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Immunostimulatory Effect of *Cayratia trifolia* L. Domin on Granulocytes and Natural Killer Cells in Tuberculosis-Induced Rats

Efek Imunostimulator *Cayratia trifolia* L. Domin terhadap Granulosit dan Sel Pembunuh Alami pada Tikus Model Tuberkulosis

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ABSTRACT

Pulmonary tuberculosis (TB) remains a major public health problem in Indonesia and a leading cause of death from infectious diseases, partly because of impaired host immunity. Galing (*Cayratia trifolia* L. Domin) has been reported to exert immunomodulatory effects in bacterial infections. This study evaluated the immunostimulatory effect of galing leaf and stem extracts on granulocytes and natural killer (NK) cells in a TB-induced rat model. Leaf and stem extracts were administered orally to 28 Wistar rats at doses of 400 and 500 mg/kg body weight for seven days, followed by induction with TB ESAT-6 antigen. Granulocyte counts were measured using a hematology analyzer, whereas NK cells were quantified by ELISA. Galing extract significantly increased granulocyte and NK cell expression compared with the negative control ($p < 0.05$). These findings indicate that galing extract has potential as an immunostimulatory agent in pulmonary TB by enhancing granulocyte and NK cell responses.

ABSTRAK

Tuberkulosis (TB) paru masih menjadi masalah kesehatan utama di Indonesia dan salah satu penyebab utama kematian akibat penyakit infeksi, antara lain karena melemahnya sistem imun penderita. Tumbuhan galing (*Cayratia trifolia* L. Domin) telah dilaporkan memiliki efek imunomodulator pada infeksi bakteri. Penelitian ini mengevaluasi efek imunostimulator ekstrak daun dan batang galing terhadap granulosit dan sel pembunuh alami pada tikus model TB. Ekstrak daun dan batang diberikan secara oral kepada 28 tikus Wistar pada dosis 400 dan 500 mg/kg BB selama tujuh hari, kemudian diinduksi dengan antigen TB ESAT-6. Jumlah granulosit diukur menggunakan hematology analyzer, sedangkan sel pembunuh alami diukur dengan ELISA. Ekstrak galing secara signifikan meningkatkan ekspresi granulosit dan sel pembunuh alami dibandingkan kontrol negatif ($p < 0,05$). Temuan ini menunjukkan bahwa ekstrak galing berpotensi sebagai agen imunostimulator pada TB paru melalui peningkatan respons granulosit dan sel pembunuh alami.

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1. INTRODUCTION

Tuberculosis (TB) remains a major global health challenge. The World Health Organization (WHO) has targeted a 90% reduction in TB incidence between 2015 and 2035; however, challenges in therapeutic management and prevention continue to hinder the achievement of this target. TB is among the most prevalent infectious diseases worldwide, including in Indonesia, where its incidence remains high and continues to increase. The etiological agent of TB, *Mycobacterium tuberculosis* (Mtb), has infected humans for thousands of years (World Health Organization, 2024). Approximately 90% of individuals infected with TB develop latent infection, whereas about 5% progress to active TB within the first two years after infection, and another 5% may later develop active disease and become potential sources of transmission (Tobin & Tristram, 2025). Progression to active TB is strongly influenced by impaired immune function, including immunosenescence in the elderly, genetic immune deficiencies, human immunodeficiency virus infection, diabetes mellitus, dysregulated cytokine production involving tumor necrosis factor, interferon-gamma, and interleukin-12, phagocytic dysfunction, leukopenia, and reduced natural killer (NK) cells and granulocytes. These immune components are essential elements of the nonspecific immune response that influence susceptibility to Mtb infection and TB pathogenesis (Van Crevel et al., 2002; Zhuang et al., 2024).

