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Effects of Fermented *Crescentia cujete* and Enrofloxacin on Inflammation in Pneumonic Pasteurellosis Rats

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ABSTRACT

Pneumonic pasteurellosis (PP) is a respiratory disease caused by *Pasteurella multocida* (*P. multocida*) with broad host susceptibility and zoonotic potential. Pasteurellosis is classified as a strategic infectious animal disease and a neglected tropical disease in Indonesia, highlighting the need for effective supportive therapies. Fermented *Crescentia cujete* L. (FCC) contains bioactive compounds with anti-inflammatory and antioxidant activities. The present study aimed to evaluate the anti-inflammatory effects of FCC combined with enrofloxacin on hematological parameters and the immune expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) in an induced PP rat model. Twenty male Sprague-Dawley rats, three months old and weighing 250-300 g, were divided into five groups, including healthy control (P1), untreated infected rats (P2), rats treated with 20 mg/kg body weight (BW) of enrofloxacin (P3), rats given 20 and 30 mg/kg BW of enrofloxacin and ibuprofen, respectively (P4), and rats administered 20 and 5.92 mg/kg BW of enrofloxacin and FCC (P5), respectively. After a 7-day acclimatization, rats were infected intratracheally with *P. multocida* on day 8, and treatments were administered orally for seven days starting on day 11. Hemoglobin, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) decreased significantly in P2 compared to P1, with no difference between P2 and P3. Although hemoglobin, MCH, and MCHC improved in P4 and P5 toward control levels, most variables remained significantly different from P1. Leukocyte and neutrophil counts were significantly elevated in P2 compared to P1; however, P5 demonstrated a declining trend toward normal values and did not differ significantly from P1. Pulmonary GM-CSF and IL-6 immune expression increased markedly in P2 compared to P1. Groups P3 and P4 exhibited elevated cytokine expression compared to P1, without significant differences from P2. In contrast, P5 indicated a significant reduction in GM-CSF and IL-6 expression compared to P2, reaching levels comparable to those of P1. The FCC combined with enrofloxacin improved hematological status and modulated inflammatory cytokines in PP. The present findings indicated that FCC could be a potential supplementary treatment for managing inflammation and aiding hematological recovery in bacterial infection pneumonia.

Keywords: *Crescentia cujete*, Granulocyte Macrophage-Colony Stimulating Factor, Hematology, Interleukin-6, *Pasteurella multocida*

INTRODUCTION

Pneumonic pasteurellosis (PP) is pneumonia caused by *Pasteurella multocida* (*P. multocida*), a Gram-negative facultative anaerobic bacterium (Wilson and Ho, 2013). Pneumonic pasteurellosis can infect different animals and livestock, causing fowl cholera and lower respiratory tract infections in cattle and pigs, which are potentially zoonotic. Transmission to humans occurs through bites or scratches from cats or dogs (Guan et al., 2020). In Indonesia, Pasteurellosis is classified as a strategic animal infectious disease under the Ministry of Agriculture Regulation No. 4026/Kpts/OT. 140/4/2013a and is also listed as a neglected tropical disease (Ministry of Health of the Republic of Indonesia, 2020).

Pasteurellosis targets erythrocytes and leukocytes, particularly monocytes, neutrophils, and macrophages (Kubatzky, 2012), leading to excessive inflammation and tissue damage (Yang et al., 2022). Pneumonic pasteurellosis increases expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the type II pneumocyte, macrophages, and granulocyte cells, triggering inflammation (Wilkie et al., 2012). Inflammation is further characterized by the release of pro-inflammatory cytokines, specifically interleukin-6 (IL-6; Aliyu et al., 2022). Elevated IL-6 expression can exacerbate pneumonia (Yang et al., 2022).

The World Health Organization recommended treating PP with antibiotics combined with anti-inflammatory medicines such as ibuprofen, particularly for acute respiratory syndromes associated with severe inflammation and pneumonia (WHO, 2022). However, these combinations have side effects such as antibiotic resistance (Bindu et al., 2020), oxidative stress (Maigoda, 2021), impaired bacterial elimination (Peternel, 2013), lung tissue damage, leading to

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the development of infections (Abbasifard and Khorramdelazad, 2020), and increased risk of hospitalization costs (MacLeod et al., 2021). The mentioned limitations highlighted the need for alternative therapies. Alternative treatments, such as herbal remedies, are necessary to reduce the side effects of medicines.

One herbal alternative is the berenuk fruit (calabash, *Crescentia cujete L.*). The calabash fruit is widely recognized as a medicinal plant with different uses, including anti-inflammatory and antioxidant properties (Gonzales et al., 2023). Calabash contains alkaloids, flavonoids, saponins, tannins, and polyphenols (Atmodjo, 2019). One procedure to increase the bioactive compounds in herbs is fermentation. The fermented *Crescentia cujete* (FCC) contains several bioactive compounds, including antioxidants, choline, phytonadione, alpha-tocopherol, and retinol (Prakoso et al., 2024). Therefore, the present study aimed to analyze the anti-inflammatory effects of FCC on hematology and immune expression of GM-CSF and IL-6 in the lungs of induced PP rats.

MATERIALS AND METHODS

Ethical approval

The present study was approved by the Ethical Committee for Research on Animals, Faculty of Dentistry, University Airlangga, Surabaya, Indonesia, with the ethical approval registration number of 0511/HRECC.FODM/IV/2025.

