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Original Research Article

Modulation of Cytokine Imbalance by *Phaleria macrocarpa* Leaf Extract in LPS-stimulated RAW 264.7 Macrophages

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ABSTRACT

Chronic inflammation is strongly associated with excessive production of pro-inflammatory cytokines. Mahkota Dewa (*Phaleria macrocarpa*) is a traditional medicinal plant recognized for its anti-inflammatory potential. Nevertheless, its specific role in modulating pro- and anti-inflammatory cytokines within immune cells remains inadequately explored. This study aimed to investigate the anti-inflammatory activity of *P. macrocarpa* leaf extract by assessing its regulatory effects on pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-*β*) in RAW 264.7 macrophage cells stimulated with lipopolysaccharide (LPS). The cells were treated with *P. macrocarpa* leaf extract at 9, 18, and 36 µg/mL, none of which exhibited cytotoxic effects. Cytokine levels were measured using flow cytometry. The findings demonstrated a significant reduction ($p<0.05$) in IFN-γ and TNF-α levels at 18 and 36 µg/mL of *P. macrocarpa* leaf extract. Furthermore, IL-10 levels increased in a dose-dependent manner, while the highest TGF-*β* expression was observed at 18 µg/mL. The findings revealed that *P. macrocarpa* leaf extract exerts significant anti-inflammatory effects by modulating cytokine production in LPS-induced macrophages. These results suggest its broader therapeutic potential in managing inflammation-related diseases, warranting further molecular and clinical investigation.

Keywords: Anti-inflammatory, Cytokines, Inflammation, Homeostasis, Macrophages.

Introduction

Inflammation is a complex biological response to infection or cell damage, mediated by activation of immune cells and release of pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-6 (IL-6). This process is mainly regulated by transcription factors, notably nuclear factor-kappa B (NF-κB), which modulate the expression of various inflammatory mediators.[1] To maintain immune homeostasis and prevent tissue damage, anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-*β*) counteract the inflammatory response.[2] A balanced interaction between pro- and anti-inflammatory cytokines is essential for an appropriate immune response.[1,2] Disruption of this balance can lead to chronic inflammation, which is implicated in diseases such as rheumatoid arthritis, cancer, diabetes, neurodegenerative disorders, and asthma.[3] To address these inflammation-related conditions, nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, aspirin, and paracetamol are widely used due to their effectiveness in relieving symptoms.[4,5,6] However, long-term use of NSAIDs is often associated with various side effects, including gastrointestinal and renal side effects.[7]

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Consequently, alternative treatments with lower side effects are essentially needed. In this context, plant-based therapies have garnered increasing attention for their potential anti-inflammatory properties and historical use in traditional medicine.[8] As a result, the exploration of herbal bioactive compounds for drug development is currently a major focus of pharmaceutical research.[9,10]

One promising plant that has potential as an anti-inflammatory agent is Mahkota Dewa (*Phaleria macrocarpa* Boerl.). *P. macrocarpa* is a medicinal plant species native to Papua Island, Indonesia, and widely distributed in Southeast Asian, including Indonesia, Singapore, and Malaysia.[11] Traditionally, its leaves have been used to treat various diseases, including diabetes, rheumatism, digestive issues, and cancer.[12] The leaves of this plant are known to have high antioxidant activity due to their rich flavonoid, phenolic, and terpenoid content.[13-15] The results of the DPPH assay showed that the ethanol extract of *P. macrocarpa* leaf had strong antioxidant activity with an IC_{50} value of 73.91 ppm.[16] Aminullah et al.[17] reported that *P. macrocarpa* leaf extract significantly reduced tumor volume in mice without causing toxic effects on the kidneys or liver. Christina et al.[18] further supported its anticancer potential through an *in silico* study showing inhibition of Bcl-2 and stimulation of caspase-3 and Bax. The anti-inflammatory activity of *P. macrocarpa* leaves has also been observed in a mouse model of dextran sodium sulfate (DSS)-induced colitis.[19] Furthermore, strong antioxidant activity has been reported in its pericarp and mesocarp extract against DPPH, ferric ions, and nitric oxide. These extracts were shown to significantly inhibit nitric oxide production in LPS/IFN-γ-stimulated RAW 264.7 macrophage cells, suggesting notable anti-inflammatory effects.[13] A recent study also revealed that *P. macrocarpa* leaves decrease the level of IL-6 and TNF-α in diabetic rats.[20]