Enhancing immune function through immunostimulatory agents is therefore a relevant strategy for addressing immune dysregulation in TB. Nevertheless, clinically proven immunomodulators for TB remain limited, highlighting the need to identify novel immunostimulatory candidates, particularly those derived from natural resources. One promising candidate is the galing plant (*Cayratia trifolia* L. Domin), which has been reported to possess several pharmacological activities relevant to infectious and inflammatory conditions (Shukla et al., 2022; Maheshwari et al., 2022). Previous studies have demonstrated that galing exhibits antidiabetic and antioxidant activities (Yusuf et al., 2018; Do et al., 2025), antibacterial effects (Gupta et al., 2012; Ilyas et al., 2019), anti-inflammatory properties (Yusuf et al., 2021; Yusuf et al., 2025), and immunomodulatory or immunostimulatory effects in bacterial infection and breast cancer models (Ilyas et al., 2025; Yusuf et al., 2019).

The traditional use and bioactive composition of galing further support its potential as an immunomodulatory agent. Empirically, this plant has been used to treat typhoid fever, relieve cough, reduce inflammation, and support physical well-being through immune-related mechanisms (Kumar et al., 2011; Do et al., 2025; Yusuf et al., 2025). Recent studies have shown that galing enhances nonspecific immunity by stimulating macrophage phagocytic activity during bacterial infection and modulating cytokines such as interferon-gamma, as well as regulating macrophage expression in breast cancer models (Ilyas et al., 2025; Ilyas et al., 2023a; Yusuf et al., 2025). These activities may be associated with its secondary metabolites, including flavonoids, alkaloids, tannins, terpenoids, and saponins (Kumar et al., 2011; Yusuf et al., 2018; Yusuf et al., 2025), as well as compounds such as (+)-ar-turmerone, abscisic acid, keracyanin, and 12-oxo-phytodienoic acid (Park et al., 2012; Mokdad-Bzeouich et al., 2016; Santamarina et al., 2021; Maheshwari et al., 2022; Zhang et al., 2022; Orellana-Paucar, 2024). These compounds are presumed to contribute to the pharmacological activity of galing, particularly its immunomodulatory effects, although further validation in relevant in vivo models is required.

Despite these findings, scientific evidence on the immunomodulatory effect of galing in TB remains unavailable. In particular, its effect on granulocytes and NK cells, two important components of innate immunity involved in early defense against TB, has not been clearly investigated. Therefore, this study aimed to evaluate the immunostimulatory effect of ethanolic leaf and stem extracts of galing on granulocyte and NK cell expression in a TB antigen-induced rat model. This approach was used to provide preliminary evidence regarding the potential of galing as a natural immunostimulatory candidate for supporting immune responses in TB.

2. METHODS

2.1. Sample Preparation and Extraction

Fresh galing (*Cayratia trifolia* L. Domin) plant samples were collected from Kambu Village, Kambu District, Kendari City, Southeast Sulawesi Province, Indonesia. The plant materials were cleaned, air-dried at room temperature, and pulverized to obtain powdered simplicia from the leaves and stems. A total of 700 g of leaf powder and 3,600 g of stem powder were macerated using 96% ethanol as the extraction solvent, with solvent volumes of 1,500 mL and 7,200 mL, respectively. Maceration was carried out at room temperature for 72 hours with occasional agitation. The extracts were filtered repeatedly until exhaustive extraction was achieved. The combined filtrates were concentrated under reduced pressure at 50°C using a rotary evaporator (Stuart RE300, United States) to obtain crude ethanolic extracts (Ilyas et al., 2022; Ilyas et al., 2025).

2.2. Immunostimulatory Assay

A total of 28 healthy male Wistar rats were randomly allocated into seven treatment groups. Animal handling was conducted in accordance with the Regulation of the Indonesian Food and Drug Authority (BPOM RI) Number 18 of 2021 concerning guidelines for preclinical pharmacodynamic testing of traditional medicines. The use of experimental animals was approved by the Health Research Ethics Committee of LPPM, Universitas Halu Oleo (Approval No. 0999/UN29.20.1.2/PG/2024). The treatments were administered orally once daily for seven consecutive days according to the predetermined dosing volumes (Ilyas et al., 2021a; Ilyas et al., 2023b). The treatment groups were assigned as follows:

Normal control (KN): received no extract and no TB ESAT-6 antigen induction. Negative control (K–): received 0.5% Na-CMC and TB ESAT-6 antigen induction. Positive control (K+): received

commercial *Phyllanthus niruri* extract® at 0.922 mg/kg BW and TB ESAT-6 antigen induction. K1: received leaf extract at 400 mg/kg BW and TB ESAT-6 antigen induction. K2: received leaf extract at 500 mg/kg BW and TB ESAT-6 antigen induction. K3: received stem extract at 400 mg/kg BW and TB ESAT-6 antigen induction. K4: received stem extract at 500 mg/kg BW and TB ESAT-6 antigen induction.

