

Regulation of anti-inflammatory and antioxidant responses by methanol extract of *Mikania cordata* (Burm. f.) B. L. Rob. leaves via the inactivation of NF- κ B and MAPK signaling pathways and activation of Nrf2 in LPS-induced RAW 264.7 macrophages

Eunjeong Kang^a, Junho Lee^a, Sumin Seo^b, Salah Uddin^c, Sangwoo Lee^d, Sang Beom Han^b, Sayeon Cho^{a,*}

^a Laboratory of Molecular and Pharmacological Cell Biology, College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

^b Laboratory of Biomedical Mass Spectrometry, College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

^c Ethnobotanical Database of Bangladesh, Tejgaon Dhaka, 1208, Bangladesh

^d International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 34141, Republic of Korea

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ABSTRACT

Mikania cordata (Burm. f.) B.L. Rob. has been traditionally used in tropical countries throughout Asia and Africa to treat gastric ulcers, dyspepsia, and dysentery. However, the mechanisms responsible for its anti-inflammatory and antioxidant activities are not fully understood. Therefore, this study sought to investigate the anti-inflammatory and antioxidant effects of methanol extracts of *M. cordata* (MMC) on inflammation and oxidative stress in lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages and elucidate its underlying regulatory mechanism. MMC significantly suppressed the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂) in LPS-stimulated RAW 264.7 macrophages by downregulating the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) at both the mRNA and protein levels. Moreover, MMC effectively reduced the mRNA expression levels and production of pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α (TNF- α). These suppressive effects of MMC on pro-inflammatory mediators and cytokines were mediated through the inhibition of transforming growth factor beta-activated kinase 1 (TAK1), which subsequently blocked the activation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs). MMC also upregulated the nuclear factor erythroid-2-related factor 2 (Nrf2) by inducing the degradation of Kelch-like ECH-related protein 1 (Keap1), an Nrf2-specific E3 ligase. Accordingly, MMC enhanced Nrf2 target gene expression of anti-oxidative regulators such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1). However, it had minimal effect on the DPPH radical scavenging capacity in vitro. Collectively, these findings demonstrate that MMC holds promise as a potential therapeutic agent for alleviating inflammation-related diseases and oxidative stress.

Abbreviations: MMC, methanol extract of *Mikania cordata*; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; I κ B, inhibitor of NF- κ B; ERK, extracellular signal-regulated kinase; TLR, toll-like receptor; AP-1, activator protein 1; ERK, extracellular signal-regulated kinase; TAK1, transforming growth factor- β -activated kinase 1; NO, nitric oxide; iNOS, inducible NO synthase; COX-2, cyclooxygenase-2; TNF- α , tumor necrosis factor- α ; PGE₂, prostaglandin E₂; IL-6, interleukin-6; IL-1 β , interleukin-1 beta; Keap1, Kelch-like ECH-related protein 1; GFP, green fluorescent protein; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; ANOVA, one-way analysis of variance; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-2-related factor 2; NSAIDs, non-steroidal anti-inflammatory drugs.

* Corresponding author.

E-mail address: sycho@cau.ac.kr (S. Cho).

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1. Introduction

Inflammation is a physiological process that plays a crucial protective role against harmful stimuli [1], which contributes to over half of all deaths caused by inflammation-related diseases [2]. Dysregulation of inflammation has been linked to several chronic conditions (e.g., cancer, cardiovascular diseases, diabetes, rheumatoid arthritis, chronic obstructive pulmonary disease) and acute conditions such as acute lung injury [1]. Conventional treatments often involve non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids [3]. However, these options come with drawbacks such as gastrointestinal ulcers, hyperglycemia, and steroid withdrawal symptoms [3]. This motivates the search for natural compounds mimicking NSAID effects with fewer side effects.

During inflammation, macrophages, which are key components of the innate immune system, act as the first line of defense against pathogens. Particularly, macrophages are activated by external stimuli such as lipopolysaccharide (LPS) [4]. LPS binding to toll-like receptor 4 (TLR4) induces dimerization of the TLR4-MD2 complex, activating intracellular Toll/interleukin (IL)-1R homology domains and recruiting adaptor molecules such as myeloid differentiation factor 88 [5–7]. This triggers innate immune responses in macrophages, including antigen presentation and phagocytosis [6]. Additionally, macrophages produce pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E₂ (PGE₂) via inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively, as well as cytokines such as interferon-gamma, tumor necrosis factor (TNF)- α , IL-6, IL-10, IL-12, and IL-1 β [8]. TLR4-mediated inflammation is initiated through the activation of transcriptional pathways, primarily involving nuclear factor-kappa B (NF- κ B) and activator protein-1 (AP-1) as key transcription factors in macrophages [9].

NF- κ B and AP-1 signals are activated via distinct cascades. The inhibitor of NF- κ B (I κ B) kinase (IKK) complex generally promotes NF- κ B and I κ B phosphorylation [10]. Unphosphorylated I κ B sequesters NF- κ B subunits p50 and p65 in the cytoplasm [10]. Phosphorylation of I κ B α at Ser-32/36 leads to ubiquitin-dependent protein degradation, translocating NF- κ B to the nucleus, and activating its transcriptional activity. Among the various phosphorylation sites of NF- κ B, Ser-536 of NF- κ B p65 is highly inducible in response to inflammatory stimuli [11]. Furthermore, phosphorylation of this site is associated with increased NF- κ B activity through the TLR4 signaling pathway [12]. AP-1 is activated by mitogen-activated protein kinase (MAPK) signaling pathways involving extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) 1/2/3, and p38 [13–15]. Sequential phosphorylation of MAPK kinase and MAPK kinase generally activates each MAPK [16,17]. Both NF- κ B and MAPK signaling pathways are activated by transforming growth factor- β -activated kinase 1 (TAK1) [15].

Furthermore, hyperactivated macrophages release reactive oxygen species (ROS) along with pro-inflammatory mediators and cytokines [18]. Although a moderate ROS level is necessary for proper redox signaling, cell development, and differentiation, excessive ROS production leads to oxidative stress, which plays a crucial role in chronic inflammation associated with various diseases [19,20]. Kelch-like ECH-related protein 1 (Keap1)/nuclear factor erythroid 2-related factor (Nrf2) is one of the most common antioxidant signaling pathway [20]. Under oxidative stress, Keap1 undergoes degradation or a conformational change that leads to the dissociation of Nrf2 from Keap1 [21,22], promoting its nuclear translocation and inducing the expression of antioxidant genes such as heme oxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase (NQO1) [23]. Nrf2 and antioxidant genes collectively reduce inflammation by scavenging ROS and detoxifying hazardous substances, underscoring the significance of identifying natural compounds that activate Nrf2.

Plant-based natural products have a long history of clinical trials and are a prominent focus in clinical research [24]. *Mikania cordata* (Burm. f.) B.L. Rob., a rapidly growing woody perennial plant widely distributed

across Eastern Africa and Southeast Asia [25,26], has been traditionally used to treat several conditions such as coughs, swelling, wounds, dysentery, and itching [27]. Phytochemical analyses have revealed that the members of the genus *Mikania* contain terpenes, tannins, sterols, and flavonoids, which are known for their anti-inflammatory effects [25]. Ethanol extracts of *M. cordata* leaves have also been reported to possess several therapeutic properties, including antibacterial, anti-diarrheal, anti-ulcer, anti-cancer, and anti-nociceptive activities [28–31]. The roots of this plant have also been reported to possess anti-inflammatory and antipyretic effects in rodents, in addition to functioning as a central nervous system depressant [25,27,32,33]. Moreover, the entire plant exhibits antiemetic and anthelmintic effects [34].

Despite its longstanding use in traditional medicine and the findings of recent research, the ethnopharmacological anti-inflammatory and antioxidant effects of *M. cordata* have not been investigated at the molecular level. Hence, the objective of this study was to assess the anti-inflammatory and antioxidant properties of methanol extracts of *M. cordata* (MMC). Additionally, this study aimed to elucidate the precise molecular mechanisms of this extract on anti-inflammatory and antioxidant signaling pathways in LPS-stimulated RAW 264.7 macrophages.