Herbal preparation

Crescentia cujete fruit was obtained from the garden of Wijaya Kusuma University, Surabaya, Indonesia. The fruit was washed, cleaned, and peeled. The fermentation of calabash fruit was carried out using a mixture of water, fruit, sugar, and pectinase (Pectinex Ultra AF-P, Novozymes, London, England). The components were combined at a ratio of 1,000 mL of water, 400 grams of fruit pulp, 40 grams of sugar, and 40 mL of pectinase. The mixture was stored at 25°C for 30 days, stirred manually every 24 hours, and, after fermentation was complete, stored at 4°C (Wilujeng et al., 2023).

Study design and animals

A total of 20 male Sprague-Dawley rats, 3 months old and weighing 250-300 g, were randomly divided into five groups, with four rats per group. The treatment groups included control healthy rats (P1) and infected rats with PP, which did not receive any treatments (P2). The third group included the PP rats that were treated with 20 mg/kg body weight (BW) of enrofloxacin (P3), the fourth group consisted of PP rats that were given 20 mg/kg BW of enrofloxacin and 30 mg/kg BW of ibuprofen (P4), and the fifth group was the PP rats that received 20 mg/kg BW of enrofloxacin and 5.92 mg/kg BW of FCC (P5; Prakoso et al., 2024). The rats were acclimated to the environment for seven days at 20-30°C, with a light cycle, and were fed BR-1 (Japfa Comfeed, Indonesia) food and water *ad libitum*. *Pasteurella multocida* was induced intratracheally on day eight post-adaptation (PA). Treatment was administered orally using an oral tube from days 11 to 18 PA.

Infection induced by *Pasteurella multocida*

The *P. multocida* used was derived from a collection of clinical isolates kept at the Laboratory of Microbiology, Faculty of Health, University of Muhammadiyah Sidoarjo, Indonesia. Induction using a *P. multocida* suspension was equated to a 0.5 McFarland standard (Himedia R092, Indonesia) with a density of 10^8 CFU. Inoculation of *P. multocida* was performed intratracheally with 100 µl (Duplessis et al., 2021). The procedure was performed once on day eight PA, with a severe acute infection degree reached on day 11 PA. The successful establishment of PP was characterized by signs of acute fever, severe fibrinous-fibrinopurulent bronchopneumonia, fibrinous pleurisy, and septicemia (Abdullah et al., 2014).

Blood sample examination

On day 19 PA, the rats were anesthetized using ketamine at a dose of 40 mg/kg BW (Ket-A-100®; Struck et al., 2011), and blood samples were collected by using a microhematocrit through the retroorbital plexus of rats and placed in a container containing ethylene diamine tetraacetic acid (EDTA). Blood was stored in a refrigerator at 4°C for up to 24 hours for further analysis. Blood was analyzed for total erythrocytes, total leukocytes, hemoglobin, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leukocyte differential, and platelets. Hematology profile analysis was performed using a vet automatic hemoanalyzer (Wheisman AC310, Indonesia).

Lung sample examination

Lung samples were collected on day 19 after treatment. Before lung collection, the rats were euthanized using cervical dislocation.

Immunohistochemistry

The lung sample slides were dehydrated using graded alcohols, xylene, and phosphate-buffered saline, and then blocked with skim milk 1% in PBS (Prakoso et al., 2021). For antigen retrieval, the slides were incubated in Bond Epitope Retrieval Solution (RE7119, Leica Biosystems, USA) at 98°C for 20 minutes, immediately followed by a rinse with cold water. Next, endogenous peroxidase activity was quenched by incubating the slides with hydrogen peroxide 4% (Peroxidase Block, RE7101, Leica Biosystems, USA) for five minutes, followed by rinsing with PBS. This step was followed by a protein-blocking stage, where the slides were treated with 0.4% casein in PBS (Protein Block, RE7102, Leica Biosystems, USA) for five minutes, then rinsed with PBS. The slides were then incubated with the primary antibodies, anti-GM-CSF (sc-32753, Santa Cruz Biotechnology Inc, USA) and anti-IL-6 (z03034, Santa Cruz Biotechnology Inc, USA), for 30 minutes, followed by a PBS rinse. The anti-IL-6 antibody was diluted 1:100, while the anti-GM-CSF antibody was diluted 1:250 in primary antibody diluent (AR9352, Leica Biosystems, USA). After the primary antibody step, Rabbit anti-mouse IgG, which was diluted in 10% animal serum (Post Primary Antibody, RE7111, Leica Biosystems, USA), was applied for 30 minutes, and the slides were rinsed with PBS. Subsequently, the slides were incubated with anti-rabbit poly-HRP IgG containing 10% animal serum (Novolink Polymer, RE7112, Leica Biosystems, USA) for 30 minutes, followed by rinsing with PBS. For signal visualization, the slides were treated with diaminobenzidine (DAB) chromogen for 5 minutes, rinsed with tap water, and then counterstained using hematoxylin (RE7107, Leica Biosystems, USA) for 30 seconds. Finally, the slides were rinsed with tap water for five minutes, after which they were dehydrated, cleared, and mounted (Prakoso et al. 2020). The slides were stained with mouse antibodies, anti-IL-6 (Cat. No. z03034; Santa Cruz Biotechnology Inc., USA) and anti-GM-CSF (Cat. No. sc-32753; Santa Cruz Biotechnology Inc., USA), following the staining protocol outlined by Prakoso et al. (2020). The anti-IL-6 antibody was diluted 1:100, and the anti-GM-CSF antibody was diluted 1:250 in the primary antibody diluent (Cat. No. AR9352; Leica Biosystems, USA).

