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Immunostimulant Effects of Bitter Leaves (*Gymnanthemum amygdalinum* (Del.) Sch.Bip.ex Walp.) Leaf Extract

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ABSTRACT

In Indonesia, the widespread impact of COVID-19 has led to an increased interest in exploring natural immunostimulants as complementary therapies. One promising candidate is Gymnanthemum amygdalinum, commonly known as bitter leaf. Bitter leaf is a medicinal plant that can be used as an immunostimulant because it contains flavonoid compounds. This study aims to investigate the immunostimulatory effects of Bitter leaf extract obtained using the MAE method, particularly in enhancing immune function and the effective dosage in rats. The ethanolic extract of bitter leaf was made using the microwave-assisted extraction (MAE) method, which can potentially extract high levels of flavonoid compounds compared to other extraction methods in vivo. There were as many as 25 rats, which were divided into five treatment groups: the negative control was treated with CMC Na 0,5% (w/v); the positive control was treated with 4,5 mg/200 g body weight (BW) of Echinacea purpurea. The treatment groups from Dose 1; Dose 2; and Dose 3 were treated with an ethanol extract of bitter leaf at 14 mg/200 g BW, 28 mg/200 g BW, and 56 mg/200 g BW. Immunostimulant effect testing was carried out using the carbon clearance method, and the number of leukocytes was counted. In carbon clearance testing, each group was given a carbon solution intravenously; blood was taken at 5, 10, and 15 minutes via the tail vein, and its absorbance was measured using UV-Vis 675 nm. The count of leukocytes was observed using the chamber of a hemocytometer through a microscope. The research results showed that administration of doses I, II, and III provided an immunostimulant effect. Based on the results, the ethanolic extract from a bitter leaf from Dose III (56 mg/200 g BW) provided a stronger immunostimulant effect in rats based on the phagocytosis index parameters of (9.005) and the number of leukocytes of (8,370/µL).

***Keywords*:** Gymnanthemum; Immunostimulants; MAE; Rats; In vivo

INTRODUCTION

The COVID-19 pandemic has presented unprecedented challenges to global health systems, emphasizing the urgent need for effective strategies to enhance immune function and mitigate viral infections. In Indonesia, the widespread impact of COVID-19 has led to an increased interest in exploring natural immunostimulants as complementary therapies. One promising candidate is *Gymnanthemum amygdalinum*, commonly known as African leaf or bitter leaf, a plant renowned for its medicinal properties in traditional African medicine. Increasing immunological function is one strategy to combat COVID.[1] Immunostimulants are part of immunomodulators that have a

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mechanism to correct an abnormal immune system by increasing the number of leukocytes, both specific immune cells such as lymphocytes and non-specific immune cells such as neutrophils.[2,3]

Gymnanthemum amygdalinum is rich in bioactive compounds such as flavonoids, alkaloids, saponins, and tannins, which have been shown to possess various pharmacological activities, analgesic,[4] anti-inflammatory,[5] anticancer, prevention of heart disease, antihypertensive,[6] reduce cholesterol, prevent stroke, regulate blood sugar,[7] reduce the digestive disorders, and the weight,[8] including immunomodulatory effects. The results of research from Tambusai (2018),[9] show that the ethanol extract from African leaves has immunostimulant activity in mice by increasing the immune system at doses of 100, 200, and 400 mg/kgBB.

These compounds can enhance both innate and adaptive immune responses, making them potential candidates for boosting immune function during viral infections like COVID-19. The effectiveness of these bioactive compounds, however, is significantly influenced by the extraction method used one of which is to potentially stimulate the body's immune system as a candidate for herbal medicine.[9,10]

Microwave-assisted extraction (MAE) has emerged as an innovative and efficient technique for extracting bioactive compounds from plant materials. MAE offers several advantages over conventional extraction methods, including reduced extraction time, lower solvent consumption, and higher yields of target compounds. By utilizing microwave energy, MAE facilitates the rapid and efficient release of bioactive compounds, which could enhance the immunostimulatory properties of *Gymnanthemum amygdalinum*.[11]

This study aims to investigate the immunostimulatory effects of Bitter leaf extract obtained using the MAE method, particularly in enhancing immune function. By optimizing the MAE conditions to maximize the yield of immunostimulatory compounds, this research seeks to provide valuable insights into the potential of *Gymnanthemum amygdalinum* as a natural immunostimulant.