Although previous studies have highlighted the anti-inflammatory activities of *P. macrocarpa*, its role in modulating cytokine profiles remains underexplored. To our knowledge, no studies have been performed on pro-inflammatory cytokines IFN-γ and TNF-α and anti-inflammatory cytokines IL-10 and TGF-*β* in macrophages

simultaneously. Evidence on its ability to restore the balance between pro- and anti-inflammatory cytokines in immune cell models is still limited. The study focused on the leaves of *P. macrocarpa* due to their stronger ability as anticancer and anti-inflammatory agents with low toxicity, as reported by Christina et al.[11] and Lestari et al.[20] Therefore, this study aimed to investigate the anti-inflammatory potential of *P. macrocarpa* leaves extract by focusing on its ability to suppress pro-inflammatory cytokines (IFN-γ and TNF-α) and enhance anti-inflammatory cytokines (IL-10 and TGF-*β*).

Materials and Methods

Plant collection and extraction

The extraction method for *P. macrocarpa* leaves followed previous research by Christina et al.[11] The fresh leaves of *P. macrocarpa* were obtained from the UPT Laboratorium Herbal Materia Medica, Batu (geographical coordinates: 7°52'02.8"S and 112°31'20.5"E) in September 2019. Taxonomic identification was carried out by the UPT. Balai Materia Medica Batu, Indonesia, with specimen number. 074/384A/102.7/2020. The fresh leaves were dried at 50°C for 2 days and milled into powder. Powdered leaves were extracted with 95% ethanol absolute (1:10 w/v) for 24 h in the dark at room temperature. The filtrate was filtered and concentrated under reduced pressure at 50ºC using a rotary evaporator (IKA® RV 10, IKA Works (Asia) Sdn Bhd, Malaysia). The evaporated extract was stored in a closed tube at -20°C before use.

Preparation of treatment medium

The media treatment was prepared based on previous research by Djati et al.[21] with modifications to the type of plant extract and concentration of the media treatment. The stock of extract was prepared by dissolving 40 mg of extract in 1 mL of dimethyl sulfoxide (DMSO) (EMSURE®, Merck KGaA, Germany) with a final DMSO concentration in the treatment medium of 0.09%. The treatment medium was prepared by dissolving the stock solution in DMEM high glucose medium (Sigma-Aldrich, Co., Merck KGaA, Germany) enriched with 10% Fetal Bovine Serum (FBS) (certified US, Gibco™, Thermo Fisher Scientific, USA) and 1% 10,000 U/ml penicillin–streptomycin (Gibco™, Thermo Fisher Scientific, USA). The concentrations of *P. macrocarpa* leaves used were at 9 µg/mL (1/2x IC_{50}), 18 µg/mL (IC_{50}), and 36 µg/mL (2x IC_{50}). The IC_{50} values of *P. macrocarpa* leaves in LPS-induced RAW 264.7 were determined in a preliminary experiment through NO assay and cell toxicity assay.[22]

Cell culture and treatment

The cell culture and treatment methods were based on previous studies conducted by Djati et al.[21] and Dwijayanti et al.[9] The RAW 264.7 cell line (Elabscience®, Catalog No: CL-0190, Elabscience Biotechnology Inc., USA) with passage numbers 19 to 30 was cultured using DMEM high glucose medium (10% FBS, 1% penicillin-streptomycin). Cells in healthy conditions were grown in 24-well plates with a cell density of 1×10^5 cells/well. The cells were observed to be evenly distributed using a microscope. Next, the cells were incubated in a CO_2 5%, 37 °C incubator for 24 h. The negative control group was only DMEM medium, while the positive control group was exposed to 4 µg/mL LPS. The treatment groups were exposed to different concentrations of *P. macrocarpa* leaf extract at 9, 18, and 36 µg/mL with the addition of 4 µ/mL LPS for 24 h. After treatment, cells were harvested with 75 µL of 0.25% trypsin-EDTA (Gibco™, Thermo Fisher Scientific, USA) and incubated for 5 min to detach the cells from the plate. Harvested cells were centrifuged at 2,500 rpm for 5 min at 10°C. The obtained pellet was processed for flow cytometry analysis.

