



ANTI-INFLAMMATORY EFFECTS OF *Cleistocalyx nervosum* VAR. *paniala* FRUIT EXTRACT ON LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN MURINE MACROPHAGE (RAW 264.7) CELLS

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ABSTRACT

Non-communicable diseases (NCDs) are a leading cause of mortality worldwide, and chronic inflammation plays a critical role in their pathogenesis. Although conventional anti-inflammatory drugs are widely used, their long-term use is associated with adverse effects, highlighting the need for safer therapeutic alternatives. Plant-derived phytochemicals have attracted considerable interest due to their anti-inflammatory properties. *Cleistocalyx nervosum* var. *paniala*, or Ma-Kiang, is a native Thai berry that has been reported to possess anti-inflammatory potential. However, its effects on lipopolysaccharide (LPS)-induced inflammation in murine macrophage (RAW 264.7) cells have not been studied. Therefore, this study aimed to investigate the anti-inflammatory effects of the 90% ethanolic extract of Ma-Kiang fruit (MKE) in LPS-induced RAW 264.7 macrophages. Based on the preliminary MTT assay results, non-cytotoxic concentrations ranging from 25 to 100 µg/mL, with 100 µg/mL representing the maximum non-cytotoxic concentration, were selected for subsequent anti-inflammatory assays. Cells were pre-treated with MKE at 25, 50, and 100 µg/mL for 1 h, followed by stimulation with LPS (10 ng/mL) for 24 h. MKE at 50 and 100 µg/mL significantly ($p < 0.05$) attenuated LPS-induced inflammation in a dose-dependent manner. The strongest inhibitory effect was observed at 100 µg/mL, reducing the production of NO (67%), IL-6 (65%), TNF- α (54%), IL-1 β (63%), MCP-1 (68%), and PGE2 (76%), along with the downregulation of iNOS and COX-2 expression. These findings demonstrate that MKE exerts significant anti-inflammatory effects; however, because this study was conducted in vitro, further investigations in animal models and human studies are required to confirm its efficacy.

Keywords: non-communicable diseases, *Cleistocalyx nervosum* var. *paniala*, inflammatory response, anti-inflammation, murine macrophage (RAW 264.7) cells

Received: 17 April 2026; Revised: 31 July 2026; Accepted: 7 August 2026

Introduction

Non-communicable diseases (NCDs), including cancer, cardiovascular diseases, and diabetes, are leading causes of mortality worldwide, including in Thailand.¹⁻³ Prolonged exposure to unhealthy lifestyle factors, such as smoking, alcohol consumption, psychological stress, and a high-fat diet, has been identified as a major contributor to the development of these chronic conditions.^{1,2} These risk factors are closely associated with increased production of inflammatory mediators during sustained inflammatory responses. Inflammation is a protective response to harmful stimuli; however, persistent or dysregulated inflammation leads to excessive production of reactive oxygen species (ROS) and inflammatory mediators, contributing to tissue damage and the development of chronic diseases. Macrophages play a central role in this process. Lipopolysaccharide (LPS), a component of Gram-negative bacteria, is widely used to induce inflammation in experimental models. LPS activates macrophages via nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, leading to increased production of ROS and pro-inflammatory mediators, including tumor necrosis factor- α (TNF- α), interleukins such as IL-6 and IL-1 β , monocyte chemoattractant protein-1 (MCP-1), as well as upregulation of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), resulting in enhanced production of nitric oxide (NO) and prostaglandin E2 (PGE2).⁴⁻⁶ Although conventional anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), are widely used, their long-term use is associated with adverse effects and limited efficacy in chronic low-grade inflammation.⁷⁻⁹ Therefore, safer alternatives are needed. Plant-derived phytochemicals, including phenolic acids, flavonoids, carotenoids, and anthocyanins, have demonstrated antioxidant and anti-inflammatory activities by modulating ROS and inflammatory mediators, highlighting their potential role in preventing and managing chronic diseases.^{10,11}