2. Materials and methods

2.1. MMC preparation

M. cordata was collected in Junadpur village, Noakhali district, Chittagong, Bangladesh, and identified in the Ethnobotanical Database of Bangladesh. A voucher specimen (accession number KRIB 0043252) was deposited at the herbarium of the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Korea). The collected *M. cordata* specimens were dried at room temperature, cut into suitable sizes, and ground with a grinder. For extraction, 1 L of high-performance liquid chromatography (HPLC) grade methanol was added to 100 g of ground sample. The mixture underwent 20 min of sonication and was then allowed to rest for 2 h at 45 °C. This process was repeated for three consecutive days. The sample was then filtered through a cotton-plugged funnel. A portion of the filtrate was concentrated under reduced pressure at 45 °C and stored in a refrigerated bottle. Finally, a total of 7.11 g of MMC was obtained by freeze-drying. For subsequent experiments, the extract was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The final concentration of DMSO in the experiments did not exceed 0.1 % in the medium.

2.2. Cell culture

RAW 264.7 macrophages were purchased from ATCC (VA, USA). RAW 264.7 macrophages and HEK 293 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, CA, USA) supplemented with 10 % fetal bovine serum (Invitrogen, CA, USA) and 1 % penicillin/streptomycin (Life Technologies, NY, USA). The cells were incubated in a humidified incubator containing 5 % CO₂ at 37 °C.

2.3. Antibodies and reagents

LPS and DMSO were purchased from Sigma-Aldrich (MO, USA). The following antibodies were used in this study: mouse monoclonal anti-iNOS (cat no. sc-7271), goat polyclonal anti-COX-2 (cat no. sc-1745), mouse monoclonal anti-JNK (cat no. sc-7345), mouse monoclonal anti-phospho (p)-I κ B α (Ser32/36; cat no. sc-8404), mouse monoclonal anti-I κ B α (cat no. sc-1643), mouse monoclonal NF- κ B p65 (cat no. sc-8008), mouse monoclonal IKK β (cat no. sc-8014), mouse monoclonal anti-p38 (cat no. sc-7972), mouse monoclonal STAT3 (cat no. sc-8019), and mouse monoclonal HO-1 (cat no. sc-390991) antibodies were purchased from Santa Cruz Biotechnology, Inc. (California, USA); rabbit polyclonal anti-p38 (Thr180/Tyr182; cat no. 9211), rabbit polyclonal

ERK1/2 (cat no. 9102), rabbit polyclonal anti-p-JNK (Thr183/Tyr185; cat no. 9252), rabbit monoclonal anti-p-ERK1/2 (Thr202/Tyr204; cat no. 9106), rabbit polyclonal anti-TAK1 (cat no. 4505), rabbit monoclonal anti-p-TAK1 (T184/187; cat no. 4508), rabbit monoclonal anti-p-IKK α / β (Ser176/180; cat no. 2697), rabbit monoclonal anti-p-NF- κ B p65 (Ser536; cat no. 3033), rabbit monoclonal anti-NQO1 (cat no. 62262), rabbit monoclonal anti-p-STAT3 (Tyr705; cat no. 9145), rabbit monoclonal anti-Keap1 (cat no. 8047), and rabbit monoclonal anti-Nrf2 (cat no. 12721) antibodies were purchased from Cell Signaling Technology Inc. (Massachusetts, USA); polyclonal anti-rabbit IgG Fc-HRP (cat no. LF-SA8002) and polyclonal anti-mouse IgG Fc-HRP (cat no. LF-SA8001) were from AbFrontier (Seoul, Korea). Enzyme-linked immunosorbent assay (ELISA) kits for detecting TNF- α (cat no. 88-7324) were purchased from Ebioscience (California, USA). IL-1 β (cat no. 88-7013-88) and IL-6 (cat no. 88-7064-88) mouse uncoated ELISA kits were purchased from Invitrogen (California, USA). The PGE₂ Express ELISA kit for PGE₂ detection (cat no. 500141) was purchased from Cayman Chemical (Michigan, USA). Squalene (cat no. 442785) and α -linolenic acid (ALA; cat no. 62160) were purchased from Sigma-Aldrich. Phytol (cat no. BD83109) was purchased from BLD Pharmatech Ltd. (Shanghai, China). Tert-butylhydroquinone (TBHQ; cat no. 112941), SP600125 (cat no. S5567), and U0126 (cat no. U120) were purchased from Sigma-Aldrich. SB203580 (cat no. S1076) was purchased from Selleckchem (Texas, USA). Hydroxychavicol (A2947) was purchased from TCI America (Portland OR, USA).

2.4. HPLC analysis

Prior to chromatographic analysis, the freeze-dried extract of 23.4 mg of MMC was mixed in 550 μ l of ether and macerated under sonication for 5 min. MMC was purged with nitrogen gas and reconstituted with isopropyl alcohol (IPA). For the HPLC-ultraviolet (UV) analysis of squalene, the extract solution was diluted 2 times with an initial ratio of mobile phase. ALA and phytol were analyzed by LC-mass spectrometry (MS)/MS using a 4000-fold diluted solution of 0.1 % acetic acid in water and IPA (1:9, v/v). The freeze-dried stock of MMC was dissolved in a suitable solution for each experiment. MMC and standard solutions were filtered through a hydrophilic 0.2 μ m PTFE membrane filter before injection. Chromatographic analyses were performed using an Agilent 1260 Infinity system (Santa Clara, USA) and a Nexera X2 UPLC system coupled with a Shimadzu 8060 QQQ system (Kyoto, Japan). Phenomenex Kinetex C18 column (4.6 mm $\times$ 250 mm, 5 μ m) and Agilent Poroshell 120 EC-C18 (2.1 $\times$ 100 mm, 2.7 μ m) were used for UV and MS, respectively. UV detection was conducted at 254 nm, and the HPLC and LC-MS/MS flow rates were 1.0 and 0.2 ml/min, respectively. Mobile phase A and B in HPLC-UV were water and acetonitrile (ACN), respectively, whereas mobile phase A and B in LC-MS/MS were 0.01 % acetic acid in water and IPA/ACN (50:50, v/v), respectively. The injection volume for both experiments was 5 μ l. The gradient conditions were as follows: HPLC-UV: 0 min 10 % (B), 0–3 min isocratic at 10 % (B), 3–10 min 10–95 % (B), 10–12 min isocratic at 95 % (B), and re-equilibration with 10 % for 3 min. LC-MS/MS: 0 min 10 % (B), 0–3 min isocratic at 10 % (B), 3–8 min 10–20 % (B), 8–10 min 20–98 % (B), 10–12 min isocratic at 98 % (B), and re-equilibration with 10 % for 3 min. The column temperatures were 25 $^{\circ}$ C for HPLC-UV and 50 $^{\circ}$ C for LC-MS/MS. The optimal multiple reaction monitoring conditions were optimized using electrospray ionization in negative ion mode with the following parameters: 300 $^{\circ}$ C DL temperature, 400 $^{\circ}$ C heat block temperature, 2 L/min nebulizing gas flow, and 10 L/min drying gas flow.

2.5. Cell viability assay

RAW 264.7 macrophages and HEK 293 cells were seeded in 96-well plates (5×10^4 cells/well) and incubated overnight at 37 $^{\circ}$ C. The cells were pre-treated with different concentrations of MMC (0, 50, 100, 200, and 400 μ g/ml) or vehicle (DMSO) for 2 h, followed by stimulation with

or without LPS (1 μ g/ml) or phorbol 12-myristate 13-acetate (PMA) (50 nM) at 37 $^{\circ}$ C for 24 h. Cell viability was assessed using the EZ-Cytox cell viability assay kit (DoGenBio, Seoul, Korea). After diluting the EZ-Cytox solution to 1/20 of the culture solution volume, it was added to the plates and incubated at 37 $^{\circ}$ C for 30 min. The absorbance of each group was measured at 450 nm and 650 nm (A_{450} - A_{650}) using a Synergy H1 Microplate Reader (BioTek Instruments, Inc., Vermont, USA).

2.6. NO production assay

RAW 264.7 macrophages were seeded in 96-well plates (5×10^4 cells/well) and incubated overnight at 37 $^{\circ}$ C. The cells were pre-treated with MMC (50, 100, and 200 μ g/ml) or vehicle (DMSO) for 2 h, followed by treatment with or without LPS (1 μ g/ml) at 37 $^{\circ}$ C for 24 h. The cultured medium (100 μ l) was transferred to a new 96-well plate and 100 μ l of Griess reagent (0.1 % N-(1-naphthyl) ethylenediamine, 2.5 % phosphoric acid (H₃PO₄), 1 % sulfanilamide in distilled water) was added to each well. Sodium nitrite was used to generate a standard curve. After 10 min of incubation at room temperature, the absorbance at 540 nm was measured using a Synergy H1 Microplate Reader.