Determination of pro-inflammatory (IFN-γ and TNF-α) and anti-inflammatory cytokines (TGF-β and IL-10)

The flow cytometry method refers to previous research by Djati et al.[21], which has been modified. The obtained pellet was fixed with 50 µL intracellular fixation buffer (eBioscience™, Invitrogen, Thermo Fisher Scientific, USA) at 4ºC. After fixation for 20 min, cells were homogenized with 500 µL permeabilization buffer (Invitrogen, Thermo Fisher Scientific, USA) and centrifuged at 2,500 rpm for 5 min at 10°C. The obtained pellets were stained with following antibodies (dilution ratio 1:100) Fluorescein Isothiocyanate (FITC)-conjugated IFN-γ/Interferon gamma Antibody (G-30, sc-57208, Santa Cruz Biotechnology Inc., USA), Phycoerythrin (PE)-conjugated TNFα Antibody (TN3-19.12, sc-12744), Alexa Fluor®647-conjugated TGF-*β*1 Antibody (3C11, sc-130348, Santa Cruz Biotechnology Inc., USA) and Alexa Fluor®647-conjugated IL-10 (A-2, sc-365858, Santa Cruz Biotechnology Inc., USA) for 20 min. The number of events required was 20,000 cells for each sample. Gating was performed by overlaying the control and treatment groups to identify and characterize specific cell populations in the sample. The relative number of IFN-γ, TNF-α, TGF-*β*, and IL-10 was quantified using a flow cytometer (BD FACS Calibur™, San Jose, CA, USA) and analyzed using BD CellQuest™ Pro software version 7.5.3 (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All data are presented as mean ± standard deviation (SD) from three independent experiments (n=3). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated by Levene's test before conducting parametric analysis. Statistical differences between treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p-value < 0.05 was considered statistically significant. Data analysis was performed using IBM SPSS version 29 (IBM Corp., Armonk, NY, USA).

Results and Discussion

Effects of P. macrocarpa leaves ethanol extract on pro-inflammatory cytokines

The study demonstrated that LPS induction significantly ($p<0.05$) increased IFN-γ expression to 46.05 ± 2.22% compared to a negative control group without LPS (11.55 ± 1.28%). The ethanol extract of *P. macrocarpa* leaves significantly reduced the expression of IFN-γ and TNF-α cytokines in LPS-stimulated RAW 264.7 cells (Figure 1A). Treatment with *P. macrocarpa* leaf extract at concentrations of 18 and 36 µg/mL significantly ($p<0.05$) decreased IFN-γ levels to 38.37 ± 2.06% and 28.71 ± 3.36%, respectively. However, a lower concentration (9 µg/mL) of *P. macrocarpa* leaves extract did not result in a significant reduction of IFN-γ (46.56 ± 6.27%). At extract concentrations of 18 and 36 µg/mL, the reduction in IFN-γ levels was not accompanied by cell death, indicating that the decrease at higher concentrations was not due to herbal toxicity.

Similarly, TNF-α levels increased markedly following LPS stimulation (54.18 ± 6.75 %) compared to the negative control (9.14 ± 0.58 %). Administration of the *P. macrocarpa* leaves extract reduced TNF-α expression in a dose-dependent manner, with levels decreasing to 42.11 ± 4.14% at 9 µg/mL, 31.93 ± 1.52% at 18 µg/mL, and 24.79 ± 4.95% at 36 µg/mL. All treatment groups showed statistically significant differences ($p < 0.05$) compared to the LPS-only group (Figure 1B). These results reinforce the anti-inflammatory potential of *P. macrocarpa* leaf extract, particularly through its capacity to inhibit critical pro-inflammatory cytokines that play pivotal roles in regulating immune responses. The dose-dependent decrease in TNF-α observed in this study may result from direct or indirect inhibition of NF-κB, a key transcription factor involved in cytokine production. However, further analysis is needed to elucidate the underlying molecular mechanisms.