Recent studies have underscored the potential health benefits of Vernonia amygdalina. For instance, Abiola et al. (2023) highlighted its anti-inflammatory and immune-boosting effects[12], while Habtamu and Melaku (2018) reported on its antioxidant and antimicrobial properties.[13] Additionally, Zhang et al (2019) demonstrated the effectiveness of MAE in optimizing the extraction of bioactive compounds from medicinal plants.[14] The immunostimulatory ability of bitter leaf extract can be further investigated with the support of these investigations, especially in light of the COVID-19 post-pandemic and its potential to boost immunity.

This research aims to build on these findings by specifically focusing on the immunostimulatory effects of Bitter leaf extract obtained through MAE. By optimizing the extraction process and evaluating the immunomodulatory effects, this study will use the carbon clearance method and parameters for leukocyte count to perform an immunostimulant test on male white rats. The goal of this research is to further the creation of natural immunostimulants that can maintain immune function in the event of viral pandemics like COVID-19.

METHODS

Study Design

This research was conducted at the Pharmacology and Pharmacognosy Laboratory, Pharmacy Study Program, Pakuan University Bogor in September-November 2021.

Materials, Chemicals, and Reagents

The fresh Bitter leaf that was utilized came from Bogor's Cariu village, West Java. The laboratory glass tools (Pyrek®), aluminum foil, mesh panels 40, maseration bejana, glass cylinders (PYREK®), dryers

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(Thermo®), grinders, scissors, hemocytometers, filament paper, crusher (Tyreck®), measuring pumpkin (Tyrex®), microscopes, object glass, Oven (Memmert®), micro pipettes, drop pipettes (TYREX®), UV-VIS spectroscopic photometry (THERMO®), injection sputters, probe needles, stopwatch (Xiaomi®), eppendorf tubes, reaction tubes (Thyrex ®), tube reactions (Typrek ®), irrigation (Vulkan®), and drinking place The following materials were used in this study: pellets, male white rats strain sparague Dawley (SD), absolute methanol, emersial oils, physiological NaCl 0.9%, wood powder, product from Phytopharmaca Echinacea purpurea, Pelican B-17 brand Chinese ink, aquades, acetate acid, CMC Na.0,5%, Acid Dipotassium (K2EDTA), Ferric chloride (FeCl3) 3%, H2SO4, gelatin 10%, gelatin 1%, chloroform, methanol, sodium chloride (NaCl) 10%, sodium carboxymethyl cellulose, Bouchardat's reagent, Dragendorff's reagent, Wagner's reagent, Turk's reagent, magnesium powder, ethanol 96%, and Giemsa.

Sample Preparation

Preparing simplicia

After being well cleaned and cut, the wet Bitter leaf is dried in an oven set to approximately 400°C. They are then rinsed under running water. Dry simplicia separated from dirt that might have been polluted during the drying process or leaves that were rubbed. The powder simplicia is then obtained by smoothing the dried simplicia using a grinder and in the foot using a 40 mesh design. To prevent contamination, simplicia powder is kept in a sterile container that is securely closed.

Extraction

Following the preparation of the simplicia powder, eight Erlenmeyers were filled with 25 g of the powder and 250 mL of ethanol. The Erlenmeyers were then placed in an 800-watt microwave for six minutes. The sample is periodically microwave-irradiated for one minute on and two minutes off to prevent the temperature from rising to 800°C, which could damage the target compound. The macerate is then combined and concentrated using a rotary evaporator at a temperature of no more than 400°C until a thick extract is obtained. After the extraction findings were filtered and allowed to come to room temperature, the yield of the thick extract was calculated.

Phytochemical Screening

The confirmatory qualitative phytochemical screening of plant extract was performed to identify the main classes of compounds (flavonoids, saponins, alkaloids, and tannins) present in the extract following standard protocols.[15]

Test for Flavonoids

In 5 mL of 96% ethanol, 0.5 g of the material is dissolved. Next, a little amount of 0.2 mg of magnesium powder is added to 2 mL of the sample solution. Afterward, the solution is gently agitated and 10 drops of strong hydrochloric acid are added to the tube's side.