2.7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity of MMC was determined using DPPH. MMC was diluted with 100 % methanol to different concentrations. Samples (100 μ l) and 0.1 mM of DPPH solution (100 μ l) were added to a 96-well plate and incubated at 37 $^{\circ}$ C for 30 min in the dark. The absorbances were measured at 517 nm, and the DPPH scavenging activity was calculated as $[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{Methanol}}) / \text{Abs}_{\text{Methanol}}]$.

2.8. ELISA

RAW 264.7 macrophages were seeded in 96-well plates (4.5×10^4 cells/well) and incubated overnight at 37 $^{\circ}$ C. Macrophages pre-treated with MMC (50, 100, and 200 μ g/ml) for 2 h were then stimulated with LPS (1 μ g/ml) at 37 $^{\circ}$ C for 24 h. Culture supernatants were collected by centrifugation at 3,000 rpm for 1 min at room temperature. The protein expression of the inflammatory mediators IL-1 β , IL-6, and TNF- α levels in the cell supernatants was measured using sandwich ELISA with mediator-specific monoclonal antibodies according to the manufacturer's instructions. For the detection of IL-1 β , IL-6, and TNF- α , 96-well ELISA plates were pre-coated with capture antibodies overnight at 4 $^{\circ}$ C. After washing, the plate was washed at least three additional times with 1X phosphate-buffered saline containing 5 % Tween 20 and blocked with 1X assay diluent at room temperature for 1 h. Next, 100 μ l of each sample was added and incubated at room temperature for 2 h. Following the incubation period, a biotinylated detection antibody solution was added at room temperature for 1 h. Subsequently, the plate was treated with HRP-streptavidin solution at room temperature for 30 min, after which 100 μ l of 3,3',5,5'-tetramethylbenzidine was sequentially added with appropriate incubation at room temperature for 10 min under dark conditions. The reaction was stopped by adding 50 μ l of 1 M H₃PO₄ to each well, after which absorbance was measured at 450 nm using a Synergy H1 Microplate Reader. Cytokine concentrations were calculated based on a standard curve. A 96-well plate pre-coated with goat anti-mouse IgG was used for PGE₂ production. The plate was incubated with PGE₂ express acetylcholinesterase tracer, PGE₂ monoclonal antibody, and either standards or samples for 1 h at room temperature on a shaker. The plate was covered with plastic film and washed at least three times with the provided wash buffer to remove unbound reagents, followed by washing and development with Ellman's reagent for 1 h. The absorbance at 405 nm was measured and the values were analyzed using a four-parameter logistic fit.

2.9. Cell lysis and immunoblotting analysis

RAW 264.7 macrophages treated with MMC (50, 100, and 200 µg/ml) or vehicle (DMSO) at 37 °C for 2 h and were stimulated with or without LPS (1 µg/ml) at 37 °C for 3 min (for IKKα/β, IκBα, NF-κB, and TAK1), 15 min (for MAPKs), 4 h (for STAT3) or 24 h (for iNOS and COX-2). To detect the protein levels of Nrf2, Keap1, HO-1, and NQO1, RAW 264.7 macrophages were only treated with MMC (50, 100, and 200 µg/ml) or vehicle (DMSO) at 37 °C for 24 h. The cells were washed at least two times with PBS (pH 7.4) and lysed in a buffer containing 20 mM Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl pH 8.0), 150 mM sodium chloride, 0.5 % Triton X-100, 1 % glycerol, 1 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. The lysates were centrifuged at 13,000 rpm at 4 °C for 20 min. The obtained supernatants were transferred to new tubes.

For immunoblotting, the protein concentration was quantified using the Bradford protein assay (Bio-Rad, California, USA) according to the manufacturer's instructions. The cell lysate mixtures were combined with an equal volume of 5 X sodium dodecyl sulfate (SDS)-polyacrylamide sample buffer containing 250 mM Tris-HCl (pH 6.8), 10 % SDS, 50 % glycerol, 5 % β-mercaptoethanol, and 0.01 % bromophenol blue and boiled at 100 °C for 5 min. The samples were then separated in 10 % or 12 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare, Wiscorsin, USA). The membranes were blocked with 1X Tris-buffered saline with 0.05 % Tween 20 (TBST) solution containing 5 % non-fat dried skim milk, followed by incubation with primary antibodies at 4 °C overnight. The primary antibodies were diluted in 5 % non-fat dried skim milk or 5 % bovine serum albumin in TBST solution at a 1:1000 ratio. Each membrane was washed more than three times with 1X TBST and incubated with a secondary antibody at room temperature for 2 h. The secondary antibodies were diluted in 5 % nonfat-dried skim milk in TBST at a 1:5000 ratio. The target protein bands were visualized using enhanced chemiluminescence (ECL) immunoblotting detection reagent (Pierce, Illinois, USA). The protein levels were scanned and analyzed using the LabWorks software (UVP Inc., California, USA).

2.10. Transfection and luciferase (Luc) reporter assay

HEK 293 cells were seeded in a 12-well plate and incubated overnight at 37 °C. The cells reached 70 % confluency on the day of transfection. Transfection was carried out using polyethylenimine to introduce pNF-κB-Luc, pAP-1-Luc cis-reporter, or NQO1/antioxidant response elements (ARE)-Luc reporter plasmids, together with the gWIZ-green fluorescent protein (GFP) plasmid. These reporter plasmids incorporate specific response elements associated with individual proteins, consequently leading to luciferase reporter gene expression. The transfected cells were then incubated overnight at 37 °C. GFP was used as an internal control to normalize the luciferase reporter gene values. Next, the transfected cells were pre-treated with various concentrations of MMC for 2 h, after which they were treated with PMA and incubated for an additional 24 h at 37 °C. Finally, the cells were lysed with cell culture lysis reagent (Promega, Madison, USA) and analyzed using luciferase assay reagent (Promega, Madison, USA). Luminescence and fluorescence values were measured using a multiwell plate reader (Synergy H1; Luminometer filter).

2.11. Preparation of RNA and cDNA synthesis

RAW 264.7 macrophages were seeded in a 6-well plate and incubated overnight at 37 °C. RAW 264.7 macrophages were pre-treated with MMC (50, 100, and 200 µg/ml) for 2 h. After pre-treatment, the cells were stimulated with LPS (1 µg/ml) for an additional 3 h (for iNOS, COX-2, TNF-α, IL-6, and IL-1β) or 24 h (for HO-1 and NQO1), after which they were decanted. Total RNA was extracted from the cells using the

Labozol reagent (Cosmogentech, Seoul, Korea), after which the mixture was transferred into Eppendorf tubes. Chloroform was then added and the samples were centrifuged at 13,000 rpm for 15 min. The top aqueous layer was carefully transferred into Eppendorf tubes and iso-propanol was added to these tubes. The mixture was then mixed via gentle inversion and centrifuged at 13,000 rpm for 10 min. After centrifugation, the pellet was washed with 75 % ethanol. The tubes were then centrifuged once again at 5000 rpm for 5 min. The dried pellet was dissolved in diethylpyrocarbonate-treated water. The RNA concentration and purity were quantified using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a TOPscript cDNA synthesis kit (Enzynomics, Daejeon, Korea).

2.12. Reverse transcription polymerase chain reaction (RT-PCR)

The cDNA was amplified with the following PCR cycling conditions: initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The gene expression levels were normalized to the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences of RT-PCR used in this study are listed in Table 1.

2.13. RT-quantitative PCR (RT-qPCR)

The synthesized cDNA was mixed with dyne qPCR 2x premix following the manufacturer's instructions and amplified using the CFX Connect real-time thermal cycler (Bio-Rad, Hercules, CA, USA). The amplification conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 39 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s. The SYBR fluorescence values for the PCR amplicon were normalized using the GAPDH gene as a reference. The gene expression levels were displayed as a ratio by referring to the control group as 100 %, following the $2^{-\Delta\Delta C_q}$ method. The primer sequences used in this study are listed in Table 2.

2.14. Immunofluorescence analysis

To identify the in situ expression of NF-κB, RAW 264.7 macrophages were seeded on a confocal dish at a density of 2×10^4 cells/well. The macrophages were then pre-treated with 200 µg/ml of MMC for 2 h and then treated with 1 µg/ml of LPS for an additional 3 min. After washing the macrophages three times with 300 µl of PBS, they were fixed with 4 % formaldehyde for 15 min at 4 °C. Afterward, the macrophages were washed once again with PBS and blocked with 2 % BSA in PBS for 1 h at room temperature. After blocking, the macrophages were washed three

Table 1
Primer sequences used in RT-PCR.