The immune expression of GM-CSF and IL-6 was photographed by the pathologist from the Laboratory of Pathology, Faculty of Veterinary Medicine, University of Gadjah Mada. The image was captured five times in different fields at 400X magnification. The image was analyzed using Image J software (NIH, USA) and reported as a percentage of immunoreactive area.

Enzyme-linked immunosorbent assays

The GM-CSF and IL-6 levels were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits with the product catalog numbers of MBS3808110 for GM-CSF and MBS2021530 for IL-6 (MyBiosource, USA). The wells were coated with a capture antibody and incubated overnight at 4°C, then washed and blocked with BSA. The antigen was then added to bind the capture antibody and incubated at 37°C. After washing, a primary detection antibody and an enzyme-conjugated secondary antibody were sequentially added, each followed by incubation and washing. Finally, a substrate was introduced to produce a measurable color change, indicating that antigen-antibody binding had occurred, as per the manufacturer's standard procedure.

Data analysis

Normally distributed and homogeneous data were analyzed using SPSS software version 26. For normally distributed data, the Duncan post hoc test was applied. Data that were not normally distributed and/or not homogeneous were analyzed using the Kruskal-Wallis test and the Mann-Whitney U test. The Kruskal-Wallis test was used at a 95% confidence level.

RESULTS

Hemoglobin levels showed a significant decrease in P2 compared to P1 ($p < 0.05$), but there was no difference was observed when compared to P3 ($p > 0.05$). Groups P4 and P5 indicated an increase in hemoglobin levels, approaching the values observed in P1; however, it was statistically different from the control group ($p < 0.05$; Table 1). The MCH level in P2 decreased significantly compared to P1 ($p < 0.05$), but did not differ significantly compared to P3 ($p > 0.05$). Groups P4 and P5 demonstrated MCH levels within the range of P1, yet still showed a significant difference compared to P1 ($p < 0.05$; Table 1). The MCHC level in P2 decreased significantly compared to P1 ($p < 0.05$), but no significant difference was observed compared to P3 ($p > 0.05$). Groups P4 and P5 exhibited increasing MCHC levels, approaching the range observed in P1. Specifically, P4 indicated no significant difference compared to P1 ($p > 0.05$), whereas P5 remained significantly different in comparison to P1 ($p < 0.05$; Table 1). The leukocyte level in P2 was significantly higher than in P1 ($p < 0.05$), but did not differ significantly from P3 and P4 ($p > 0.05$). Group P5 demonstrated a decreasing trend in leukocyte levels compared to P2 and did not exhibit a statistically significant difference compared to

P1. ($p > 0.05$; Table 1). The neutrophil value in P2 was significantly higher than that in P1 ($p < 0.05$), but did not differ significantly compared to P3 ($p > 0.05$). Although groups P4 and P5 demonstrated a decreasing trend in neutrophil levels, they differed significantly compared to P1 ($p < 0.05$; Table 1).

Table 1. Mean and standard deviation of hematological results of Sprague Dawley rats on day 19 of the study

Parameter	P1	P2	P3	P4	P5
RBC ($\times 10^6$ cells/mm^3)	5.72 ± 0.19^a	5.77 ± 0.33^a	5.90 ± 0.21^a	5.71 ± 0.18^a	5.89 ± 0.30^a
Hemoglobin (g/dL)	14.43 ± 0.60^a	10.91 ± 1.47^b	10.50 ± 0.55^b	13.21 ± 0.45^c	12.40 ± 2.17^c
PCV (%)	42.18 ± 1.09^a	41.81 ± 1.19^a	41.23 ± 0.82^a	42.94 ± 1.72^a	42.00 ± 1.45^a
MCV (fL)	73.71 ± 3.17^a	72.56 ± 4.95^a	69.92 ± 2.40^a	75.25 ± 3.47^a	71.49 ± 5.01^a
MCH (Pg)	25.22 ± 1.39^a	18.86 ± 1.89^b	17.81 ± 1.06^b	23.14 ± 0.66^c	21.12 ± 4.14^c
MCHC (%)	34.25 ± 2.13^a	26.09 ± 3.33^b	25.50 ± 1.82^b	30.82 ± 2.03^a	29.64 ± 5.90^a
Platelets (cell/mm^3)	5.10 ± 0.27^a	5.19 ± 0.09^a	5.20 ± 0.25^a	4.95 ± 0.20^a	5.17 ± 0.28^a
Leukocyte ($\times 10^3$ cell/mm^3)	6.10 ± 0.38^a	7.25 ± 0.38^b	7.16 ± 0.42^b	6.76 ± 0.23^b	6.57 ± 0.41^a
Neutrophils ($\times 10^3$ cell/mm^3)	1.67 ± 0.18^a	2.60 ± 0.17^b	2.42 ± 0.13^b	2.01 ± 0.21^c	1.95 ± 0.16^c
Eosinophils ($\times 10^3$ cell/mm^3)	0.15 ± 0.07^a	0.12 ± 0.06^a	0.10 ± 0.04^a	0.11 ± 0.06^a	0.08 ± 0.06^a
Basophils ($\times 10^3$ cell/mm^3)	0.03 ± 0.03^a	0.00 ± 0.00^a	0.05 ± 0.03^a	0.06 ± 0.05^a	0.01 ± 0.03^a
Lymphocytes ($\times 10^3$ cell/mm^3)	3.93 ± 0.37^a	4.29 ± 0.29^a	4.36 ± 0.29^a	4.31 ± 0.21^a	4.29 ± 0.24^a
Monocytes ($\times 10^3$ cell/mm^3)	0.30 ± 0.07^a	0.21 ± 0.12^a	0.19 ± 0.06^a	0.25 ± 0.11^a	0.23 ± 0.05^a