Test for Saponins

A reaction tube should be filled with 0.5 g of the sample that has been weighed. After adding 10 milliliters of hot water, let it cool, then give it a good shake for ten seconds. The creation of steady foam that lasts for at least a minute indicates a successful outcome. After dissolving the sample in warm water, give it a good 10 seconds of vigorous shaking. When hydrochloric acid is added, a successful outcome will yield steady foam.

Test for Alkaloids

After dissolving a sample of 0.5 g extract in 2N H2SO4, the extract is examined using the Dragendorff and Mayer alkaloid reagents. If the Dragendorff reagent produces a reddish-orange precipitate and the Mayer reagent produces a white precipitate, the experiment is considered successful.

Test for Tannins

Five milliliters of hot distilled water are used to dissolve a sample of around 200 mg of each Bitter leaf extract, which is then agitated. The mixture is cooled,

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centrifuged, and filtered after being exposed to a 10% sodium chloride solution. Next, one milliliter of the filtrate is treated as follows: a) After adding 3 mL of a 10% gelatin solution, the precipitate's existence is seen. b) After adding 3 milliliters of a 3% ferric chloride solution, any color shift to blue-black or green-brown is noted. c. After adding 3 milliliters of sodium chloride and gelatin (gelatin solution 15 in 10% sodium chloride), any precipitate should be visible.

Immunostimulant test

Acclimatization of experimental animals

All mice were adapted to the environment for 7 days. Experimental animals were only given food and water every day.

Carbon Clearance method

Making Carbon Solution

An immunostimulant activity test was carried out using the Carbon clearance method, 1.6 mL of Chinese pelican B-17 ink in 25 mL of 0.9% physiological NaCl solution.[16]

Sample Preparation

A total of five groups of rats, each with five animals, were used in this experiment. Oral administration of the test preparation was administered once daily for six days to every experimental animal. Rats were given 0.1 mL of carbon solution via the tail vein on the seventh day. Blood was then drawn at five and fifteen minutes using a 50 µl capillary tube per mouse, and the absorption was recorded using UV-Vis at a wavelength of 675 nm. The rats were lysed with 4 mL of 1% sodium carbonate. Fourteen Utilizing the following formula, the phagocytosis index (Ph), the phagocytosis constant (K), and the blood carbon half-life (t ½) were determined:

$$K = \frac{\text{In OD1} - \text{In OD2}}{t2 - t1}$$

Note:
K: Phagocytosis constant
In OD1: 5th minute absorption
In OD2: 15th minute absorption
t1: Starting time (at the minutes to 5)
t2: End time (at the minutes to 10)

$$t_{1/2} = \frac{0{,}693}{k}$$

Note:
t ½: Carbon elimination half-life
K: Phagocytosis constant

$$IF = \frac{\text{Phagocytosis Constant of Extract}}{\textit{Phagocytosis Constant of Negative Control}}$$

Note:
IF = Phagocytosis Index

Leukocyte Count

0.5 mL of blood was added with 10% K2EDTA. A total of 10 µL of blood was added with 10 µL of Turk's reagent, put into an Eppendorf tube, and shaken for 3 minutes. Then the solution is dropped into the hemocytometer counting chamber. Leave it for 2 minutes for the leukocytes to settle, then the leukocytes are counted using a microscope.[17]

$$SDP = NI \times WP \times 2{,}5$$

Note:
∑SDP: Number of white blood cells
WP: Number of white blood cells in 4 boxes
NI: Amount of dilution

Data Analysis

Data analysis used analysis of variance was the statistical test that was employed, with a 95% confidence level ($\alpha < 0.05$) and a completely randomized design. The SPSS 24.0 for Windows software was used to process research data.