Cleistocalyx nervosum var. *paniala* (Ma-Kiang), a fruit of the Myrtaceae family, is native to Thailand and is widely distributed in Southeast Asia.^{12,13} It is characterized by its vibrant purple color and high antioxidant content, particularly anthocyanins.¹²

Previous studies have demonstrated that Ma-Kiang fruit extract exhibits various biological activities, including inhibition of cancer cell growth^{14,15} and attenuation of inflammation associated with metabolic disorders, in part through modulation of inflammatory mediators such as IL-1 β .¹⁶⁻¹⁸ In addition, Ma-Kiang-derived products have been reported to improve lipid profiles and enhance antioxidant status in rats with dyslipidemia.¹⁹ Moreover, ripe fruit extract of *C. nervosum* var. *paniala* has been shown to prevent hydrogen peroxide-induced DNA damage in human monocyte U937 cells, further supporting its antioxidant and cytoprotective properties.²⁰ In addition to these general health benefits, accumulating evidence has highlighted its anti-inflammatory potential. Extracts of *C. nervosum* var. *paniala* have been reported to suppress inflammatory mediators and attenuate inflammation-associated disorders, in part by modulating inflammatory signaling pathways, including NF- κ B.^{17,18} These findings suggest that Ma-Kiang may be a promising natural source for the prevention and management of inflammation-related diseases.

However, the effects of Ma-Kiang fruit extract on LPS-induced inflammation in RAW 264.7 cells have not yet been studied. Therefore, this study aimed to investigate the anti-inflammatory effects of Ma-Kiang fruit extract in LPS-induced RAW 264.7 macrophages.

Materials and methods

1. Chemicals and reagents

All supplements for RAW 264.7 cells were obtained from Gibco (Grand Island, NY 14072, USA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). All ELISA kits were from BioLegend

(San Diego, CA, USA). The antibodies iNOS (D6B6S) (#13120), COX-2 (#4842), and β -actin (13E5) (#4967) for Western blot were purchased from Cell Signaling Technology (Danvers, MA, USA).

2. Sample preparation and extraction

Ma-Kiang fruits were obtained from the Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG) and harvested in Lampang province, Thailand, during July–August. Botanical identification and authentication of the plant material were performed by Assistant Professor Dr. Thaya Jenjittikul, Department of Plant Science, Faculty of Science, Mahidol University, and a voucher specimen (No. 9428) was deposited at Suan Luang Rama IX Herbarium, Bangkok, Thailand. Only ripe and undamaged fruits were selected. The fruits were washed with deionized (DI) water, and the pulp was separated and homogenized. The homogenized samples were lyophilized to dryness, ground into powder, vacuum-sealed in aluminum foil, and stored at -20°C until used. For extraction, the freeze-dried powder (1 g) was macerated with 90% ethanol at a 1:15 (w/v) ratio at room temperature overnight. The mixture was then centrifuged at 4,600 rpm for 10 min, and the supernatant was collected. The extraction was repeated twice, and the combined extracts were concentrated under reduced pressure using a rotary evaporator (Büchi Rotavapor, USA) at 40°C . The residue was re-dissolved in 95% ethanol and dried under a nitrogen stream. The final extract (MKE) was stored at -20°C until use. The extraction yield was 32% (w/w, dry weight basis).

3. Cell culture

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA 20110-2209, USA). RAW 264.7 cells were cultured in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), along with 100 units/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin. They were maintained in an environment with 5%

CO_2 at 37°C . The cells reached more than 90% confluence before use for experimentation.