Note: P1: Control, P2: Induced PP without therapy, P3: Induced PP with enrofloxacin 20 mg/kgBB, P4: Induced PP with enrofloxacin 20 mg/kgBB and ibuprofen 30 mg/kgBB, P5: Induced PP with enrofloxacin 20 mg/kgBB and FCC 5.92 mg/kgBB, PCV: Packed cell volume, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration. [a, b, and c] different superscript letters indicate significant differences in a row ($p \leq 0.05$).

The present study indicated that GM-CSF immune expression was localized in type II pneumocytes in the lung interstitium of rats in the control and PP-infected groups (Figure 1A). The expression level in P1 appeared relatively low compared to P2, P3, P4, and P5. Group P2 demonstrated an increase in GM-CSF immune expression compared to the control group (Figure 1B). Groups P3 and P4 indicated a reduction in GM-CSF immune expression compared to P2, but with a qualitatively higher intensity than in P1 (Figures 1C and D). The GM-CSF immune expression in P5 was relatively low compared to P2, P3, and P4, and was qualitatively lower than the control group (Figure 1E). The treatment exerted a significant effect on the pulmonary GM-CSF immune expression after PP induction (Table 2). Group P2 indicated a significant increase after induction compared to the control group ($p < 0.05$). Groups P3 and P4 exhibited a significant increase in GM-CSF immune expression after treatment compared to P1 ($p < 0.05$), but did not differ significantly compared to P2 ($p > 0.05$). Although the expression level in P5 did not significantly differ from the control group ($p > 0.05$), it was significantly lower than in P2.

The present results indicated that the immune expression of IL-6 was localized in inflammatory cells in bronchial-associated lymphoid tissue (BALT) and the lung interstitium in the PP groups (Figure 2). Qualitatively, the level of IL-6 immune expression in P2 was higher than in P3, P4, and P5 (Figure 2B). Group P3 indicated multifocal immune expression of IL-6 in the interalveolar septa, which was lower than P2 (Figure 2C). Groups P4 and P5 demonstrated a gradual decrease of IL-6 immune expression compared to P2 and P3, as illustrated qualitatively in Figures 2D and 2E. The treatment affected the IL-6 immune expression in the lungs following PP induction (Table 2). Group P2 indicated a significant increase post-induction compared to the control group ($p < 0.05$; Table 2). Groups P3 and P4 demonstrated a significant increase after therapy compared to P1 ($p < 0.05$), and there was no significant difference compared to P2 ($p > 0.05$; Table 2). The result of the IL-6 immune expression level in P5 did not differ significantly compared to the control group ($p > 0.05$), but there was a significant difference compared to P2 ($p < 0.05$; Table 2).

Table 2. Mean and standard deviation of immunohistochemistry results of Sprague Dawley rats on day 19 of the study

Parameter	P1	P2	P3	P4	P5
GM-CSF	7.57 ± 5.88^a	17.62 ± 2.24^b	13.14 ± 2.94^b	8.39 ± 3.59^c	5.84 ± 1.87^a
IL-6	6.68 ± 1.51^a	19.53 ± 2.02^b	17.92 ± 2.49^b	11.27 ± 2.62^c	8.50 ± 3.32^a

Note: P1: Control, P2: Induced PP without therapy, P3: Induced PP with enrofloxacin 20 mg/kgBB, P4: Induced PP with enrofloxacin 20 mg/kgBB and ibuprofen 30 mg/kgBB, P5: Induced PP with enrofloxacin 20 mg/kgBB and FCC 5.92 mg/kgBB, GM-CSF: Granulocyte macrophage-colony stimulating factor, IL-6: Interleukin-6. [a, b, and c] different superscript letters indicate significant differences in a row ($p \leq 0.05$).