The findings of this study showed that the ethanol extract of *P. macrocarpa* leaves significantly lowered the levels of the pro-inflammatory cytokines IFN-γ and TNF-α in LPS-stimulated RAW 264.7 macrophages (Figure 1).

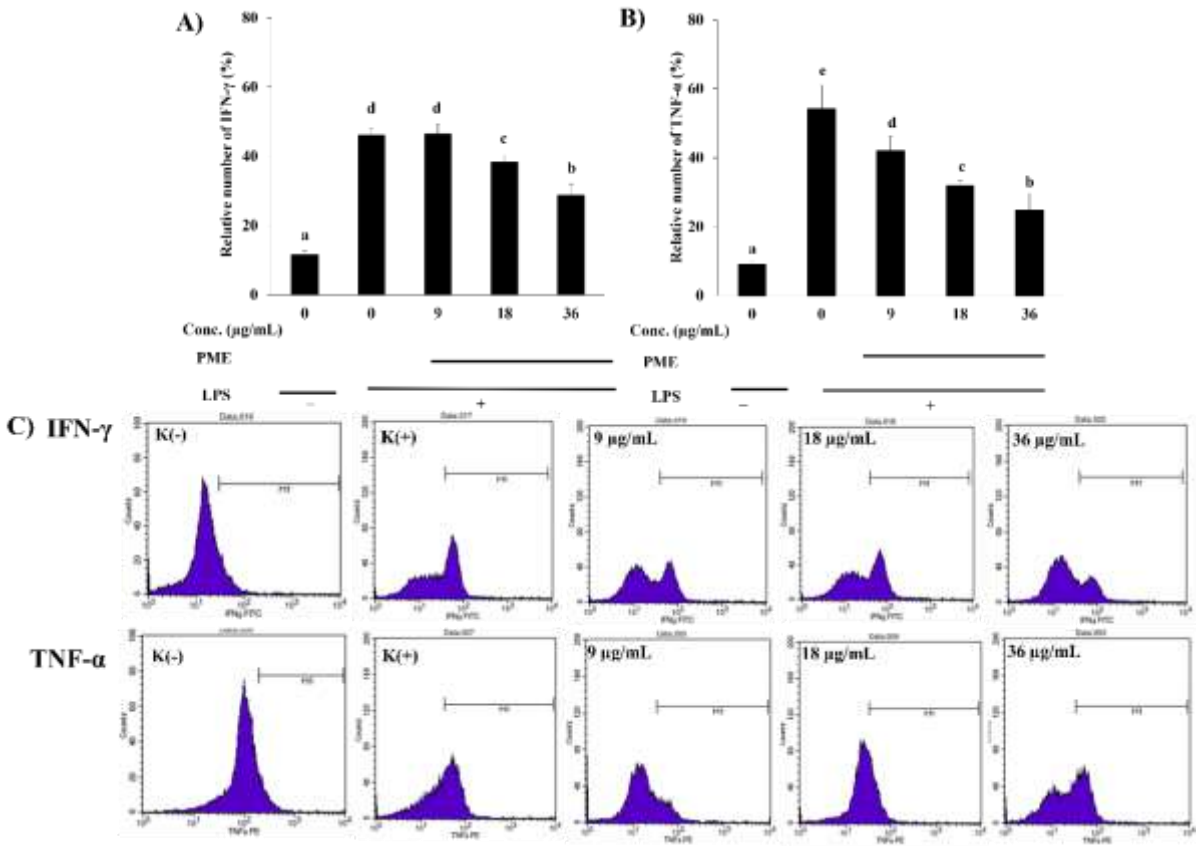

Figure 1: Effects of *P. macrocarpa* leaves ethanol extract on production of IFN-ɣ (A) and TNF-α (B) in LPS-stimulated RAW 264.7 cells. FCM histogram of these cytokines (C). Note: LPS: Lipopolysaccharide; K(-): untreated cells (without LPS induction); K(+): treated cells with LPS induction. PME: treatment with *P. macrocarpa* leaves ethanol extract at concentrations of 9, 18, and 36 µg/mL. Different subsets indicate statistically significant differences between groups ($p < 0.05$)