4. Cytotoxicity of MKE on the cell viability of RAW 264.7 cells

RAW 264.7 cells were seeded into a 48-well plate at a density of 300,000 cells/well and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 24 h. Cells were incubated with MKE at 10, 50, 100, 200, and 500 $\mu\text{g/mL}$ containing 0.5% DMSO (v/v) in DMEM for 24 h, and cell viability was determined using the MTT assay. The final concentration of DMSO was maintained at 0.5% in all treatment groups, including the vehicle control. Cell viability analysis confirmed that 0.5% DMSO did not induce cytotoxicity, suggesting that the observed biological effects were attributable to MKE rather than the solvent. Three independent experiments were performed, and the highest non-cytotoxic concentration was used in subsequent experiments. After MKE treatment, cells were washed with PBS and incubated with MTT solution (0.5 mg/mL in PBS) for 3 h. The formed formazan crystals were dissolved in DMSO, and absorbance was measured at 540 nm using a microplate reader. Cell viability was calculated relative to the control group. To minimize potential interference from the extract, blank wells containing culture medium and the corresponding extract concentration without cells were included and processed identically. The absorbance obtained from these blanks was subtracted from the sample readings before data analysis.

5. Evaluation of the effects of LPS on cell viability and NO production in RAW 264.7 cells

RAW 264.7 cells were seeded into a 48-well plate at a density of 300,000 cells/well and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 24 h. Cells were incubated with LPS at 10, 50, 100, 500, and 1,000 ng/mL for 24 h. NO levels and cell viability were determined using the Griess reagent and MTT assay, respectively. DMEM was used as a control. Three independent experiments were performed, with each treatment conducted in

triplicate. The lowest LPS concentration that significantly increased NO production without inducing cytotoxicity was selected for subsequent experiments.

6. Evaluation of the anti-inflammatory effects of MKE in LPS-stimulated RAW 264.7 cells

RAW 264.7 cells were seeded at a density of 300,000 cells/well in 48-well plates for the determination of cell viability and inflammatory marker levels, and at a density of 1,500,000 cells/well in 6-well plates for the measurement of iNOS and COX-2 expression. After incubation for 24 h, cells were pretreated with MKE at 25, 50, and 100 µg/mL (non-toxic) for 1 h, then treated with LPS at 10 ng/mL. After 24 h of incubation, cells were washed with cold PBS, and cell lysates were collected to assess iNOS and COX-2 expression. Culture media were collected to determine NO secretion using the Griess reagent, and TNF-α, IL-6, IL-1β, MCP-1, and PGE2 were measured using ELISA kits. Experiments were performed in three independent experiments.

7. Western blot analysis

Cell protein lysates were prepared using a lysis buffer containing protease and phosphatase inhibitors, followed by centrifugation at 14,000 × g for 10 min at 4 °C. The supernatants were collected, and protein concentrations were determined using a BCA assay. Equal amounts of protein (40 µg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk and incubated overnight at 4 °C with primary antibodies (1:1000), followed by incubation with secondary antibodies (1:2000). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system and captured on X-ray film. Band intensities of iNOS and COX-2 were quantified using ImageJ software and normalized to β-actin as a loading control. Results were expressed relative to the control group.

8. Enzyme-Linked Immunosorbent Assay (ELISA) test

The secretion of IL-6, TNF-α, IL-1β, MCP-1, and PGE2 in the culture medium was quantified using ELISA kits according to the manufacturer's instructions. Briefly, 96-well plates were coated with capture antibodies overnight at 4 °C and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Samples or standards were added, incubated at 25 °C for 2 h, and then incubated with biotinylated detection antibodies. The immune complexes were subsequently incubated with streptavidin–horseradish peroxidase (HRP) and visualized using a tetramethylbenzidine (TMB) substrate. The reaction was stopped with 2 M H₂SO₄, and absorbance was measured at 450 nm using a microplate reader.

9. Statistical analysis

Statistical analysis was performed using SPSS Statistics version 30 (IBM Corp., Armonk, NY, USA). All experiments were performed in three independent experiments, with each treatment condition tested in triplicate wells. Data are presented as mean ± standard deviation (SD) from three independent experiments. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Scheffé's post hoc test. A *p*-value < 0.05 was considered statistically significant.