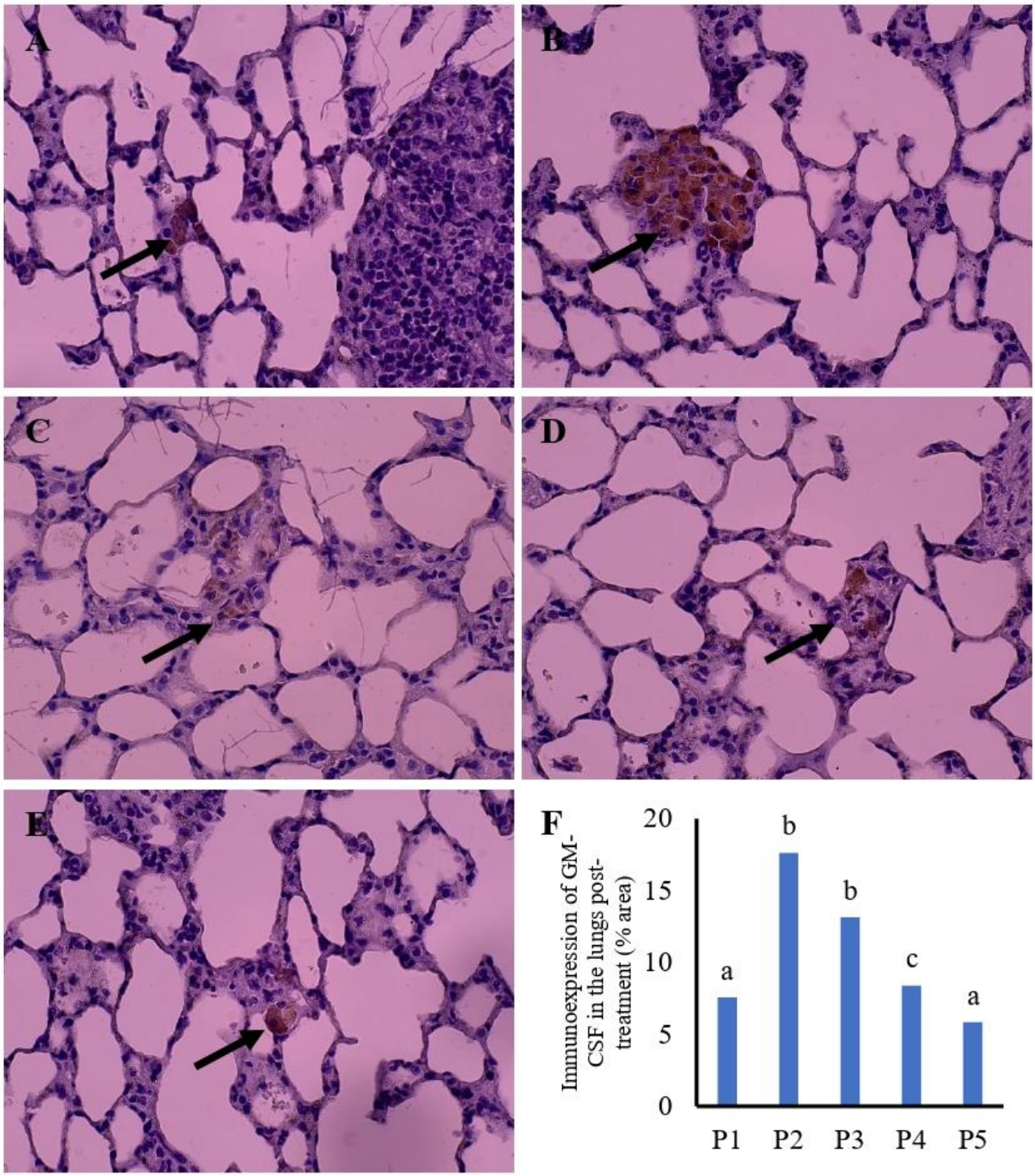

Figure 1. Granulocyte macrophage-colony stimulating factor (GM-CSF) immune expression in 3-month-old male rats with pneumonic pasteurellosis, post-treatment. **A**: GM-CSF immune expression in the lungs was localized to type II pneumocytes in the lung interstitium of group control (P1), **B**: untreated Group (P2), **C**: enrofloxacin Group (P3), **D**: enrofloxacin and ibuprofen Group (P4), **E**: enrofloxacin and FCC Group (P5), **F**: Mean pattern of GM-CSF immune expression post-treatment. IHC anti-GM-CSF antibody, DAB, 400× (A-E).

The ELISA results indicated that the treatment affected GM-CSF and IL-6 levels in the lungs after PP induction ($p < 0.05$; Figure 1A and B). The results indicated that the GM-CSF levels in P2 increased significantly compared to the control group ($p < 0.05$). Groups P3 and P4 exhibited a significant increase in GM-CSF expression compared to the control group ($p < 0.05$) and were not significantly different compared to P2 ($p > 0.05$). Group P5 did not differ significantly from the control group ($p > 0.05$), but exhibited a significantly lower value than P2 ($p < 0.05$), indicative of a successful reversion to near-normal levels. The level of IL-6 in the lungs after treatment indicated that IL-6 levels in P2 were significantly higher than in the control group ($p < 0.05$, Figure 1B). Group P3 indicated a significant increase

compared to P2 and the control group ($p < 0.05$). Group P4 indicated a significant decrease compared to the P2 ($p < 0.05$) and was not significantly different from the control group ($p > 0.05$). Group P5 did not differ significantly from the control group ($p > 0.05$) and was significantly lower than P2 ($p < 0.05$), indicating a successful restoration of IL-6 levels to near-normal values.