RAW 264.7 is a murine macrophage cell line widely employed as an in vitro model for investigating inflammatory processes, given the central role of macrophages in orchestrating the onset, progression, and resolution of inflammation. Upon activation by LPS, these cells release a range of pro-inflammatory mediators, such as prostaglandins, nitric oxide (NO), TNF-α, IL-6, IFN-γ, and IL-1β, primarily through stimulation of the NF-κB signaling pathway.[23]

Although the extract effectively suppresses IFN-γ production at 18 and 36 µg/mL, no significant reduction was observed at the lowest dose. This condition may be due to the concentration of bioactive compounds in the extract being insufficient at low doses to modulate the macrophage signaling pathway and suppress IFN-γ production.[24] IFN-γ is a pro-inflammatory cytokine critical for innate and adaptive immunity, and it can stimulate NO production and enhance macrophage activation.[25] Interestingly, IFN-γ levels can be regulated by TGF-*β*.[26] However, the regulation of IFN-γ by *P. macrocarpa* appears to be context- and cell-dependent. For instance, *P. macrocarpa* ethanol extract could stimulate IFN-γ secretion in Natural Killer cells.[27] This duality suggests that different immune cells may respond differently to the same extract, possibly due to the diverse range of bioactive compounds in *P. macrocarpa*.

TNF-α is a central pro-inflammatory cytokine predominantly secreted by M1 macrophages, playing a critical role in promoting inflammation, recruiting immune cells, and contributing to tissue injury when dysregulated. In the present study, treatment with *P. macrocarpa* extract following LPS induction significantly reduced TNF-α levels across all tested concentrations. Persistent overexpression of TNF-α has been implicated in the pathogenesis of autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus.[28,29] Moreover, Kusmardi et al.[30] demonstrated that *P. macrocarpa* leaf extract suppressed excessive TNF-α expression in the colonic mucosa, further supporting its anti-inflammatory potential. The consistent decrease in TNF-α levels observed in this study implies that *P. macrocarpa* leaf extract may exert anti-inflammatory effects by inhibiting NF-κB-mediated cytokine release.

Effects of P. macrocarpa leaves ethanol extract on anti-inflammatory cytokines

The findings revealed no significant difference in TGF-*β* levels between the LPS-stimulated group (0.02 ± 0.02%) and the untreated control group (0.01 ± 0.02%), suggesting that LPS-induced inflammation alone was insufficient to trigger anti-inflammatory cytokine production. In contrast, administration of the ethanol extract of *P. macrocarpa* leaves significantly ($p < 0.05$) increased TGF-*β* expression. The highest production of TGF-*β* was observed in cells after treatment with *P. macrocarpa* leaves ethanol extract at 18 µg/mL (65.85 ± 2.10%), which was significantly higher than at 9 µg/mL (38.26 ± 3.74%) and 36 µg/mL (23.68 ± 5.26%) (Figure 2A). This suggests that an intermediate concentration (18 µg/mL) induced the highest TGF-*β* expression, with diminished effects at the highest dose.

Similarly, IL-10 levels remained low under LPS-induced inflammation (0.65 ± 0.22%) compared to the control (0.25 ± 0.18%), indicating no significant endogenous anti-inflammatory response. However, treatment with *P. macrocarpa* leaf extract resulted in a dose-dependent elevation of IL-10 levels, with percentages of 41.77 ± 4.14% at 9 µg/mL, 79.42 ± 1.13% at 18 µg/mL, and 89.57 ± 2.57% at 36 µg/mL (Figure 2B). Each of these increases was statistically significant compared to the LPS-only control group ($p<0.05$). These findings further support the dual action of the extract, suppressing pro-inflammatory cytokines while simultaneously enhancing anti-inflammatory cytokine production, therefore highlighting its promise as a natural immunomodulatory compound. The observed decrease in pro-inflammatory cytokines may be partially driven by the upregulation of anti-inflammatory mediators.