Primer name		Sequence (5'-3')
iNOS	Forward	GCATGGAACAGTATAAGGCCAAACA
	Reverse	GTTTCTGGTGCATGTCATGAGCAA
COX-2	Forward	CAAGCAGTGGCAAAGGCCTCCA
	Reverse	GGCACTTGCAATTGATGGTGGCT
TNF-α	Forward	GTGCCAGCCGATGGGTGTACC
	Reverse	AGGCCACAGTCCAGGTCACTG
IL-6	Forward	TCTTGGGACTGATGCTGGTGAC
	Reverse	CATAACGCACTAGGTTTGCCGA
IL-1β	Forward	AGCTGTGGCAGCTACCTGTG
	Reverse	GCTCTGCTTGTGAGGTGCTG
GAPDH	Forward	GTCTTCACCACTGGAGAAGG
	Reverse	CCTGCTTCACCACTTCTTGAT
HO-1	Forward	CCTTCCCGAATCATCGACAGCC
	Reverse	GCAGTCTCTCAAACAGCTCAA
NQO1	Forward	AGCCAATCAGCGTTCCGTAT
	Reverse	GCCTCCTTACATGGCGTAGTT

Table 2
Primer sequences used in RT-qPCR.

Primer name		Sequence (5'–3')
iNOS	Forward	CGA AAC GCT TCA CTT CCA A
	Reverse	TGA GCC TAT ATT GCT GTG GCT
COX-2	Forward	GAT GCT CTT CCG AGC TGT G
	Reverse	GGA TTG GAA CAG CAA GGA TTT
TNF- α	Forward	CTG TAG CCC ACG TCG TAG C
	Reverse	TTG AGA TCC ATG CCG TTG
IL-6	Forward	TCT AAT TCA TAT CTT CAA CCA AGA GG
	Reverse	TGG TCC TTA GCC ACT CCT TC
IL-1 β	Forward	TTG ACG GAG CCC AAA AGA T
	Reverse	GAT GTG CTG CTG CGA GAT T
GAPDH	Forward	GCT CTC TGC TCC TCC TGT TC
	Reverse	ACG ACC AAA TCC GTT GAC TC
HO-1	Forward	CCT TCC CGA ACA TCG ACA GCC
	Reverse	GCA GCT CCT CAA ACA GCT CAA
NQO1	Forward	AGC CCA GAT ATT GTG GCC G
	Reverse	CCT TTC AGA ATG GCT GGC AC

times with PBS and incubated overnight in a confocal dish with NF- κ B primary antibody (1:100) at 4 °C. The confocal dish was washed three more times with PBS and then incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:500) for 1 h at room temperature. After washing with PBS, the cells were counterstained with 1 mg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 3 min and washed again with PBS. Immunofluorescence images were captured using a Zeiss Laser Scanning Confocal Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.15. Statistical analysis and experimental replicates

All experiments were independently performed in triplicate. All numerical data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments. Differences between experimental groups were assessed via one-way ANOVA and Dunnett's multiple comparison test using the GraphPad Prism 3.0 software (GraphPad Software, CA, USA). Statistically significant values were defined as *** $p < 0.001$ compared to non-treated control groups, and ^a $p < 0.05$, ^b $p < 0.01$, and ^c $p < 0.001$ compared to the LPS-treated group. Non-significant differences represent as "n.s." on the graph.

3. Results

3.1. Effects of MMC on cell viability and morphological changes of RAW 264.7 macrophages

A previous study demonstrated the presence of bioactive compounds, including squalene, ALA, and phytol, in *M. cordata* [35]. Therefore, we investigated their content in MMC using HPLC analysis.

The UV spectra and retention times of MMC were compared with those of standard compounds (Fig. S1A–C), thus confirming the presence of squalene, phytol, and ALA in MMC. The calculated percentages for these bioactive compounds in MMC are as follows: squalene 3.26 %, phytol 0.33 %, and ALA 0.47 %. The retention times of squalene, phytol, and ALA in the HPLC chromatogram were 4.321 min, 9.318 min, and 9.201 min, respectively.

Before studying the anti-inflammatory properties of MMC, we assessed its cytotoxic effects on RAW 264.7 macrophages using cell viability assays. The cells were treated with various concentrations of MMC with or without LPS stimulation. As shown in Fig. 1A, MMC did not exhibit cytotoxicity at concentrations up to 200 μ g/ml regardless of the presence of LPS. Although 200 μ g/ml of MMC showed approximately 80 % cell viability, this ratio is considered non-cytotoxic to the cells [36]. However, above 200 μ g/ml, MMC showed cytotoxicity in both LPS-stimulated and unstimulated groups when compared to non-treated control groups. Therefore, in this study, we investigated the anti-inflammatory effects of MMC on LPS-induced inflammation in RAW 264.7 macrophages using concentrations up to 200 μ g/ml. Furthermore, the cytotoxicity of bioactive compounds in MMC, including squalene, ALA, and phytol, was evaluated, and no cytotoxic effects on cell viability were observed for any of the tested concentrations (Fig. S2). These concentrations of bioactive compounds were selected based on previous analysis of the ethanolic extract of *M. cordata* [35].

Additionally, the cell morphology of RAW 264.7 macrophages upon MMC treatment in the presence or absence of LPS was examined via optical microscopy. As shown in Fig. 1B, non-treated RAW 264.7 macrophages displayed a round form, whereas LPS-treated macrophages exhibited a distinct dendritic morphology with pseudopodia. However, co-treatment with LPS and 200 μ g/ml of MMC for 24 h resulted in cells exhibiting a rounded shape with fewer pseudopodia. The morphological changes in the MMC-treated group resembled those of the non-treated control group, indicating that MMC suppresses the LPS-induced formation of pseudopodia.

3.2. Inhibitory effects of MMC on the production of NO and PGE₂ in LPS-stimulated RAW 264.7 macrophages via regulating the expression of iNOS and COX-2

To evaluate the anti-inflammatory effects of MMC, we measured its inhibitory effects on the production of NO and PGE₂, both of which are well-known inflammatory mediators, in LPS-stimulated RAW 264.7 macrophages. NO production was significantly increased by LPS stimulation but significantly reduced by MMC in a dose-dependent manner compared to the LPS-only treated group (Fig. 2A). Similarly, MMC treatment led to a reduction in PGE₂ production, albeit less dramatic than the reduction in NO production (Fig. 2B). NO and PGE₂ production are mediated by iNOS and COX-2, respectively. Therefore, we

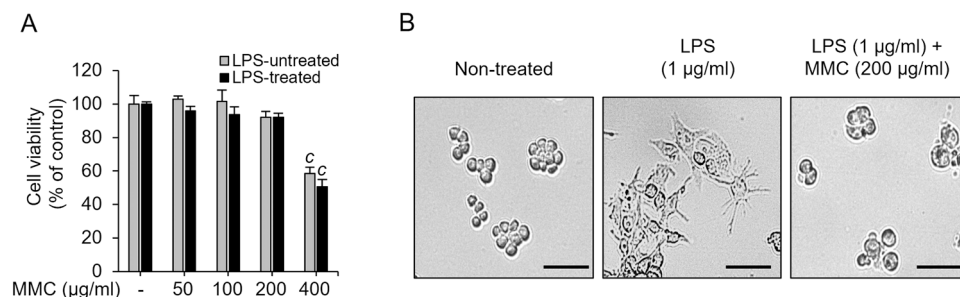


Fig. 1. Effects of MMC on the cell viability and morphology of LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pre-treated with (A) various concentrations of MMC or (B) 200 μ g/ml of MMC for 2 h, followed by treatment with or without LPS (1 μ g/ml) for an additional 24 h. (A) Cell viability was assessed using the EZ-Cytox assay kit, and the bars represent the percentage of viable cells after 24 h of treatment with MMC concentrations ranging from 0 to 400 μ g/ml. The values represent the mean $\pm$ SD. ^c $p < 0.001$ (compared to non-treated control group). (B) Morphological changes were observed using a light microscope at 10 $\times$ magnification. Scale bar, 50 μ m.