Blood samples were collected through intracardiac puncture 24 hours after induction with TB Early Secreted Antigenic Target 6 kDa (ESAT-6) antigen in the relevant treatment groups. The ESAT-6 antigen (MedikBio®, Indonesia) is a recombinant protein derived from *Mycobacterium tuberculosis* strain CDC 1551/Oshkosh. Blood samples were transferred into EDTA-containing vacutainer tubes and centrifuged at 3,500 rpm for 15 minutes to obtain plasma (Ilyas et al., 2021b; Muhammad et al., 2022).

2.3. Granulocyte Cell Count Assay

Granulocyte cell counts were determined using a hematology analyzer (Mindray BC-2600®, China). Hematological data were generated automatically by the instrument and recorded from the printed output (Ikhram et al., 2022; Rosidah et al., 2020).

2.4. Natural Killer Cell Quantification Assay

Natural killer (NK) cell quantification was performed using a rat-specific NK cell ELISA kit. Plasma samples were analyzed using an ELISA reader. Briefly, 50 µL of standard solution and 40 µL of plasma sample were dispensed into designated wells according to the treatment groups, followed by the addition of 10 µL of anti-NK cell antibody into each sample well. Streptavidin-HRP was then added to both sample and standard wells. The mixtures were homogenized, the plate was sealed, and incubation was performed at 37°C for 60 minutes.

After incubation, the plate was washed five times using 0.35 mL of wash buffer for 1 minute per wash. Subsequently, 50 µL of substrate solutions A and B were added to each well and incubated at 37°C for 10 minutes under dark conditions. The reaction was terminated by adding 50 µL of stop solution to each well, resulting in a color change from blue to yellow. Absorbance was measured at 450 nm using an ELISA reader (iMark™, United States) within 10 minutes after the reaction was stopped. NK cell counts were calculated based on the standard curve using the linear regression equation $y = 0.0042x + 0.4503$, with $R^2 = 0.9925$ (Grudzien & Rapak, 2018; Qin et al., 2024).

2.5. Data Analysis

Granulocyte and NK cell data were analyzed using IBM SPSS Statistics version 27 (IBM, United States). Data were analyzed using one-way analysis of variance (ANOVA) at a 95% confidence level, with α set at 0.05. Intergroup differences were further evaluated using Tukey's post hoc test. Statistical significance was defined as $p < 0.05$.

3. RESULTS AND DISCUSSION

The extract was administered orally for seven consecutive days to allow its bioactive compounds to progressively modulate the immune response. This duration was selected based on previous studies reporting that immunostimulatory agents administered for 5–14 days can induce measurable innate and adaptive immune responses and stimulate immune cell activity, including natural killer (NK) cells (Liu et al., 2021; Schepetkin et al., 2023).

This study evaluated the immunostimulatory effects of ethanolic leaf and stem extracts of *C. trifolia* by assessing granulocyte and NK cell expression in ESAT-6 antigen-induced Wistar rats. The ESAT-6 antigen induction model and the selected immunological parameters were used because they are relevant to innate immune responses associated with tuberculosis (TB) (Cooper, 2015; Joslyn et al., 2022). In this study, ESAT-6 antigen at a dose of 0.01 mg/mL was used to induce an immune response related to TB exposure and to evaluate the potential of *C. trifolia* extract to enhance innate immune defense.

The innate immune response is important in the early control of *M. tuberculosis*. Previous findings have shown that *M. tuberculosis* can evade phagocytic elimination mediated by granulocytes, including neutrophils, and by NK cells through mechanisms involving NF-κB signaling. This immune evasion may inhibit immune cell apoptosis and promote the persistence of chronic infection (Soldevilla et al., 2023). Therefore, granulocytes and NK cells are relevant parameters for evaluating the immunostimulatory potential of natural product-based candidates in TB-related immune models.