Results

1. Effects of MKE on the cell viability of RAW 264.7 cells

To evaluate the cytotoxicity of MKE in RAW 264.7 cells, cell viability was assessed following treatment with MKE at 10–500 µg/mL for 24 h using the MTT assay. MKE at 10, 50, and 100 µg/mL did not exhibit cytotoxic effects (Figure 1). In contrast, the higher concentrations of 200 and 500 µg/mL significantly decreased cell viability in RAW 264.7 cells compared to the control (Figure 1). Therefore, 100 µg/mL was identified as the maximum non-cytotoxic concentration, and concentrations of 25, 50, and 100 µg/mL were selected for subsequent anti-inflammatory assays.

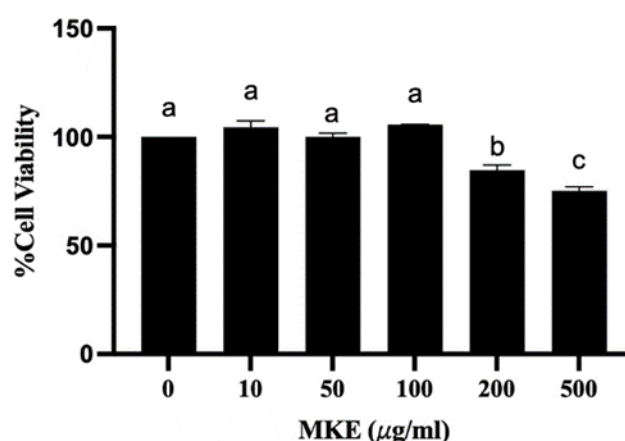


Figure 1 Effects of MKE on cell viability of RAW 264.7 cells. Cells were treated with various concentrations of MKE (10–500 µg/mL) for 24 h. DMEM was used as a control. Cell viability was measured by the MTT assay. Graphs represent average cell viability (mean $\pm$ SD; $n = 3$). Different superscript letters (a-c) above the bars indicate significant differences between groups at $p < 0.05$.

2. Effect of LPS on NO production and cell viability of RAW 264.7 cells

To determine the optimal LPS concentration for inflammatory stimulation in RAW 264.7 cells, NO production and cell viability were evaluated. Cells were incubated with LPS at 10, 50, 100, 500, and 1,000 ng/mL for 24 h. The results showed that LPS treatment at concentrations of 10–1,000 ng/mL significantly increased NO production compared with the control (Figure 2A). In addition, LPS at all tested concentrations did not significantly affect cell viability compared to the untreated control (Figure 2B). Based on these results, 10 ng/mL LPS was selected for subsequent experiments, as it was the lowest concentration that significantly increased NO production without affecting cell viability.

3. Effects of MKE on NO production and iNOS expression in LPS-induced RAW 264.7 cells

To evaluate the anti-inflammatory effects of MKE, key inflammatory markers, including NO production and iNOS expression, were assessed in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were pre-treated with MKE at 25, 50, and 100 µg/mL for 1 h, followed by stimulation with LPS at 10 ng/mL for 24 h. The results demonstrated that LPS

treatment significantly increased NO production and iNOS expression ($p < 0.05$) compared with the control group (Figure 3), indicating that LPS effectively induced an inflammatory response. Pre-treatment with MKE at 50 and 100 µg/mL significantly ($p < 0.05$) reduced NO production and iNOS expression, whereas no significant effect was observed at 25 µg/mL (Figure 3). Notably, MKE alone did not significantly differ from the control group. These findings suggest that MKE exerts anti-inflammatory effects by inhibiting NO production through the downregulation of iNOS expression in LPS-stimulated RAW 264.7 macrophages.

4. Effects of MKE on PGE2 secretion and COX-2 expression in LPS-induced RAW 264.7 cells

To further evaluate the anti-inflammatory effects of MKE, key inflammatory mediators involved in the COX-2 pathway, including PGE2 production and COX-2 expression, were assessed in LPS-stimulated RAW 264.7 macrophages. The results demonstrated that LPS treatment significantly increased PGE2 production and COX-2 expression compared with the control group ($p < 0.05$) (Figure 4), confirming the successful induction of an inflammatory response. Pre-treatment with MKE at

50 and 100 $\mu\text{g/mL}$ significantly ($p < 0.05$) reduced PGE2 production and COX-2 expression, whereas no significant effect was observed at 25 $\mu\text{g/mL}$ (Figure 4). Notably, MKE alone did not significantly differ from

the control group. These findings suggest that MKE exerts anti-inflammatory effects by suppressing PGE2 production through the downregulation of COX-2 expression in LPS-stimulated RAW 264.7 macrophages.