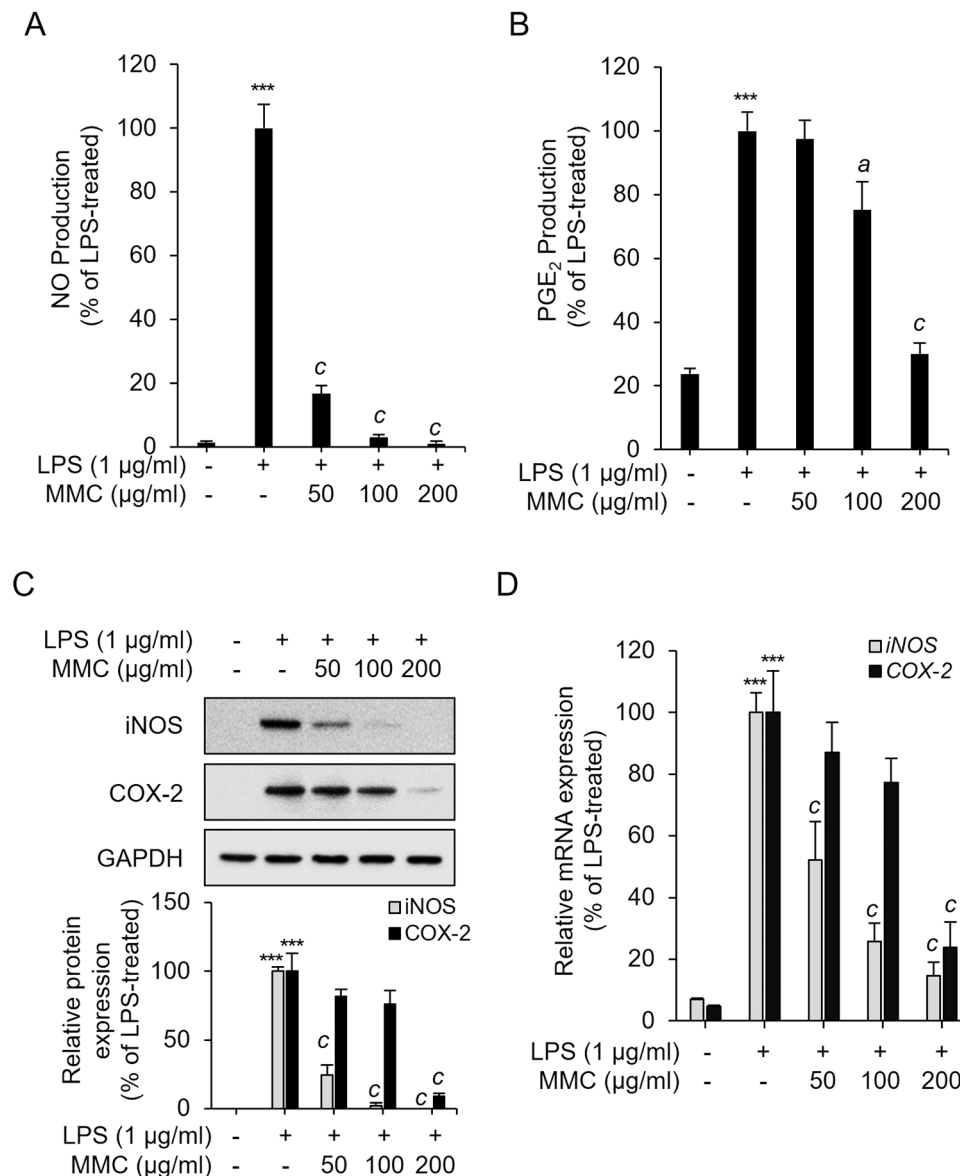


Fig. 2. Effects of MMC on the production of NO and PGE₂ and mRNA and protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pre-treated with various concentrations of MMC (50, 100, and 200 μg/ml) for 2 h, followed by treatment with or without LPS (1 μg/ml) for an additional (A, B, and C) 24 h or (D) 3 h. (A) Secreted NO levels in the cell supernatants were measured using the Griess reagent. The data were normalized to the LPS-only treated group and calculated based on a nitrite standard curve. The data represent the mean ± SD of three independent experiments. (B) PGE₂ production in the cell supernatants evaluated by ELISA. (C) After preparation of total cell lysates, immunoblot analysis was performed. The protein expression of iNOS and COX-2 was detected using an ECL system and quantified with the LabWorks software. (D) Total RNA was extracted, and reverse transcribed to cDNA. The expression levels of iNOS and COX-2 were analyzed by RT-qPCR. The mRNA and protein expression levels of iNOS and COX-2 were normalized to GAPDH and represented as bar graphs. The values represent the mean ± SD. ***p < 0.001 (compared to non-treated control group); ^ap < 0.05 and ^cp < 0.001 (compared to LPS-treated group).

investigated whether the inhibitory effect of MMC on NO and PGE₂ production resulted from the down-regulation of iNOS and COX-2. Here, we observed decreases in both mRNA and protein levels of iNOS and COX-2 (Fig. 2C, D, and S3), indicating that MMC-mediated suppression of iNOS and COX-2 expression leads to a reduction in NO and PGE₂ production. Furthermore, the reduction in iNOS expression was more pronounced compared to the decrease in COX-2 levels, which was consistent with the observed changes in NO and PGE₂ production. These results indicate a stronger regulatory effect of MMC on the transcription of iNOS than on that of COX-2.

Additionally, we investigated whether the bioactive compounds present in MMC (squalene, ALA, and phytol) were responsible for the significant reduction in NO production observed with MMC treatment. However, these three bioactive compounds only induced a slight

decrease in NO production compared to MMC alone (Fig. S4). These findings suggest that squalene, ALA, and phytol may not be the main bioactive compounds responsible for the observed inhibitory effects on NO production in MMC.

3.3. Inhibitory effects of MMC on the expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages

During the induction of the inflammatory response, the production of pro-inflammatory mediators is accompanied by the release of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which serve as critical indicators of inflammation. To further investigate the anti-inflammatory actions of MMC, we measured the effects of MMC on the mRNA and protein levels of various pro-inflammatory cytokines,

including TNF- α , IL-1 β , and IL-6, in LPS-stimulated RAW 264.7 macrophages. MMC treatment reduced the elevation of mRNA and protein levels of TNF- α , IL-1 β , and IL-6 caused by LPS stimulation (Fig. 3A, B, and S5). Interestingly, MMC treatment decreased the mRNA and protein levels of IL-1 β and IL-6 in a dose-dependent manner, whereas the downregulation of TNF- α mRNA and protein levels was less pronounced at low MMC concentrations compared to IL-1 β and IL-6 in LPS-stimulated RAW 264.7 macrophages. Moreover, MMC exhibited anti-inflammatory effects by regulating the transcriptional levels of pro-inflammatory mediators and cytokines in RAW 264.7 macrophages.

3.4. Inhibitory effects of MMC on transcriptional activities of NF- κ B and AP-1 in HEK 293 cells

MMC decreased the production of pro-inflammatory mediators and cytokines, which highlighted the need to determine the mechanisms underlying the regulation of their expression. Thus, luciferase reporter assays were conducted to quantify the transcriptional activity of NF- κ B and AP-1, two main transcription factors involved in the inflammatory response. HEK 293 cells were transfected with a luciferase reporter gene under the control of NF- κ B- or AP-1-dependent promoter activity. Given that HEK 293 cells are unresponsive to LPS because they lack TLR4, PMA was used instead of LPS to induce the transcriptional activation of NF- κ B and AP-1 [37]. As illustrated in Fig. 4A and B, luciferase activities were increased in the presence of PMA compared to the non-treated control group but significantly reduced with MMC treatment. Cell viability assays confirmed that MMC and PMA treatment did not induce cytotoxic effects on HEK 293 cells (Fig. S6). Significant reductions in NF- κ B-dependent luciferase activity were observed at every MMC concentration, whereas a significant reduction in AP-1-dependent luciferase activity was observed at 200 μ g/ml. Although the reduction in NF- κ B-dependent luciferase activity was more pronounced with MMC treatment than AP-1-dependent luciferase activity, our findings indicated that MMC exerts anti-inflammatory responses by inhibiting the transcriptional activities of NF- κ B and AP-1.