RESULTS AND DISCUSSION

This study aims to investigate the immunostimulatory effects of Bitter leaf extract obtained using the MAE method, particularly in enhancing immune function and determine the effective dose of Bitter leaf in rats. Collecting 11.5 kg of fresh Bitter leaf raw materials picked from Cariu village, Bogor, drying the Bitter leaf plant using an oven at 40°C. The temperature used is a low temperature because it is feared that the flavonoid compounds in the

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leaves will be lost in the heating process a high temperature is used because the bioactive components can undergo structural changes and produce low extract quality.[18]

The MAE method, which uses a wave energy of 2450 MHz and energy generated by 800 watts, is used to remove bitter leaves. The process of microwave extraction starts as soon as the solvent gets inside the substance. According to Alaman et al (2023), the fundamental idea of MAE extraction is that heat is channeled evenly throughout the entire material or that heat is discharged volumetrically in the irradiation medium because the material vibrates during the microwave, resulting in a uniform distribution of heat discharge throughout the material. The MAE extraction method helps break down cell walls to extract flavonoid chemicals more effectively and has the advantages of a faster extraction time extension, less solvent usage, and more appropriate extraction outcomes.[19] This process allows us to generate large amounts of flavonoids. Table 1 presents the findings from the phytochemical screening.

Table 1. Phytochemical Screening Result of Bitter Leaf Extract

Metabolites	Powder	Extract
Flavonoid	+	+
Saponin	+	+
Alkaloid	+	+
Tannin	+	+

Phytochemical tests are conducted to determine the group of compounds contained in African leaf ethanol extract qualitatively and are a specific parameter of an extract of a simplicia. Bitter leaf ethanol extract is known to contain flavonoid components, alkaloids, saponins, and tannins based on the data acquired. The outcomes are in line with the findings of phytochemical assays conducted in the Tambusai (2018)[9], Setiani (2022)[6], and Bharathamma (2024)[20] which found flavonoids, saponins, alkaloids, and tannins in powder and Bitter leaf that were extracted using ethanol.

The study employed 25 male white rats with an average body weight of 263.84 g as experimental animals. When test preparations were given to male white rats, CMC was used as the negative control. Na 0.5% suspension and a favorable control The product suspension of Echinacea purpurea from Phytopharmaca comes in three doses: dose I is 14 mg/200 g BW, dose II is 28 mg/200 g BB, and dose III is 56 mg/200 g BB. For six days, the test preparation was administered orally to each experimental animal once a day.

In this study, carbon was administered intravenously as an antigen to test the phagocytosis ability through a process known as carbon clearance. Chinese ink is utilized as carbon because of its smaller particle size, which prevents blood vessel obstructions. Leukocyte cells' process of phagocytosis causes the blood's carbon content to drop with time.[21] At five and fifteen minutes, the speed of carbon clearing is visible. With a UV-Vis Spectrophotometer that has a wavelength of 675 nm, the blood's carbon absorption value was measured.

Table 2. Average Absorbance Value

Treatment Group	Absorbance	
	Minute to 5	Minute to 15
Negative Control	0,9324	0,8886 [a]
Positive Control	1,4386	0,9862 [b.c]
Dose I	1,3564	1,195 [c]
Dose II	1,2926	1,119 [b.c]
Dose III	1,2966	0,9122 [b]

Note: Numbers followed by the same superscript letters a, b, c in different columns indicate there is no real difference

Table 2 shows that the results of each treatment vary noticeably from one another. A drop in carbon levels can be used to show how the body responds immunologically to foreign items entering the body. According to statistical testing, there is a significant difference in the effect of the positive control (Phytopharmaca product Echinacea purpurea 4.5 mg/200g

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BW) and the negative control (CMC Na 0.5%). The positive control, the phytopharmaca product Echinacea purpurea (4.5 mg/200g BW), did not significantly differ from dosage I (14 mg/200g BW), dose II (28 mg/200g BW), and dose III (56 mg/200g BW) of the bitter leaf ethanol extract suspension. According to these findings, dosages I, II, and III of the Bitter leaf ethanol extract suspension all show an immunostimulant effect. The absorbance results can be used to derive the phagocytosis constant value for each treatment, which is a measure of the phagocytosis's speed. The blood's carbon velocity value increases with a higher phagocytosis constant value, indicating a faster rate of phagocytic cell phagocytosis.[22,23]

Table 3. Average Value of Phagocytosis Constant

Treatment group	Phagocytosis Constant
Negative Control	0,00478
Positive Control	0,04504
Dose I	0,01614
Dose II	0,01732
Dose III	0,03884

According to Table 3, the positive control has the greatest phagocytosis constant value, which is followed by the African leaf ethanol extract doses I, II, and III. The results of the Phagocytosis constant can be used to calculate the half-life of each therapy. A metric used to describe how long it takes for the drug's level in plasma to cut in half during the elimination phase is called the half-life value.