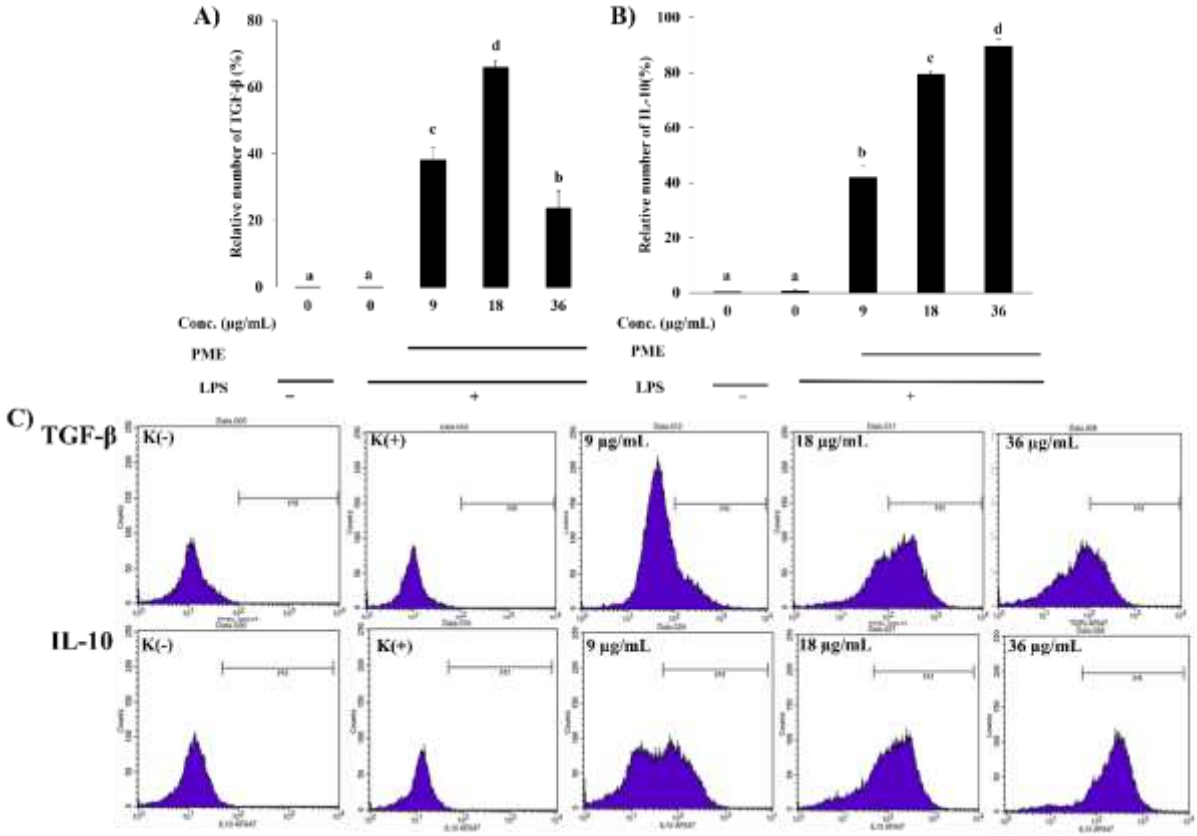

Figure 2: Effects of *P. macrocarpa* leaves ethanol extract on production of IFN-ɣ (A) and TNF-α (B) FCM histogram (C). Note: LPS: Lipopolysaccharide; K(-): untreated cells (without LPS induction); K(+): treated cells with LPS induction. PME: treatment with *P. macrocarpa* leaves ethanol extract at concentrations of 9, 18, and 36 μg/mL. Different subsets indicate statistically significant differences between groups ($p < 0.05$).

In this study, treatment with *P. macrocarpa* leaf extract significantly elevated the levels of IL-10 and TGF-*β* in LPS-stimulated RAW 264.7 macrophages (Figure 2), whereas these cytokines remained low in both untreated and LPS-only groups. These results suggest that the extract not only inhibits pro-inflammatory signaling but also actively enhances anti-inflammatory responses.