3.1. Granulocyte Cell Expression

Granulocyte cell counts were measured using a hematology analyzer, an automated instrument capable of providing rapid hematological assessment, including complete blood count parameters. This instrument requires a small sample volume, has a simple operating procedure, and provides results within a short time (Fauzi et al., 2024). The granulocyte cell counts in each treatment group are presented in **Figure 1**.

As shown in **Figure 1**, the mean granulocyte cell count increased in all extract-treated groups, with the highest count observed in the group administered *C. trifolia* leaf extract at 500 mg/kg BW. This response was comparable to that of the positive control group treated with commercial *P. niruri* extract ($p > 0.05$), which served as the reference immunostimulatory treatment. In contrast, the lowest granulocyte cell count was observed in the negative control group administered Na-CMC, which was significantly lower than that of the extract-treated groups ($p < 0.05$). This result indicates that the immune response in the negative control group depended mainly on the animals' innate immunity, as Na-CMC has no pharmacological activity in stimulating granulocyte production (Badia et al., 2024).

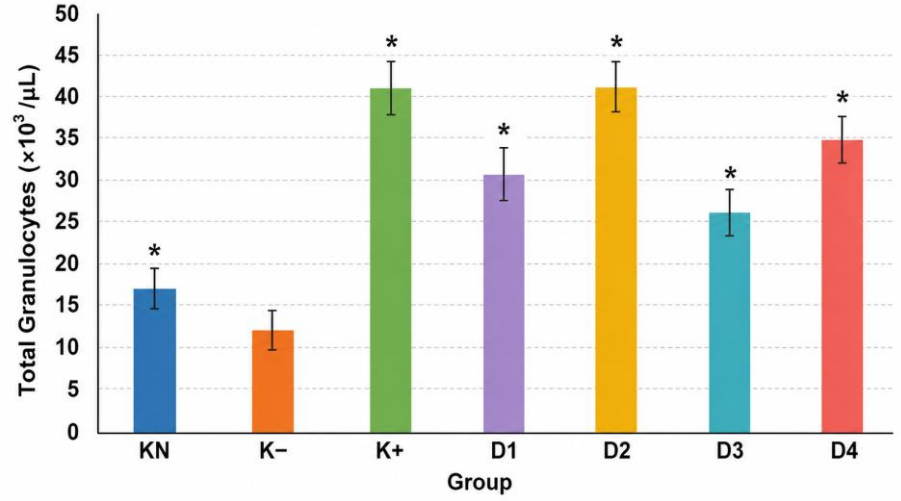

Figure 1. Total granulocyte cell expression, expressed as mean ± SD, among treatment groups. KN = normal control; K– = negative control; K+ = positive control treated with commercial *Phyllanthus niruri* extract; K1 = leaf extract of *C. trifolia* at 400 mg/kg BW; K2 = leaf extract of *C. trifolia* at 500 mg/kg BW; K3 = stem extract of *C. trifolia* at 400 mg/kg BW; K4 = stem extract of *C. trifolia* at 500 mg/kg BW. $p < 0.05$ indicates a significant difference compared with the negative control, as determined by Tukey's post hoc test.

The increase in granulocyte cell expression following *C. trifolia* extract administration may be associated with the presence of bioactive compounds with immunostimulatory activity. This finding is consistent with Yusuf et al. (2019), who reported that ethanolic leaf extract of *C. trifolia* at doses of 400 and 500 mg/kg BW enhanced macrophage phagocytic activity in bacterially infected animal models.

Previous studies have identified that *C. trifolia* contains secondary metabolites, including flavonoids, alkaloids, tannins, saponins, and triterpenoids (Yusuf et al., 2018; Thawkar et al., 2023). Flavonoids and alkaloids have been reported to increase the production of interleukin-12 (IL-12), a cytokine that promotes phagocytosis and endocytosis by granulocytic phagocytes. IL-12 also enhances the capacity of immune cells, including monocytes, lymphocytes, and neutrophils, to respond to TB infection through the secretion of cytotoxic enzymes that degrade intracellular pathogens (Shukla et al., 2022). Tannins have also been reported to exert physiological effects, including stimulation of phagocytic cells, as well as antitumor and antibacterial activities (Maheshwari et al., 2022). Collectively, these bioactive compounds may contribute to the increased granulocyte cell expression observed in this study.