Table 4. Average Value of Half-Life

Groups	Half-Life (Minutes)
Negative Control	158,67
Positive Control	15,65
Dose I	44,75
Dose II	42,04
Dose III	19,09

The quickest half-life values for immunostimulants are displayed in Table 4. An improvement in the capacity for phagocytosis is shown by a reduction in the blood's carbon elimination time. According to the data, the Bitter leaf ethanol extract suspension dose III had the shortest half-life value when compared to dose I and dose II, indicating that dose III had a very high phagocytosis process enhancement.

The use of exploration of medicinal plants has been extensive in Indonesia for a wide variety of diseases with different mechanisms of work, one of them in diseases associated with the immune system.[24] The immunostimulatory effect of the ethanol extract from Bitter leaf can be seen from the phagocyte index value obtained. A phagocytosis index >1 indicates the ability of Bitter leaf ethanol extract as an immunostimulant, an increase in the phagocytosis index indicates an improved immune system. The average value of the phagocytosis index shows phagocytic activity towards carbon particles as antigens. Figure 1 displays the average phagocytosis index results. Apart from being able to maintain the body's immune system as a preventive measure, immunostimulants are also able to work together to defend the body's balance when attacked by an infection and have a faster healing period.

Target cells for immunostimulatory activity include T and B lymphocytes, macrophages, granulocytes, and others. Where in macrophage cells various factors influence the release of the immune response, namely cytokine production, increased lysosomal enzymes, the release of nitric oxide, interleukins, and TNF-α.[25]

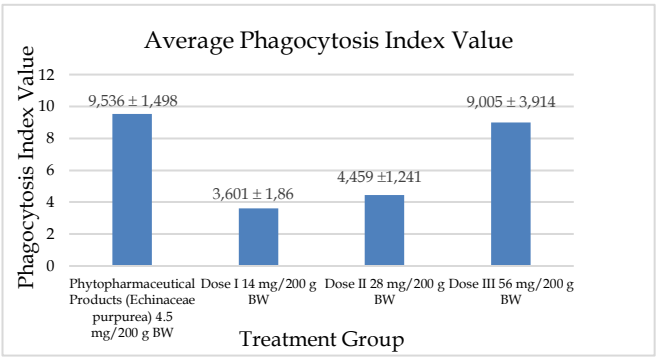

Figure 1. The average graph of the phagocytosis index

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These compounds are known for their antioxidant/anti-inflammatory capacity, and insulin-sensitizing/antihyperglycemic effects might be reduced to delay the metabolism syndrome progression.[26]

Given that the positive control, dose I, dose II, and dose III have phagocytosis index values greater than 1, Figure 1 illustrates this effect of immunostimulant medication on immune response. The phagocytosis index's average value indicates the phagocytic activity of phagocytic cells against carbon particles as antigens as a result of the injection of an ethanol extract derived from Bitter leaf. The Bitter leaf ethanol extract's phagocytosis index at doses I, II, and III demonstrates a correlation between dose increases and phagocytosis index values, i.e., a higher phagocytosis index value corresponds to a bigger concentration increase. Increased non-specific immunity and macrophage phagocytosis are indicated by a high phagocytosis index value.

Dose III has a stronger immunostimulant effect, this is because in dose III the ethanol extract of Bitter leaf contains more flavonoid secondary metabolites which play a role in increasing the phagocytosis index. According to Alara et al. (2019) stated that the higher phenolic compounds responsible for natural antioxidant could be obtained from Bitter leaf using the microwave-assisted extraction technique, which may possibly be utilized as a therapeutical source for managing several diseases.[27] The mechanism of action of flavonoids is activating Natural Killer (NK) cells to stimulate the production of IFN γ (Interferon-γ) which can activate macrophages and stimulate increased phagocytic activity.[28]