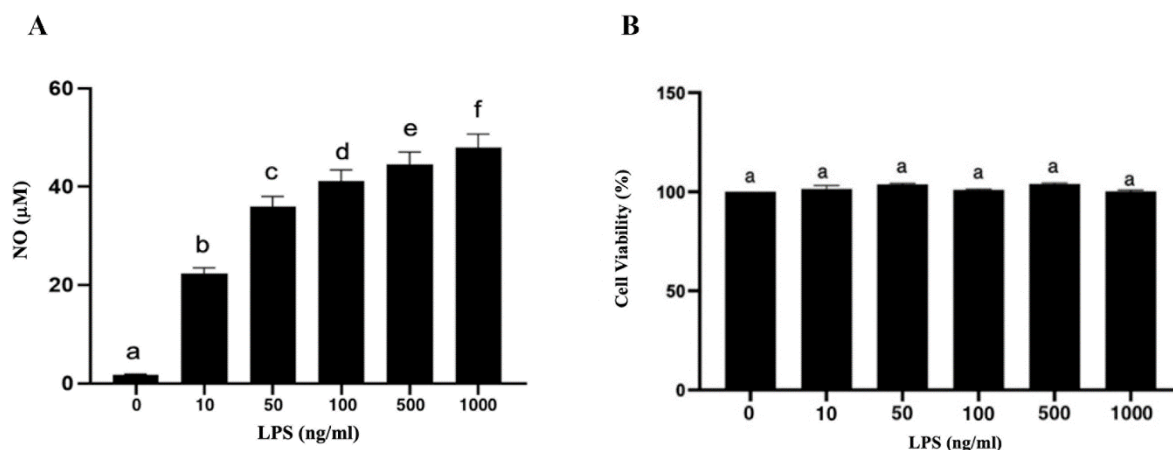


Figure 2 Effects of LPS on NO production and cell viability of RAW 264.7 cells. Cells were treated with various concentrations of LPS (10–1,000 ng/mL) for 24 h. DMEM was used as a control. (A) NO production was evaluated with Griess reagent, and (B) cell viability was measured by the MTT assay. Graphs represent average cell viability (mean $\pm$ SD; $n = 3$). Different superscript letters (a–f) above the bars indicate significant differences between groups at $p < 0.05$.