3.2. Natural Killer Cell Expression

As key effectors of the innate immune system, NK cells play an important role in eliminating pathologically transformed cells, including tumor cells and pathogen-infected cells such as those affected by *M. tuberculosis*. The cytotoxic activity of NK cells is essential during the early immune response against TB because these cells help modulate bacterial burden and limit systemic spread through the release of pro-inflammatory cytokines, particularly interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (Liu et al., 2021; Yao et al., 2025). NK cell activation also enhances macrophage function by stimulating phagocytic activity, thereby facilitating more efficient bacterial clearance (Bernink et al., 2013).

NK cell expression measured by ELISA showed significant variation among treatment groups (**Figure 2**). Administration of *C. trifolia* leaf and stem extracts at doses of 400 and 500 mg/kg BW significantly increased NK cell expression compared with the negative control group ($p < 0.05$), supporting the immunostimulatory potential of the extracts in a TB antigen-induced model. The dose of 500 mg/kg BW produced the highest response and was statistically comparable to the positive control group receiving commercial *P. niruri* extract ($p > 0.05$), as determined by post hoc analysis.

The increase in NK cell expression after administration of *C. trifolia* leaf and stem extracts indicates that the extracts may enhance innate immune responses in the ESAT-6 antigen-induced model. The response observed at 500 mg/kg BW suggests that this dose may be more effective than 400 mg/kg BW, as shown by its comparable effect to the positive control. These findings are in line with Yusuf et al. (2019), who demonstrated that ethanolic leaf extract of *C. trifolia* enhanced nonspecific immune responses through increased macrophage phagocytic activity in bacterially infected animal models.

The immunostimulatory activity observed in this study may be attributed to the bioactive constituents of *C. trifolia*, including flavonoids, tannins, alkaloids, terpenoids, and saponins (Ilyas et al., 2021a). Flavonoids have been reported to upregulate pro-inflammatory cytokines such as IFN-γ and TNF-α, which play key roles in NK cell activation and proliferation. Therefore, the present findings support the hypothesis that *C. trifolia* leaf and stem extracts may function as natural immunostimulatory agents by activating NK cells, which are important components of innate immune defense against TB-related immune challenges (Liu et al., 2021; Maheshwari et al., 2022).

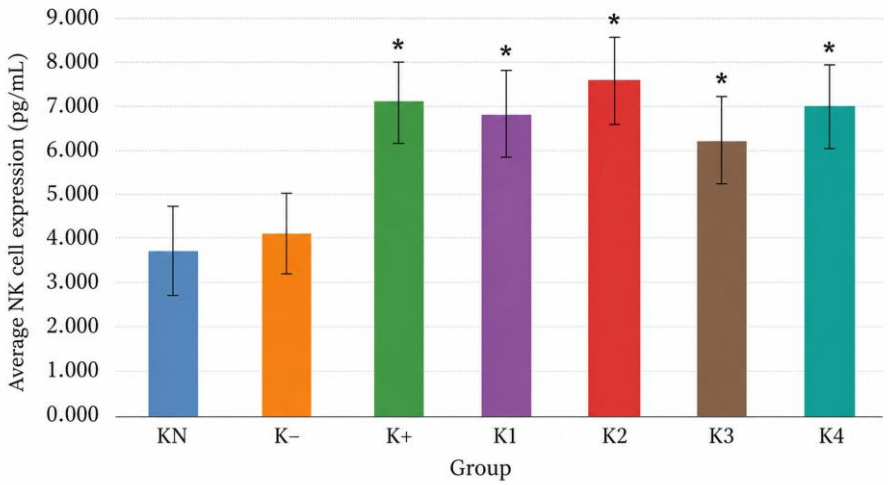

Figure 2. Mean ± SD of natural killer (NK) cell expression in each treatment group. KN = normal control; K– = negative control; K+ = positive control treated with commercial *Phyllanthus niruri* extract; K1 = leaf extract of *C. trifolia* at 400 mg/kg BW; K2 = leaf extract of *C. trifolia* at 500 mg/kg BW; K3 = stem extract of *C. trifolia* at 400 mg/kg BW; K4 = stem extract of *C. trifolia* at 500 mg/kg BW. $p < 0.05$ indicates a significant difference compared with the negative control group, as determined by Tukey's post hoc test.