IL-10 is a key regulatory cytokine that maintains immune homeostasis and prevents excessive tissue damage by inhibiting IFN-γ and TNF-α.[2,31] In a study using LPS from *B. pseudomallei*, IL-10 upregulation was associated with reductions in TNF-α and IFN-γ levels, while IL-10 neutralization led to increased pro-inflammatory cytokine production.[32] Therefore, the observed decline in pro-inflammatory cytokines in this study may be partially attributed to the upregulation of IL-10 by the extract. The increase in IL-10 expression by macrophages may also act through autocrine negative feedback to reduce IFN-γ and TNF-α, thereby strengthening the immunoregulatory loop. IL-10 produced by macrophages can downregulate the synthesis of pro-inflammatory cytokines within the same cells by modulating signaling pathways downstream of Toll-like receptors (TLRs). The presence of this feedback loop due to autocrine signaling helps maintain immune homeostasis by preventing hyperinflammation.[33]

TGF-*β* acts as an immunosuppressive cytokine that promotes macrophage polarization toward the anti-inflammatory M2 phenotype, while simultaneously suppressing the expression of pro-inflammatory mediators such as TNF-α, IL-6, IL-12, and iNOS.[34,35] In this study, the optimal increase in TGF-*β* expression was observed at 18 μg/mL, while lower and higher doses showed less effect. This may be due to the influence of specific compounds such as apigenin, which is present in *P. macrocarpa* leaves[18] and has been reported to inhibit TGF-*β* signaling in certain cancer cell lines through Smad2/3- and Src-dependent pathways.[36] Apigenin can exhibit pro-inflammatory and anti-inflammatory effects on TGF-*β* production depending on the dose and cell type. At low concentrations, apigenin shows partial inhibition of the TGF-*β* signaling pathway, such as Smad 2/3 phosphorylation and MAPK activation, which allows for certain pro-inflammatory responses. At higher concentrations, apigenin inhibits TGF-*β* more strongly across a broader range of signaling pathways, such as Smad 2/3, MAPK, and NF-κB, resulting in stronger anti-inflammatory effects.[37,38] Therefore, the decrease in TGF-*β* expression at the highest dose may be due to the modulatory effects of such compounds. Research by Shofiah et al.[22] revealed that *P. macrocarpa* bioactive compounds can inhibit PI3K/AKT and MMP9 pathways and result in decreased levels of nitric oxide (NO), inducible nitric oxide synthase (iNOS), and IL-1-*β*. This previous study supports the finding of the study that *P. macrocarpa* leaves extract has a significant anti-inflammatory activity.

The present study only evaluated cytokine expression at the cellular level using RAW 264.7 macrophages. Nevertheless, this study did not investigate the underlying molecular pathways or pinpoint the specific bioactive compounds responsible for the observed effects. Future research should incorporate *in silico* approaches, such as molecular docking and dynamic simulations, to predict interactions between the extract's constituents and the receptors of TNF-α, IL-10, TGF-β, and IFN-γ. In addition, bioactive compounds can be profiled using LC-MS/MS. Validation through *in vivo* studies with inflammatory disease modeling in experimental animals can also be conducted for further research.

Conclusion

The ethanol extract of *P. macrocarpa* leaves demonstrates potential in modulating inflammatory responses through the modulation of pro-inflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-*β*) in LPS-induced RAW 264.7 macrophage cells. At the concentration of 18 μg/mL, the extract significantly inhibited IFN-γ and enhanced TGF-*β* production. In contrast, the

reduction in TNF-α and the increase in IL-10 levels showed a clear dose-dependent relationship with extract concentration. These findings support the anti-inflammatory potential of *P. macrocarpa* leaves extract *in vitro*. However, further investigation is necessary to identify its active compounds and elucidate the molecular mechanisms underlying its immunomodulatory effects. Given the critical roles of these cytokines in the pathogenesis of chronic inflammatory diseases, their modulation may offer potential therapeutic benefits.

Conflict of Interest
The author`s declare no conflict of interest.

Authors' Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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