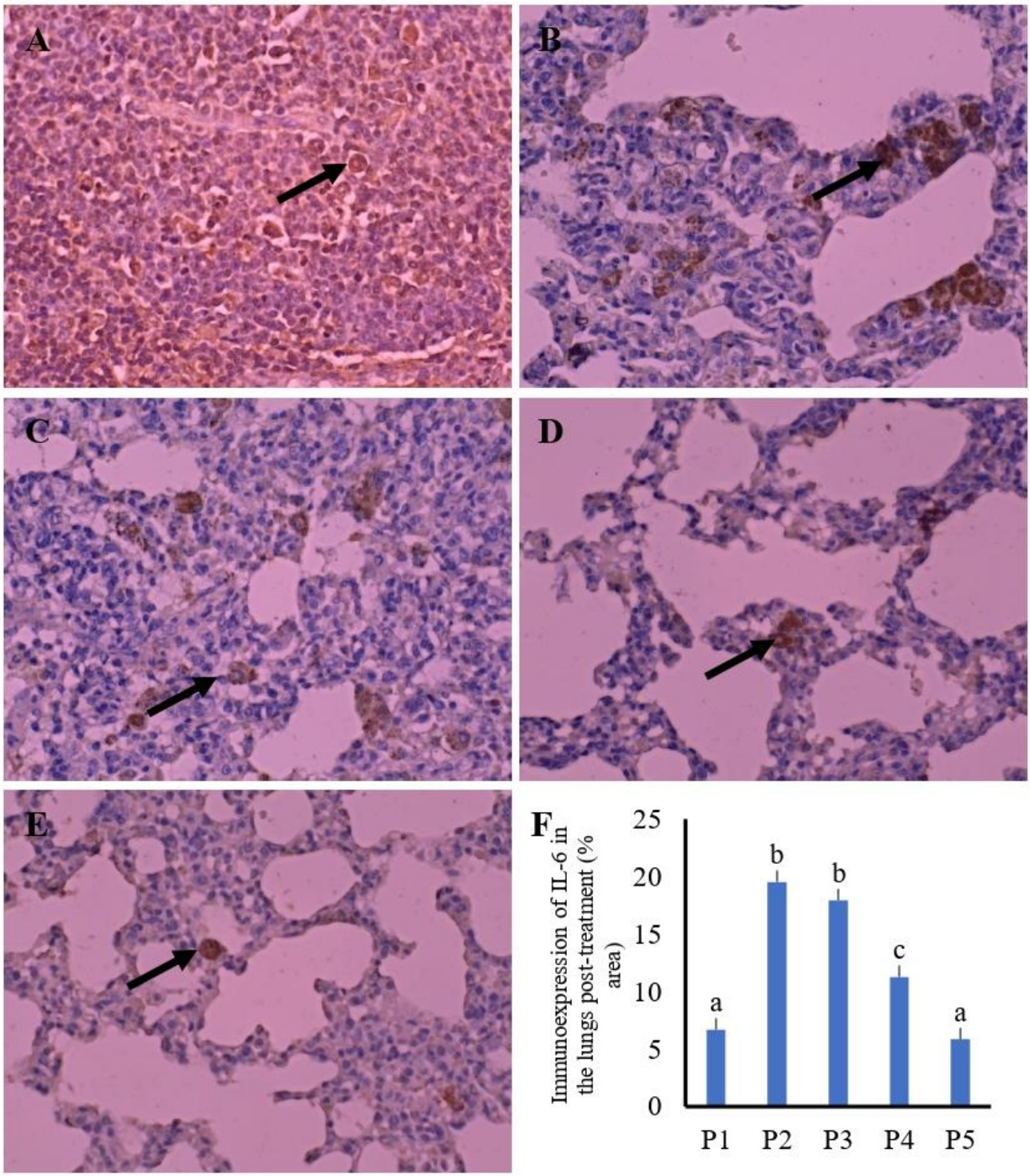

Figure 2. Interleukin-6 (IL-6) immune expression in 3-month-old male rats with pneumonic pasteurellosis, post-treatment. **A**: IL-6 immunoexpression in the lungs was localized to inflammatory cells in the bronchial associated lymphoid tissue (BALT) and in the lung interstitium of group P2, **B**: IL-6 immune expression in type II pneumocytes in the interalveolar septa of untreated group (P2), **C**: enrofloxacin Group (P3), **D**: enrofloxacin and ibuprofen Group (P4), **E**: enrofloxacin and FCC Group (P5), and figure **F**: Mean pattern of IL-6 immune expression post-treatment. IHC anti-IL-6 antibody, DAB, 400× (A-E).