Several compounds identified in *C. trifolia* may also contribute to immune modulation, including NK cell activation. (+)-ar-Turmerone has been reported to regulate T-cell and macrophage activity while suppressing pro-inflammatory cytokines such as TNF-α and IL-1β, thereby supporting a balanced immune environment that allows NK cells to function optimally against tumor or pathogen-infected cells (Park et al., 2012; Orellana-Paucar, 2024). Similarly, abscisic acid acts through the peroxisome proliferator-activated receptor gamma pathway by suppressing pro-inflammatory cytokine production and promoting anti-inflammatory cytokines such as IL-10. This mechanism may help maintain immune equilibrium and support effector immune cells, including NK cells (Magnone et al., 2009; Bassaganya-Riera et al., 2011).

Keracyanin, an anthocyanin compound reported in *C. trifolia*, has been associated with modulation of immune cell activity, including macrophages and T cells, by enhancing IL-2 and IFN-γ release, which are important for NK cell activation (Santamarina et al., 2021; Boichuk et al., 2025). In addition, 12-oxo-phytodienoic acid exhibits anti-inflammatory activity by inhibiting the NF-κB signaling pathway, a key regulator of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. By downregulating NF-κB activity, 12-oxo-phytodienoic acid may reduce systemic and localized inflammation, thereby preventing chronic inflammatory conditions that could impair effector immune cell function, including NK cells. However, the specific contribution of each compound to NK cell modulation in the present model requires further confirmation (Liu et al., 2014; Zhang et al., 2022).

Taken together, these findings suggest that *C. trifolia* has considerable potential as a natural immunostimulatory agent by enhancing granulocyte and NK cell responses through multiple immune-related mechanisms. However, this study has several limitations. First, the immunological parameters were limited to granulocytes and NK cells, whereas additional markers such as macrophage activity, cytokine profiles, T-cell subsets, and inflammatory mediators would provide a more comprehensive understanding of the immune response. Second, the study used an ESAT-6 antigen-induced model rather than direct infection with live *M. tuberculosis*, which may not fully represent the complexity of TB pathogenesis. Third, the sample size was limited, and further studies using broader immunological endpoints and more representative TB infection models are required to strengthen the evidence for the immunomodulatory activity of *C. trifolia* in TB.

4. CONCLUSION

This study demonstrates that ethanolic leaf and stem extracts of *C. trifolia* enhance granulocyte and natural killer (NK) cell expression in an ESAT-6 antigen-induced tuberculosis animal model. These findings indicate that *C. trifolia* has potential as a natural immunostimulatory agent by strengthening innate immune responses related to granulocyte and NK cell activity. However, further studies using more comprehensive immune parameters and direct *M. tuberculosis* infection models are needed to confirm its potential application as an adjunct immunostimulatory candidate for pulmonary tuberculosis

AUTHOR CONTRIBUTIONS

Conceptualization, M.I.Y., A.J., and W.O.S.; methodology, M.I.Y. and N.R.; software, M.; validation, M.I.Y., A.J., and P.; formal analysis, M.; investigation, L.L.B.; resources, [initials to be confirmed]; data curation, A.P.; writing—original draft preparation, M.I.Y.; writing—review and editing, M.I.Y. and A.P.; visualization, M.; supervision, W.O.S.; project administration, N.R. and L.L.B.; funding acquisition, M.I.Y. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

The animal study protocol was approved by the Health Research Ethics Committee of LPPM, Universitas Halu Oleo (Approval No. 0999/UN29.20.1.2/PG/2024).

INFORMED CONSENT STATEMENT

Not applicable

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ROLE OF FUNDERS

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist in improving the clarity, structure, and readability of the text. After using this tool, the authors thoroughly reviewed, edited, and verified the entire content to ensure that it accurately represents their own ideas, data, and interpretations. The authors take full responsibility for the integrity and originality of the published work.

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