3.5. Inhibitory role of MMC on TAK1-mediated MAPK and NF- κ B signaling pathways in LPS-stimulated RAW 264.7 macrophages

MMC regulated the expression of the NF- κ B and AP-1 transcription factors, which highlighted the need to identify the main upstream signaling pathways involved in this regulatory process. Therefore, we assessed the effects of MMC on the activation of NF- κ B and MAPK signaling pathways in LPS-induced RAW 264.7 macrophages using immunoblotting. Here, we investigated the effects of MMC on LPS-

stimulated phosphorylation of IKK α / β , I κ B, and NF- κ B, as well as the degradation of total I κ B. As shown in Fig. 5A, MMC strongly suppressed the LPS-induced phosphorylation of IKK α / β , I κ B α , and NF- κ B p65 when pre-treated with concentrations over 50 μ g/ml. MMC inhibited the phosphorylation of I κ B α at Ser-32/36, leading to I κ B α accumulation by preventing its degradation. MMC also suppressed the phosphorylation of NF- κ B p65 at Ser-536, which enhances its transcriptional activity and nuclear translocation. Additionally, immunofluorescence staining analysis using confocal microscopy demonstrated that NF- κ B was predominantly localized in the cytoplasm in unstimulated RAW 264.7 macrophages (Fig. 5B). Treating the cells with LPS resulted in a strong NF- κ B signal in the nucleus, which was indicative of nuclear translocation of NF- κ B. However, MMC treatment decreased this LPS-induced nuclear translocation of NF- κ B.

To investigate the involvement of the MAPK signaling pathway in the anti-inflammatory response of MMC, we examined the effects of MMC on the phosphorylation states of MAPKs in LPS-stimulated RAW 264.7 macrophages via immunoblotting. As shown in Fig. 5C, MMC treatment led to a decline in the phosphorylation of JNK, p38, and ERK. To compare the inhibitory effect of MMC on phosphorylation NF- κ B and MAPKs with known inhibitors, hydroxychavicol (5 μ g/ml), SP600125 (10 μ M), SB203580 (10 μ M), and U0126 (10 μ M) that function as inhibitors of NF- κ B, JNK, p38, and ERK respectively were employed [38, 39].

Considering that MMC contained many inactive compounds, the results showed the effectiveness of MMC in inhibiting NF- κ B and MAPK phosphorylation (Fig. S7A-D). These results confirmed that MMC inhibits the NF- κ B and MAPK signaling pathways, resulting in a reduction in the production of pro-inflammatory mediators and cytokines in LPS-stimulated RAW 264.7 macrophages. However, it is important to note that MMC exhibited a stronger inhibitory effect on NF- κ B than on MAPKs. Furthermore, we conducted additional experiments to examine the phosphorylation level of STAT3 that is activated by IL-6 [40]. Our results showed that treatment with MMC suppressed STAT3 activity by reducing IL-6 production (Fig. 5E).

Our findings demonstrated that MMC inhibited the activation of both NF- κ B and MAPK signaling pathways, suggesting that TAK1, a common upstream kinase of these signaling pathways, could be a potential target of MMC. To verify this, we assessed the phosphorylation level of TAK1 in LPS-induced RAW 264.7 macrophages via immunoblotting. As expected, TAK1 phosphorylation was increased by LPS stimulation but was significantly reduced by MMC at concentrations of 100 and 200 μ g/ml in LPS-induced RAW 264.7 macrophages (Fig. 5D). These results suggest that MMC exerts its anti-inflammatory response in LPS-stimulated macrophages by suppressing TAK1 or its upstream regulators.

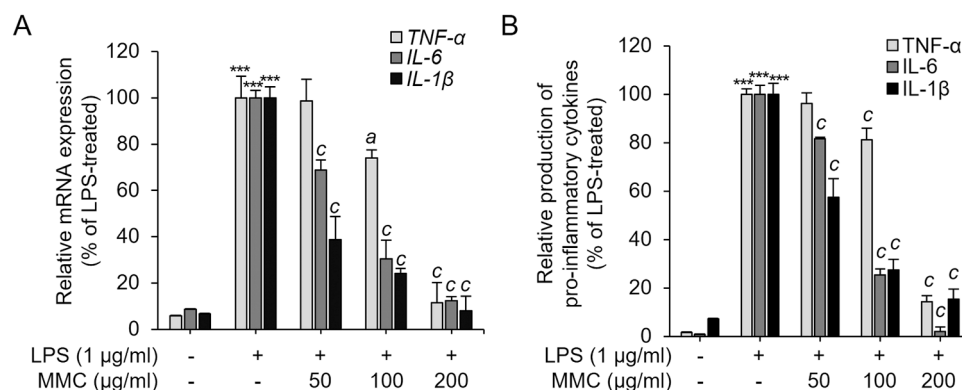


Fig. 3. Effects of MMC on LPS-induced mRNA and production of pro-inflammatory cytokines. RAW 264.7 macrophages were pre-treated with MMC (50, 100, and 200 μ g/ml) for 2 h and then stimulated with LPS for an additional (A) 3 h or (B) 24 h. (A) The mRNA expression levels of IL-6, IL-1 β , and TNF- α were analyzed by RT-qPCR. Relative mRNA expression levels were normalized to GAPDH and displayed as bar graphs. (B) The protein expression levels of IL-6, IL-1 β , and TNF- α were measured using ELISA kits and represented as bar graphs. All bar graphs represent the mean $\pm$ SD. ***p < 0.01 (compared to non-treated control group); ^ap < 0.05 and ^p < 0.001 (compared to LPS-treated group).

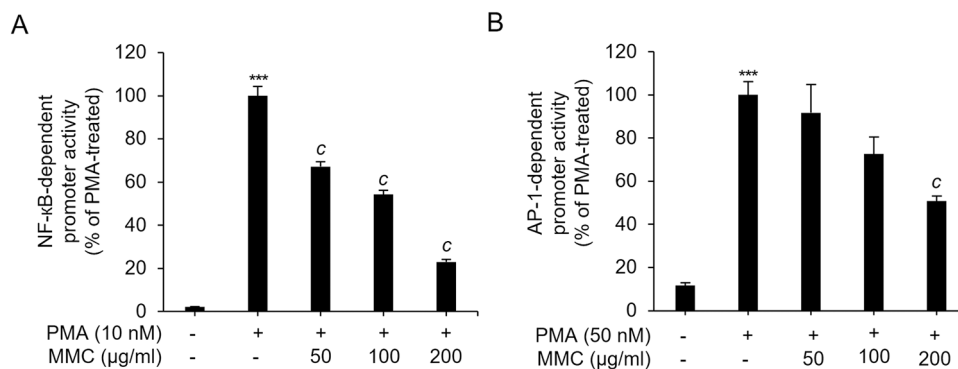


Fig. 4. Inhibitory effects of MMC on the transcriptional activation of NF- κ B and AP-1. The promoter activity of (A) NF- κ B and (B) AP-1 was measured in HEK 293 cells. Luciferase gene constructs containing NF- κ B and AP-1 binding sites, along with the gWIZ-GFP gene, were transfected into HEK 293 cells and incubated overnight. The transfected cells were pre-treated with different concentrations of MMC for 2 h and then treated with PMA for another 24 h. Luciferase values were normalized using GFP fluorescence values. The bar graphs represent the mean $\pm$ SD. *** p < 0.01 (compared to non-treated control group); ^c p < 0.001 (compared to PMA-treated group).

3.6. Activation of Nrf2 signaling pathway and induction of Nrf2-dependent antioxidant genes by MMC in RAW 264.7 macrophages

Nrf2 is another important transcription factor that protects cells against excessive ROS formation during an inflammatory response. Given that previous studies have indicated that *M. cordata* possesses potential antioxidant activity [27,41], we further examined the antioxidant activity of MMC in RAW 264.7 macrophages through the Nrf2 signaling pathway. Prior to evaluating the antioxidant effects of MMC, we assessed its radical scavenging ability via the DPPH assay. However, as illustrated in Fig. 6A, MMC exhibited minimal scavenging activity compared to quercetin, a well-known radical scavenger [42]. Despite these outcomes, we examined the antioxidant effect of MMC on RAW 264.7 macrophages by analyzing the expression levels of Nrf2 and its antioxidant-related target genes. As shown in Fig. 6B, MMC treatment increased the protein levels of Nrf2 and its target genes (HO-1 and NQO1) in a dose-dependent manner, while concurrently reducing Keap1 protein levels. To further substantiate the modulation of Nrf2 signaling by MMC, we employed TBHQ as a positive control, a well-established activator of Nrf2. MMC (200 μ g/ml) showed a similar effect as 5 μ g/ml of TBHQ (Fig. S8A), suggesting that MMC exerts strong antioxidant effects. Furthermore, RT-qPCR and RT-PCR analysis confirmed that MMC also increased mRNA levels of Nrf2 target genes HO-1 and NQO1 in a dose-dependent manner (Fig. 6C and S8B). When Nrf2 is activated, it translocates into the nucleus and binds to AREs in the promoters of target genes [43]. In the reporter assays, MMC significantly and dose-dependently increased NQO1/ARE promoter activity (Fig. 6D). However, the bioactive compounds of MMC, namely squalene, phytol, and ALA, did not significantly increase NQO1/ARE promoter activity compared to MMC (Fig. S8C). These results indicate that MMC exhibits strong antioxidant effects by regulating Keap1/Nrf2 signaling pathway, leading to the expression of its main antioxidant target genes.

