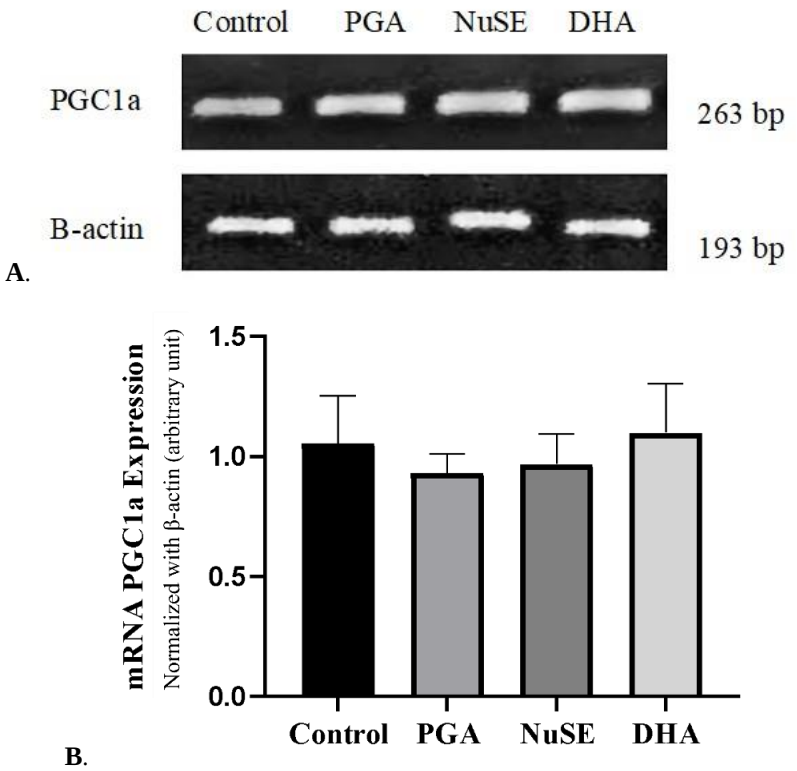

Figure 1. PGC 1 alpha mRNA expression, (A) mRNA band visualization, (B) quantification of mRNA band density (normalized with b actin). Data were presented as Mean ± SEM

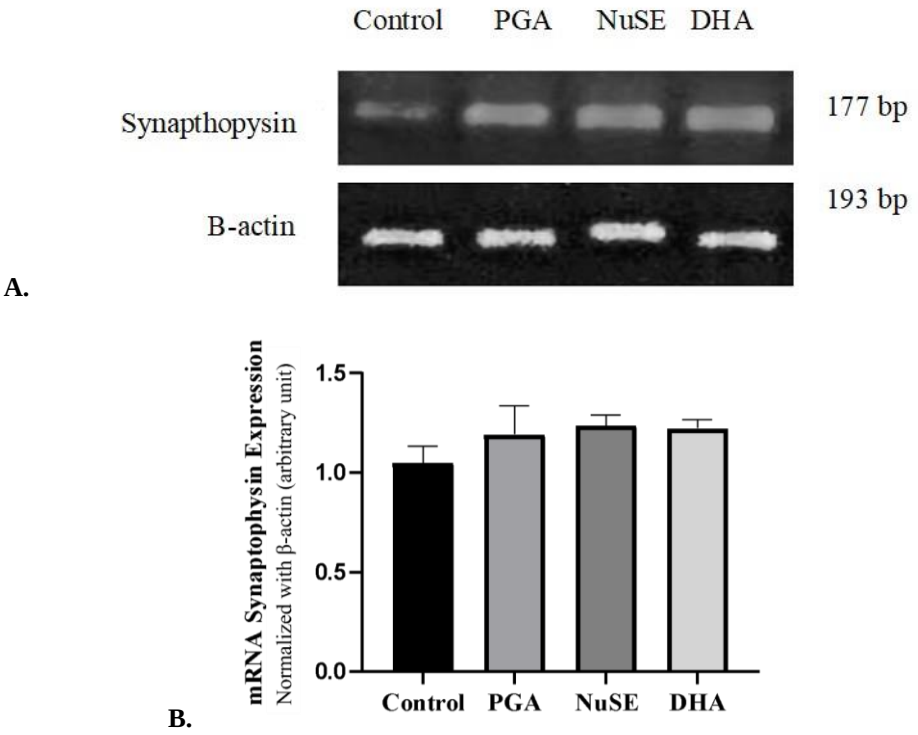

Figure 2. Synaptophysin mRNA expression, (A) Synaptophysin mRNA band visualization, (B) quantification of Synaptophysin mRNA band density (normalized with b actin). Data were presented as Mean ± SEM