Statistical analysis revealed that there is no discernible difference between the three doses of bitter leaf ethanol extract (14 mg/200 g BW, 28 mg/200 g BW, and 56 mg/200 g BW). Echinacea purpurea, 4.5 mg/200 g BW, a phytopharmaca, was used as a positive control ingredient. The chemical composition of the supplement Echinacea purpurea used for immunostimulant effects consists of glycoproteins, alkylamides, and polysaccharides. A variety of physiological functions, including immunity, are mediated by glycoproteins, which are chains of proteins and carbohydrates. Research has demonstrated the immunomodulatory and high bioavailability properties of alkylamides, a chemical molecule found in the genus Echinacea.[29]

The purpose of this test is to evaluate the effect of ethanol extract from bitter leaf on the hematopoietic system, which influences immunity. Rats administered with three different doses of Bitter leaf ethanol extract (dose I: 14 mg/200 g BW, dose II: 28 mg/200 g BW, and dose III: 56 mg/200 g BW) and the phytopharmaca product Echinacea purpurea product (4.5 mg/200 g BW) demonstrated an increase in leukocytes, suggesting that the rats have immunity that appears to fight antigens in the form of carbon injections, with the ability to increase the number of white blood cells.[29,30] Table 5 displays, after six consecutive days of therapy, the average number of blood leukocytes in rats receiving negative control, positive control, dosage I, dose II, and dose III.

Table 5. Average Value of Leukocyte Count

Groups	**Leukocyte Count** (µL)
Negative Control	6.250 ± 584,33 [a]
Positive Control	8.580 ± 426,58 [c]
Dose I	6.760 ± 250,85 [a,b]
Dose II	7.300 ± 281,73 [b]
Dose III	8.370 ± 498,87 [c]

In Table 5 it can be seen that dose III and the positive control have higher leukocyte values compared to the other groups, where the leukocyte count for dose III is 8,370/µL and the positive control is 8,580/µL, the percentage difference between dose III and the positive control is equal to 2.4%, this is because at dose III of Bitter leaf ethanol extract contains more flavonoids. The mechanism of action of flavonoids is that they can increase IL-6 levels which can stimulate hepatocytes to

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produce acute phase proteins together with CSF and stimulate progenitor cells to produce.[31]

Bitter leaves have a variety of contents that play an important role in modulating the immune system in bacterial infections. From the results of research, it is known ethanol extracts from Bitter leaf has the ability to modulate the levels of IL-6 and IL-10 as well as the number of bacteria in male Wistar rats infected with Staphylococcus aureus. The remaining components of the ethanol extract from bitter leaf, such as the terpenoids andrographolide, may have a molecular role in the decrease of IL-6. Andrographolide also contributes to the reduction of IL-6 by reducing the production of oxygen radicals by neutrophils, regulating iNOS, and blocking NFκB activation. Additionally, one of the bitter leaf's ingredients, tannin, has the capacity to suppress NFκB at p65 and its catalytic activity as well as reduce intracellular kinase phosphorylation. As a result, these activities may lower IL-6 levels.[32]

The results of the ANOVA test showed $p < 0.05$, where the number of leukocytes between groups had a significant difference. The statistical analysis revealed that there was no significant difference between dosage I (14 mg/200g BW) and dose II (28 mg/200g BW) or between dose III (56 mg/200g BW) and the positive control (Phytopharmaceutical product *Echinacea purpurea*). The immunostimulant effect of Dose III is about the same as that of the positive control, Phytopharmaca Product *Echinacea purpurea*.

CONCLUSION

In conclusion, Bitter leaves Ethanol Extract has an immunostimulating effect characterized by a phagocytosis index value > 1 at each dosage treatment. Dose III (56 mg/ 200g BB) has a stronger immuno-stimulant effect based on the phagocytotic index parameter of (9,005) and the number of leukocytes (8,370/µL).

Conflict of Interest

All authors declare no conflict of interest in this manuscript.

Authors' Declaration

LAS contributed to developing the idea, exploring the data, analyzing the data, guiding the research, and writing the article. IYW and NAR contributed to analyzing the data and writing the article. SAF contributed to conducting research, exploring data, analyzing data, and writing this article. All authors have approved the final published manuscript.

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Ethical approval

The research protocol was approved by the Local Ethical Animal Committee of FMIPA Pakuan University with number 017/KEPHP-UNPAK/07-2021.

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