4. Discussion

Numerous studies have characterized the anti-inflammatory and antioxidant properties of plant extracts. Nevertheless, despite the long-standing use of *M. cordata* in traditional medicine, the molecular mechanisms underlying its anti-inflammatory and antioxidant activities have remained largely unexplored. Therefore, our study sought to investigate the effect of MMC on LPS-stimulated RAW 264.7 macrophages, particularly in relation to inflammatory reactions and oxidative stress.

The regulation of inflammatory mediators such as iNOS-derived NO and COX-2-derived PGE₂, as well as cytokines such as IL-1 β , IL-6, and TNF- α , generally involves various intracellular signaling pathways. The

NF- κ B and MAPK signaling pathways, which regulate NF- κ B and AP-1 transcription factors, respectively, play major roles in this response [44]. Previous studies have demonstrated that these mediators and cytokines share NF- κ B and AP-1 binding regions in their promoters [45–47]. Consistent with these findings, our study revealed that MMC inhibited the transcriptional activity of NF- κ B and AP-1 in LPS-stimulated macrophages. However, the inhibition of NF- κ B transcriptional activity by MMC was more pronounced than that of AP-1. In our previous studies, we demonstrated that TNF- α and COX-2 levels are more correlated with AP-1 transcriptional activity, whereas iNOS, IL-6, and IL-1 β levels were more strongly regulated by NF- κ B [37,48,49]. Consistent with the regulation of NF- κ B and AP-1 transcriptional activity, our results indicated that MMC inhibited the mRNA and protein levels of NO, iNOS, IL-6, and IL-1 β more potently compared to TNF- α , PGE₂, and COX-2. This could be attributed to the differential binding affinities of transcription factors to the promoter region of pro-inflammatory genes. Additionally, different components in MMC may differentially regulate the transcription of pro-inflammatory genes due to the presence of various active ingredients in MMC.

HPLC chromatography results demonstrated that there were significant amounts of squalene, phytol, and ALA in the ethanolic extract of *M. cordata* [35], thus confirming the presence of these bioactive compounds in MMC. These compounds have demonstrated anti-inflammatory effects in conditions such as inflammatory bowel disease and the paw edema model [50–52]. Squalene and ALA have been shown to suppress LPS-induced NO production and TNF- α production in macrophages, thus confirming their anti-inflammatory effects [53,54]. However, when analyzing the content of these bioactive compounds present in MMC using HPLC, it was observed that their quantities were significantly lower compared to the study of the ethanolic extract of *M. cordata*. Furthermore, our study revealed that the reduction in NO production by these bioactive compounds was not as strong as that observed with MMC treatment. This suggests that other chemicals present in MMC likely play important roles in regulating LPS-mediated inflammatory responses. However, a complete phytochemical profiling of MMC has not yet been investigated, and further studies are needed.

In macrophages, NF- κ B activation by LPS is primarily mediated through the phosphorylation and subsequent degradation of I κ B α at Ser-32/36 [55]. The degradation of I κ B α leads to the translocation of NF- κ B to the nucleus, resulting in the activation of its transcriptional activity [55]. Our study demonstrated that MMC inhibited I κ B α degradation by attenuating its phosphorylation at Ser-32/36, thereby decreasing NF- κ B p65 activity in a dose-dependent manner. These findings suggest that MMC prevents the suppression of pro-inflammatory genes in LPS-stimulated RAW 264.7 macrophages by regulating the nuclear translocation of NF- κ B. Furthermore, MMC effectively attenuated not

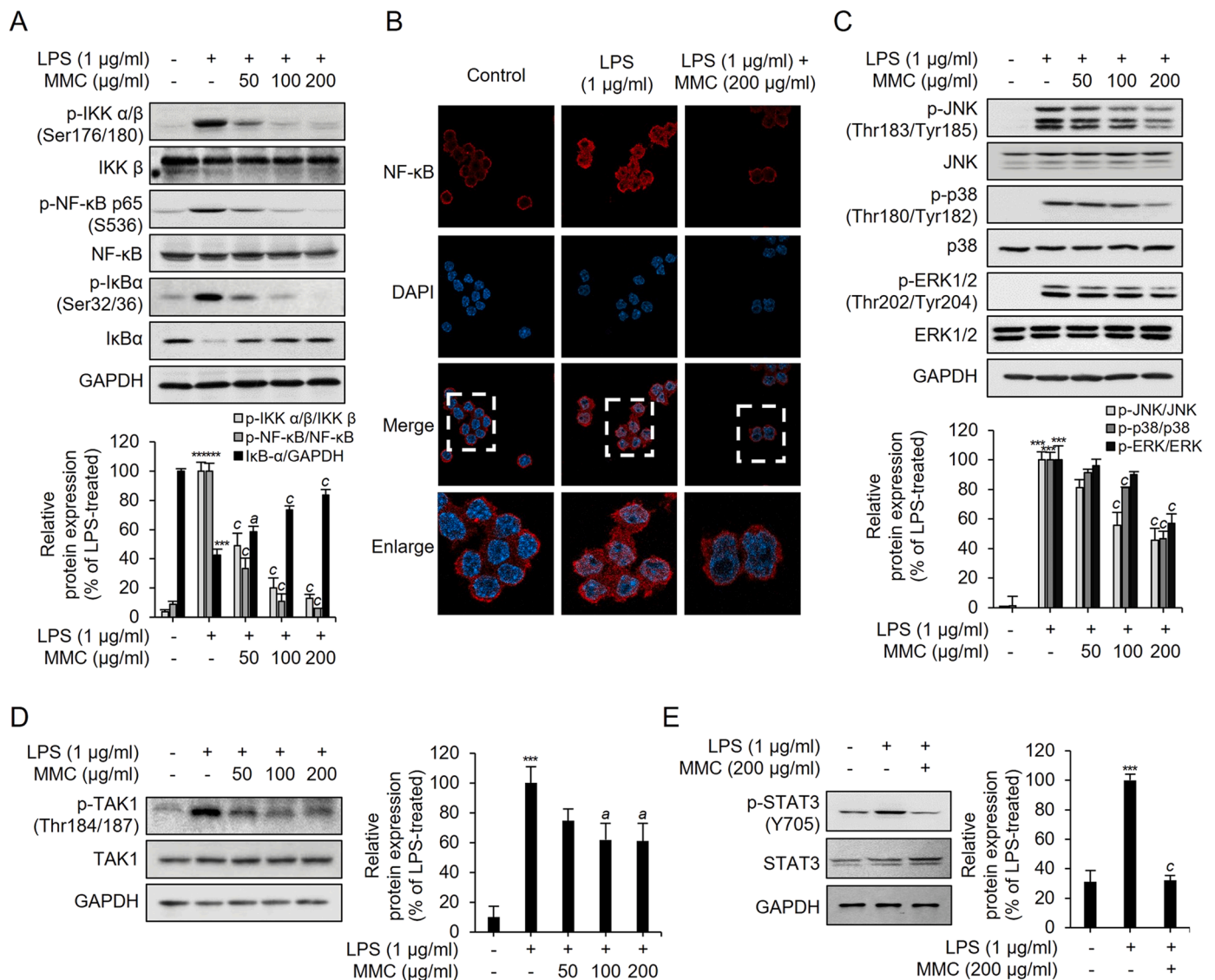


Fig. 5. Inhibitory effects of MMC on the activation of NF-κB, MAPKs, and STAT3 in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were pre-treated with MMC (50, 100, and 200 µg/ml) for 2 h and stimulated with or without LPS for (A, B, and D) 3 min, (C) 15 min or (E) 4 h. (A) Protein expression levels were measured by immunoblotting analysis. The bar graph below the panel shows the quantitative analysis of phosphorylated protein normalized to the total protein concentration. (B) NF-κB p65 subunits were probed with anti-NF-κB polyclonal primary antibody and Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody. Nuclei were stained with DAPI, and images were captured by confocal microscopy. (C, D, and E) Protein expression levels were measured by immunoblotting analysis. The bar graph below the panel shows the quantitative analysis of phosphorylated protein normalized to the total protein concentration. The values are expressed as the mean ± SD of triplicates. ***p < 0.01 (compared to non-treated control group); ^ap < 0.05 and ^cp < 0.001 (compared to LPS-treated group).