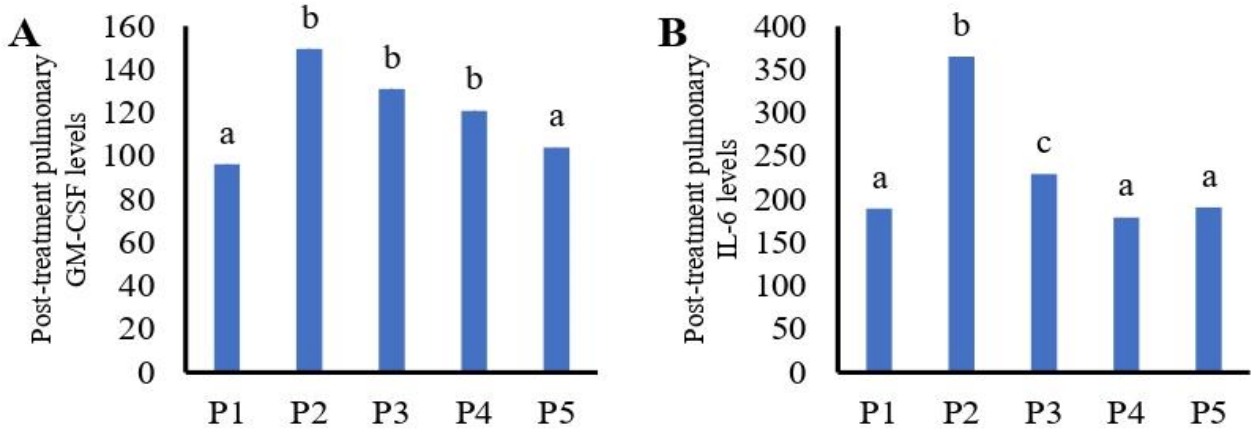

Diagram 1. Mean pattern of immune expression in 3-month-old male rats with pneumonic pasteurellosis model, post-treatment. **A**: Granulocyte macrophage-colony stimulating factor (GM-CSF), **B**: Interleukin-6 (IL-6).

DISCUSSION

Enrofloxacin acts by inhibiting DNA gyrase and topoisomerase IV and exhibits strong bactericidal activity against *Pasteurella* spp. (Hooper and Jacoby, 2016). However, its clinical efficacy in PP may be decreased due to increasing bacterial resistance and impaired drug distribution within the heavily infected lungs (Li et al., 2023). Adding ibuprofen can improve clinical outcomes by reducing inflammation. However, long-term use may decrease antibody production and pro-inflammatory cytokines such as IL-6 (Mihara et al., 2011). Inflammation can suppress protective immune responses, particularly in lungs that depend heavily on the balance between inflammation and local defense, which may lead to immunocompromised states or depletion of pulmonary immune function (Huemer, 2015). As an alternative, FCC has been shown to have potential as a natural anti-inflammatory and immunomodulatory agent capable of suppressing excessive inflammation (Prakoso et al., 2024).

Alterations in the hematological profile were observed, indicating a restorative effect on hemoglobin, MCH, MCHC, leukocytes, and neutrophils, along with decreased GM-CSF and IL-6 immune expression in male rats treated with enrofloxacin and FCC. The hematological changes might be due to FCC components, including choline, alpha-tocopherol, phytonadione, and retinol (Prakoso et al., 2024). Hemoglobin indicates the total amount of hemoglobin in the blood volume, while MCH and MCHC describe the distribution and concentration of hemoglobin at the cellular level (Yao et al., 2023); therefore, changes in hemoglobin levels would directly affect MCH and MCHC. Hemoglobin decreased considerably in rats treated with enrofloxacin and FCC (P5), consistent with the findings of Santos et al. (2003), who reported that choline alters red blood cell membrane properties, which can influence hemoglobin levels. Choline can enhance the stability and structure of hemoglobin by influencing hemostasis and erythropoiesis cell function (Huang et al., 2018). Alpha-tocopherol prevents the development of erythrocytopenia and helps maintain hemoglobin levels under oxidative stress conditions (Ivanova et al., 2011). Alpha-tocopherol protects red blood cell membranes from oxidative damage, thereby reducing hemolysis and increasing haptoglobin levels by binding free hemoglobin released due to hemolysis (Sovira et al., 2020). Phytonadione acts as an essential cofactor, enabling clotting factors to bind calcium and participate in blood clotting (Beaty, 2016). Retinol plays a crucial role in increasing hemoglobin levels by enhancing iron absorption and mobilization, supporting erythrocyte formation, and reducing inflammation (Sun et al., 2010).

The total leucocyte count in group P5 was similar to that in the control group, consistent with the findings of Daş et al. (2021), who reported that choline administration increased the number and function of leukocytes and reduced excessive inflammatory responses. Choline plays a vital role in maintaining leukocyte function and health by reducing oxidative stress and lowering leukocyte inflammatory activation (Mehta et al., 2009). It has been established that alpha-tocopherol can attenuate systemic inflammation and restore leukocyte counts elevated by excessive inflammatory responses. This observation aligns with the present findings, which revealed normalization in leukocyte parameters following alpha-tocopherol treatment (Moreira et al., 2022). The present findings align with previous studies, demonstrating that alpha-tocopherol stabilizes leukocyte counts under stress and inflammatory conditions (Sahakyan, 2024). Phytonadione has been reported to suppress the production of pro-inflammatory cytokines, thereby attenuating inflammatory responses. These anti-inflammatory effects can consequently reduce the occurrence of reactive leukocytosis, or leukocyte elevation, which typically increases during acute or chronic inflammatory conditions (Dahlberg et al., 2021). Previous studies have shown that retinol is essential for the differentiation of hematopoietic stem

cells into different leukocyte lineages. This finding agrees with the present study, indicating that retinol regulates leukocyte development and maintains hematological balance (Cañete et al., 2017).

The total neutrophils in P5 were close to the level in the control group, consistent with Zhou et al. (2018), who found that under severe inflammatory conditions, choline can reduce the number of neutrophils migrating to the site of inflammation systemically. Choline supplementation can reduce the expression of genes responsive to inflammation, modulate neutrophil activation, and reduce inflammation (Lopreiato et al., 2019). Alpha-tocopherol acts as an antioxidant, reducing oxidative stress that triggers neutrophil activation and suppressing the production of inflammatory cytokines that can trigger neutrophil recruitment (Hernandez et al., 2013; Wallert et al., 2019). Retinol has been reported to enhance neutrophil differentiation and bactericidal activity, improving host defense while limiting excessive neutrophil proliferation. The current results are consistent with previous findings demonstrating that retinol contributes to immune balance by stabilizing leukocyte responses under inflammatory conditions (Stream et al., 2024).