Augmented signal modulation at synapses can enhance neuroplasticity, thereby improving cognitive function within the brain, as evidenced by an upregulation in *synaptophysin* mRNA expression.[11,20] *Synaptophysin*, a key integral glycoprotein composing the synaptic vesicle membrane, facilitates the release of neurotransmitters into the synaptic cleft, enabling signal transmission between neurons following fusion with the plasma membrane.[20] In prior studies, *synaptophysin* has previously served as a biomarker for evaluating

synaptogenesis in cultured hippocampal neurons.[21] A previous study reported increased *synaptophysin* expression in younger rats aged 12 weeks that were administered nutmeg seed extract.[12] We observed a similar trend in this study, where *synaptophysin* expression increased statistically significantly between the nutmeg and control groups. Statistically, the increase in DHA groups also significant compared to the control group. An increase in the DHA group also accompanied the increase in *synaptophysin* expression in the nutmeg group. This study is similar to the previous study in that DHA potentially affects *synaptophysin* expression to raised.[22]

Synaptogenesis in brain plasticity can occur through the PPARγ-PGC1α pathways and other pathways, such as the MAPK/Erk pathway. This pathway leads to the transcription of protein factors, including cAMP response element-binding protein (CREB), Myc, and ribosomal S6 kinase (RSK)[23]. Another study has reported that *Myristica fragrans* have diaryl butane-type lignan which activates the AMPK pathways.[24] Conversely, another natural substance that can enhance *synaptophysin* expression is *Centella asiatica*, which contains triterpenoids and flavonoids that can increase mitochondrial expression by upregulating NRF2 expression.[25] This study is similar to another study that stated DHA potentially activates the NRF2 pathway to upregulate brain plasticity.[26] Quercetin, the most common compound found in medicinal plants such as in NuSE, positively influenced the expression of the hippocampal FoxG1/CREB/BDNF signaling pathway. BDNF regulates phosphorylation and CREB activation, which increases sympathetic neuron survival and is an essential factor in short-term and long-term memory.[27] DHA also exhibits an enhancing effect on the CREB pathway and CREB-regulated genes. The activation of CREB has been associated with promoting of neuronal plasticity and playing a crucial role in neuronal development. BDNF, which plays a crucial role in modulating neuronal plasticity within the hippocampus, is considered one of the essential regulatory genes influenced by CREB.[28] NuSE may affect many synaptogenesis pathways that increase *synaptophysin* expression, which needs more research.

Certain limitations in this study should be considered. First, PGC1α expression is regulated by PPARγ, but since macelignan has a dual agonist effect on PPARα/γ, this study did not observe the effect on PPARα. Second, this study only examined neurons in the hippocampus that needed further studies that might have implications in another region. Third, the duration used in this study is only 12 weeks; further studies are needed to reveal long-term effect of nutmeg if compared to control and DHA.

This study concludes that NuSE increased *synaptophysin* mRNA expressions compared to the control group in significance statistics but did not increase PGC1α expression significantly. The duration, concentration of the treatment groups, other compounds contained in NuSE, or the age of the rats may contribute to this result. Further studies are needed to search for another pathway besides PPARγ that may affect neuroplasticity in NuSE.

CONCLUSION

This study concludes that NuSE increased *synaptophysin* mRNA expressions compared to the control group in significance statistics but did not increase PGC1α expression significantly. The duration, concentration of the treatment groups, other compounds contained in NuSE, or the age of the rats may contribute to this result. Further studies are needed to search for another pathway besides PPARγ that may affect neuroplasticity in NuSE.

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