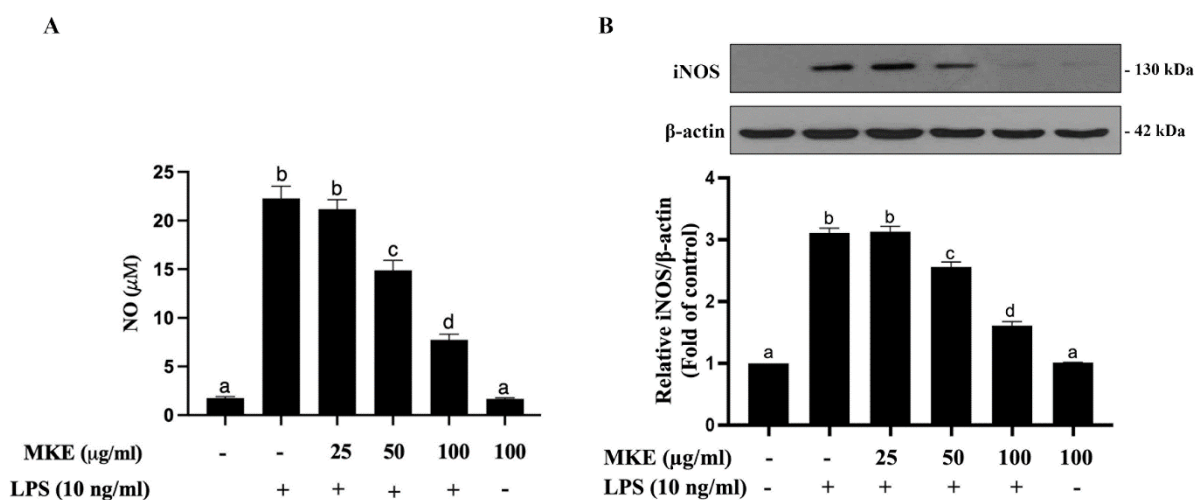


Figure 3 Effects of MKE on NO production and iNOS expression in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pre-treated with MKE at 25, 50, and 100 $\mu\text{g/mL}$ for 1 h, followed by stimulation with LPS at 10 ng/mL for 24 h. DMEM containing 0.5% DMSO was used as a control. (A) NO production was evaluated with Griess reagent, and (B) iNOS expression was determined by Western blot. Graphs represent mean $\pm$ SD ($n = 3$). Different superscript letters (a–d) above the bars indicate significant differences between groups at $p < 0.05$.

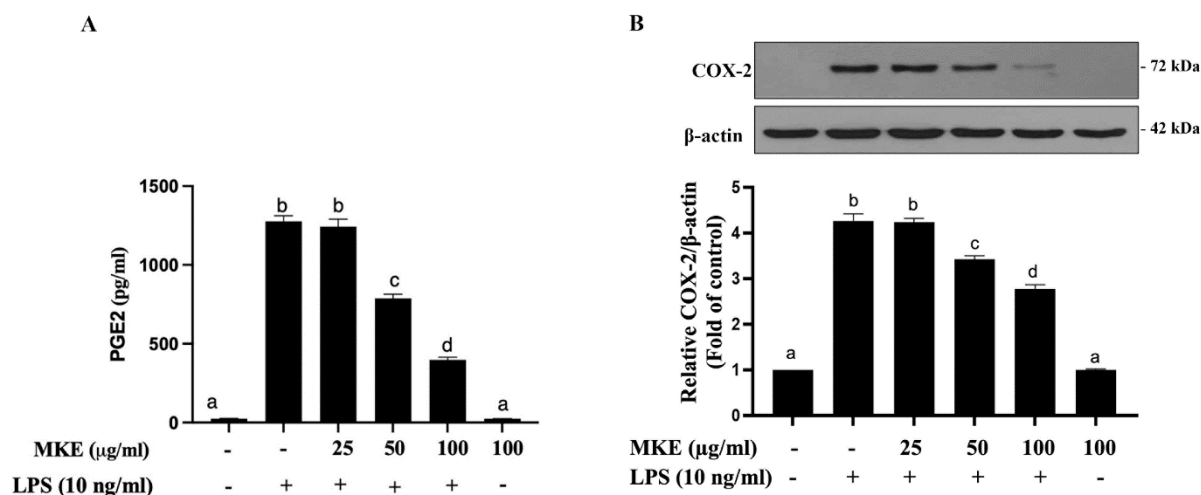


Figure 4 Effects of MKE on PGE2 production and COX-2 expression in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pre-treated with MKE at 25, 50, and 100 µg/mL for 1 h, followed by stimulation with LPS at 10 ng/mL for 24 h. DMEM containing 0.5% DMSO was used as a control. (A) PGE2 production was evaluated with an assay kit, and (B) COX-2 expression was determined by Western blot. Graphs represent the mean concentrations of PGE2 and relative COX-2 expression levels (mean $\pm$ SD; $n = 3$). Different superscript letters (a-d) above the bars indicate significant differences between groups at $p < 0.05$.