Immune expression of GM-CSF in rats with FCC therapy was reduced during the present study. Type II pneumocytes synthesize GM-CSF in response to inflammation; however, excessive production can lead to granulocyte and macrophage activation and proliferation, resulting in tissue damage and chronic inflammation (Ingelfinger et al., 2021). Choline in FCC could have suppressed the GM-CSF-mediated immune responses. This finding aligned with that of Mehta et al. (2009), indicating that choline reduced oxidative stress and inhibited the NF-κB pathway, which might indirectly modulate GM-CSF-mediated immune responses. Alpha-tocopherol has been shown to suppress NF-κB activation, thereby reducing the synthesis of pro-inflammatory cytokines (Li et al., 2012). Alpha-tocopherol can modulate immune responses across different cell types and suppress local and systemic inflammatory responses (Hu et al., 2022). Phytonadione can reduce oxidative stress, inhibit NF-κB and NLRP3 activation, and downregulate the expression of different pro-inflammatory cytokines. These mechanisms provide a plausible biological basis for the indirect effects of vitamin K in attenuating neutrophil activation and migration, as well as reducing GM-CSF levels under inflammatory conditions (Dosumu et al., 2021). Retinol can help modulate excessive immune responses and reduce the activation of inflammatory cells during severe inflammatory conditions (Spinas et al., 2015).

The immune expression of IL-6 in rats treated with enrofloxacin and FCC exhibited a reduction in IL-6 levels, nearing those of the control group. The BALT and the pulmonary interstitium synthesize pro-inflammatory cytokines, such as IL-6, in response to inflammation (Liska et al., 2013). Elevated IL-6 levels can trigger different physiological responses that may exacerbate inflammatory conditions and tissue damage (Kang and Kishimoto, 2021). Choline in FCC can suppress IL-6 immune expression, consistent with the findings of Baker et al. (2023), who reported that choline plays a crucial role in modulating the immune response through anti-inflammatory mechanisms by reducing IL-6 expression under inflammatory conditions. Choline acts as a precursor to acetylcholine, which can inhibit NF-κB activation, the primary transcription factor for pro-inflammatory cytokine genes, including IL-6 (Xia et al., 2022). Choline can reduce oxidative stress and inhibit the cholinergic inflammatory pathway (Yang et al., 2021). Alpha-tocopherol plays a crucial role in maintaining cellular membrane stability, preventing free radical damage, and reducing oxidative stress (Lemaire et al., 2010).

Alpha-tocopherol possesses antioxidant and anti-inflammatory activities that contribute to the reduction of IL-6 expression under inflammatory conditions. Alpha-tocopherol inhibited the formation of free radicals, which trigger activation of proinflammatory signaling pathways, particularly NF-κB, thereby reducing IL-6 gene transcription in immune cells such as macrophages, lymphocytes, and endothelial cells (Sharma and Vinayak, 2011). Retinol plays an important role in maintaining the integrity of the mucosal epithelium and reducing the infiltration of pro-inflammatory immune cells by inhibiting the release of pro-inflammatory cytokines such as IL-6, TNF, and macrophage inflammatory proteins (Wojtal et al., 2013). Retinol supports the regeneration of the mucosal surface and epithelium, which is vital for maintaining the structural integrity of the epithelium (Druilhe et al., 2008). Phytonadione exhibits anti-inflammatory effects by inhibiting NF-κB activation (Ohsaki et al., 2010), and its administration has been demonstrated to decrease oxidative stress. While there is no direct evidence linking this reduction to decreased IL-6 transcription or secretion in immune cells, it is biologically plausible, as oxidative stress can activate pro-inflammatory pathways such as NF-κB (Li et al., 2003).

CONCLUSION

The present study indicated that FCC improved hematological profiles, particularly hemoglobin, MCH, MCHC, leukocytes, and neutrophils, which reflected recovery responses in rats following treatment. Additionally, reduced immune expression of GM-CSF and IL-6 was observed in rats with induced PP. Future studies should be conducted to analyze the potential of FCC as an anti-inflammatory agent and its possible toxicity to ensure safety on a larger scale samples.

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Authors' contributions

Paskalis Guntur Widya Mahendra and Sitarina Widyarini designed the study. Paskalis Guntur Widya Mahendra, Yos Adi Prakoso, and Sitarina Widyarini performed the animal experimentation. Paskalis Guntur Widya Mahendra formulated, prepared, and preserved the tested compound. Paskalis Guntur Widya Mahendra and Yos Adi Prakoso performed data analysis and interpretation. Paskalis Guntur Widya Mahendra, Yos Adi Prakoso, and Sitarina Widyarini equally contributed to drafting, revising, and finalizing the submitted paper. All the authors approved the last edition of the manuscript.

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Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The present manuscript is original, conducted solely by the authors, and it has not been published elsewhere. The authors confirmed that no AI tools, including generative AI models, were used in the writing, editing, or preparation of this manuscript.

Availability of data and materials

The data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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