5. Effects of MKE on inflammatory cytokine and chemokine secretion in LPS-induced RAW 264.7 cells

To further evaluate the anti-inflammatory effects of MKE, key inflammatory mediators, including pro-inflammatory cytokines (IL-6, TNF- α , and IL-1 β) and the chemokine MCP-1, were measured in LPS-stimulated RAW 264.7 macrophages. The results showed that LPS treatment significantly increased the secretion of IL-6, TNF- α , IL-1 β , and MCP-1 ($p < 0.05$) compared with the control group (Figure 5), confirming the successful induction of an inflammatory response. Pre-treatment with MKE at 50 and 100 µg/mL significantly ($p < 0.05$) attenuated the secretion of IL-6, TNF- α , IL-1 β , and MCP-1, whereas no significant effect was observed at 25 µg/mL (Figure 5). Notably, MKE alone did not significantly differ from the control group. These findings suggest that MKE exerts anti-inflammatory effects by suppressing the secretion of IL-6, TNF- α , IL-1 β , and MCP-1 in LPS-stimulated RAW 264.7 macrophages.

Discussion

The present study demonstrates that MKE exerts significant anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages, as evidenced by dose-dependent suppression of NO, PGE2, TNF- α , IL-6, IL-1 β , and MCP-1 production, together with downregulation of iNOS and COX-2 expression. These findings support the potential of Ma-Kiang fruit as a natural anti-inflammatory agent for the prevention and management of inflammation-related NCDs.

Cleistocalyx nervosum var. *paniala*, commonly known in Thailand as "Ma-Kiang," is a native berry belonging to the Myrtaceae family, characterized by its purple pigmentation and rich anthocyanin content, particularly cyanidin-3-glucoside (C3G).^{12,13} Excessive NO production, driven by upregulation of iNOS in activated macrophages, is a hallmark of the inflammatory response.²¹ In this study, LPS markedly increased NO production and iNOS expression, whereas MKE treatment attenuated both in a dose-dependent manner, suggesting that MKE suppresses

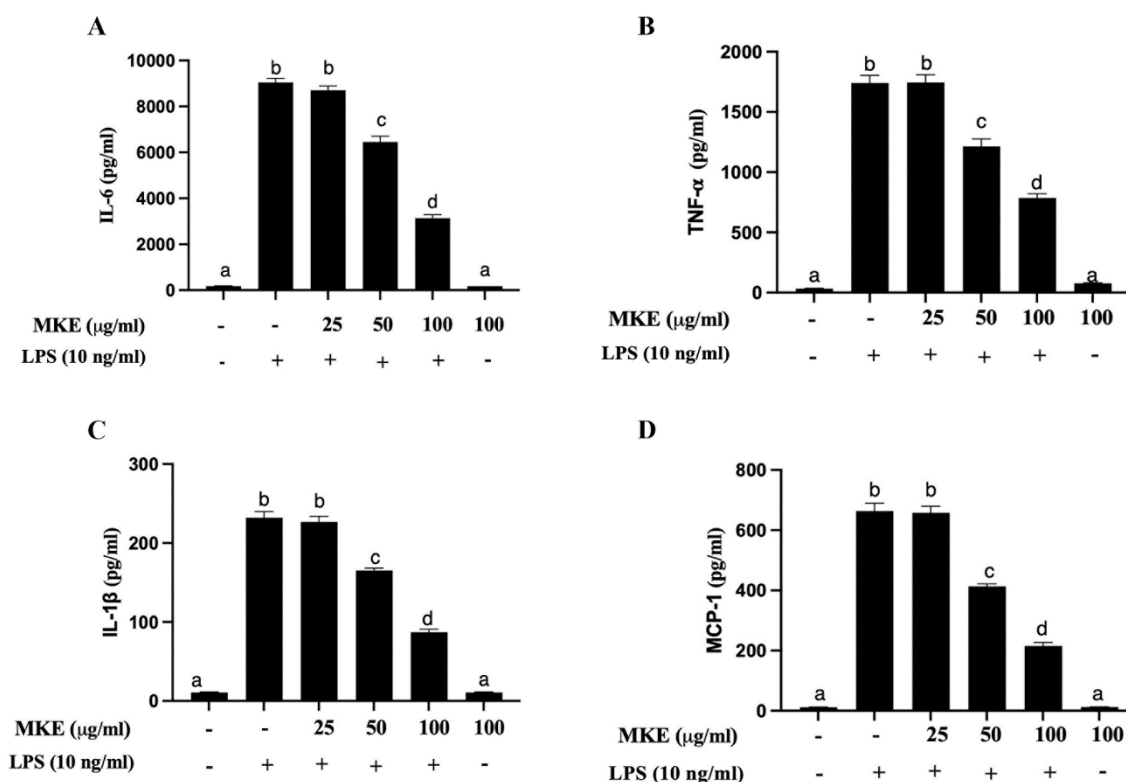


Figure 5 Effects of MKE on inflammatory cytokine and chemokine secretion in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pre-treated with MKE at 25, 50, and 100 $\mu\text{g/mL}$ for 1 h, followed by stimulation with LPS at 10 ng/mL for 24 h. DMEM containing 0.5% DMSO was used as a control. (A) IL-6, (B) TNF- α , (C) IL-1 β , and (D) MCP-1 secretion were evaluated using an ELISA kit. Graphs represent the levels of inflammatory cytokines and chemokines (mean $\pm$ SD; $n = 3$). Different superscript letters (a-d) above the bars indicate significant differences between groups at $p < 0.05$.

inflammation in part by inhibiting the iNOS/NO pathway. A parallel mechanism was observed for the COX-2/PGE2 cascade: COX-2 mediates the synthesis of PGE2, a key pro-inflammatory prostaglandin that contributes to tissue damage,²² and MKE significantly suppressed both COX-2 expression and PGE2 production in LPS-stimulated cells, indicating that MKE also modulates this downstream inflammatory pathway. Beyond these two pathways, MKE reduced the secretion of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6, and the chemokine MCP-1, which respectively drive inflammatory signaling and immune cell recruitment, pointing to a broader suppressive effect across multiple arms of the inflammatory response. These results are consistent

with earlier reports that C3G suppresses LPS-induced NO production and iNOS/COX-2 expression in RAW 264.7 macrophages; Jung et al. reported reductions of 41.9% in NO and 51.7% in PGE2 through this mechanism.²³ Similarly, anthocyanins have been shown to attenuate LPS-induced TNF- α , IL-6, and IL-1 β secretion via suppression of NF- κ B and MAPK signaling,^{24,27} pathways that regulate transcription of iNOS, COX-2, and pro-inflammatory cytokines in activated macrophages.^{4,5} Taken together, the anti-inflammatory effects of MKE observed here may involve modulation of NF- κ B and MAPK signaling, likely mediated by its anthocyanin content, particularly C3G, previously identified as a major anthocyanin in this fruit;¹³ however, NF- κ B and MAPK

activation were not directly examined in this study, and this mechanistic link warrants further investigation. Because the phytochemical profile of the MKE extract was not characterized, the contribution of anthocyanins remains inferred from prior literature rather than confirmed, and other constituents such as phenolic compounds and flavonoids may also contribute, potentially through additive or synergistic effects. Future studies should characterize the extract's active compounds and include a standard anti-inflammatory drug as a positive control to further validate these findings.

Conclusion

In conclusion, MKE exerts significant anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages by suppressing the production of key inflammatory mediators, including NO, PGE₂, TNF- α , IL-6, IL-1 β , and MCP-1. These effects are associated with the downregulation of iNOS and COX-2 expression, suggesting that MKE modulates inflammatory responses at both functional and molecular levels. Therefore, MKE may serve as a potential natural agent for the prevention and management of inflammation-related non-communicable diseases. However, further in vivo and clinical studies are required to confirm its efficacy and safety.

Acknowledgements

Biological Science and Animal Model Unit, Mahidol University, Nakhon Pathom, Thailand, and the Faculty of Medicine Ramathibodi Hospital and Institute of Nutrition, Mahidol University, Bangkok, Thailand.

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