only the NF-κB signaling pathway but also the MAPKs in LPS-stimulated RAW 264.7 macrophages. Although MMC reduced the phosphorylation levels of MAPKs, significant inhibitory effects on phosphorylated MAPKs occurred at different concentrations: p-JNK and p-p38 at 100 µg/ml and p-ERK at 200 µg/ml. Additionally, MMC effectively suppressed the phosphorylation levels of STAT3. This indicates that various active components in MMC may target these proteins separately. Notably, NF-κB and MAPK signaling pathways share a common upstream kinase, TAK1 [56]. Here, we observed the suppression of phosphorylated TAK1 with high concentrations of MMC. These results suggest that the inhibition of NF-κB and MAPKs by MMC is mediated through the inhibition of TAK1. Interestingly, despite inhibiting TAK1, MMC exhibited greater effectiveness in suppressing the NF-κB signaling pathway compared to the MAPK pathway. We speculate that TAK1 may have a greater impact on NF-κB than on MAPK. Alternatively, MMC, with its diverse array of bioactive compounds, may not only regulate TAK1 but also other upstream kinases involved in the modulation of the NF-κB signaling

pathway such as NF-κB-inducing kinases [57]. MMC might selectively target critical upstream kinases, either directly or indirectly, that are more crucial for NF-κB activation rather than MAPK activation. As the notable pathophysiological roles of TAK1 in conditions like colitis and organ dysfunction [58,59], it underscores the potential therapeutic relevance of TAK1 inhibition by MMC for TAK1-mediated diseases.

There are several limitations in this study that impact its scientific rigor. One notable limitation is the lack of investigation into the bioactive compounds in MMC. Therefore, it is imperative to identify the bioactive compounds of MMC through phytochemical profiling and to comprehensively elucidate their functions. Given the diverse range of bioactive compounds present in *Mikania cordata*, there could be other MMC target regulators that are not affected by NF-κB, MAPK, and STAT3 signaling pathways. It is also crucial to note that our study has been limited to in vitro experiments, serving as a foundational step. Further research involving in vivo experiments is imperative to validate the accuracy and applicability of our findings in a more physiologically

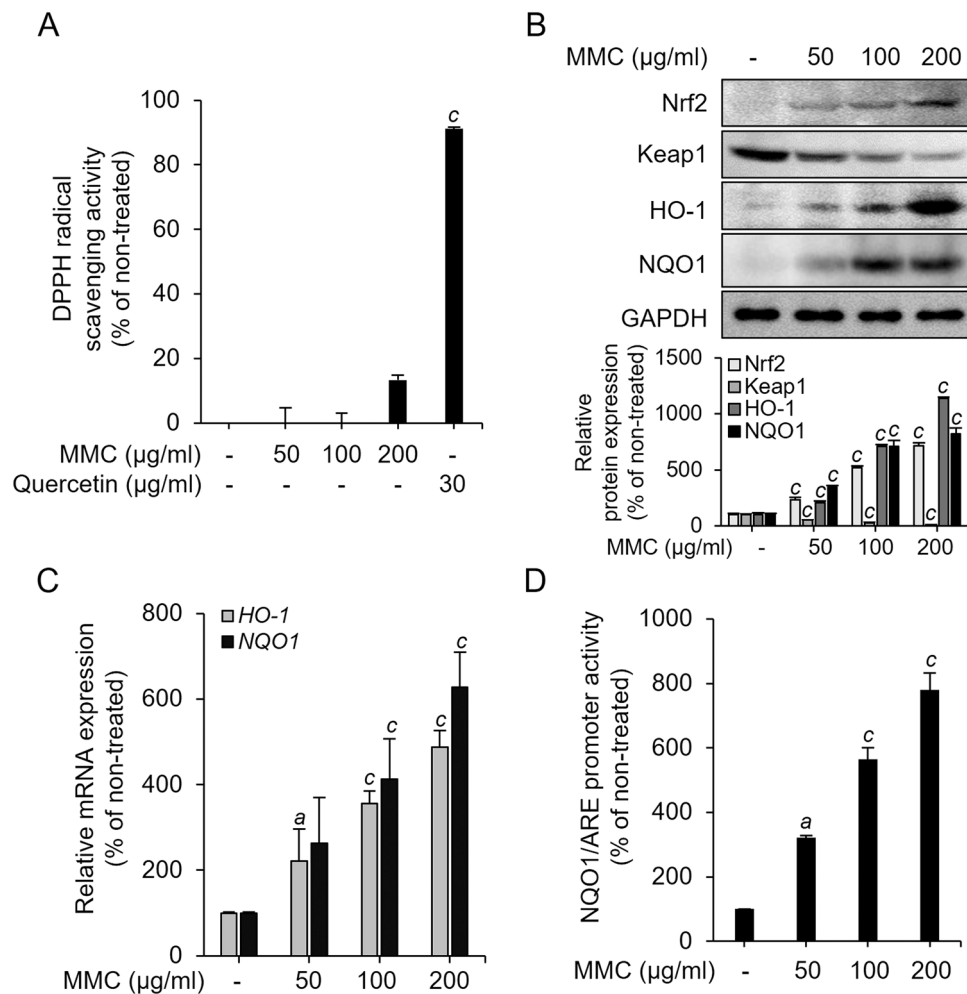


Fig. 6. Activation of Nrf2 and HO-1 expression and induction of Nrf2 target genes by MMC in RAW 264.7 macrophages. (A) The radical scavenging effect of MMC was confirmed using the DPPH assay. Methanol alone was used as a negative control and was considered as 0 %. Quercetin was used as a positive control. The DPPH radical scavenging activity is represented as the mean $\pm$ SD. (B and C) RAW 264.7 macrophages were treated with MMC (50, 100, and 200 μ g/ml) for 24 h. (B) Cells were lysed and analyzed by immunoblot analyses. The protein expression of Nrf2, Keap1, HO-1, and NQO1 was detected using an ECL system and quantified with the LabWorks software. (C) The expression levels of HO-1, NQO1, and GAPDH were analyzed by RT-qPCR. (D) HEK 293 cells were transfected with NQO1/ARE reporter gene and gWIZ-GFP. The transfected cells were then treated with MMC for 24 h. Luciferase values were normalized using GFP fluorescence values. Expression of mRNA and protein was normalized to that of the reference gene GAPDH. The relative expression levels of mRNA and protein are displayed as bar graphs. The bar graphs represent the mean $\pm$ SD. ^a $p < 0.05$ and ^c $p < 0.001$ (compared to the non-treated control group).

relevant context.

Patients with inflammatory-related diseases commonly experience excessive ROS production [60]. Previous studies have demonstrated a reciprocal relationship between abnormal ROS levels and the upregulation of inflammatory mediators and cytokines [61–63]. The Keap1/Nrf2/HO-1 signaling pathway is a well-known defense mechanism against oxidative stress [64]. Therefore, further research on the Keap1/Nrf2/HO-1 signaling pathway holds great significance for the clinical treatment of inflammation-associated diseases. This study suggests that MMC treatment induces the degradation of Keap1, leading to stabilization of Nrf2. The reduction of Keap1 by MMC may be attributed to either autophagy or proteasomal degradation [21]. Activation of Nrf2 by MMC results in the induction of mRNA and protein expressions of its main target genes, HO-1 and NQO1. Although the radical scavenging effect of MMC was low at the concentrations examined in vitro, MMC demonstrated an antioxidant effect by activating Nrf2 and inducing the expression of its target genes. Comparatively, a study by Hossain et al. reported approximately 56 % DPPH inhibition by *M. cordata* at 500 μ g/ml [65], which is a higher concentration compared to the maximum concentrations used in this study. Interestingly, previous studies have demonstrated that some of the active components of

M. cordata, including squalene, phytol, and ALA, exhibit little to no radical scavenging effects [66–68]. This observation may explain the weak DPPH radical scavenging effects observed with MMC in this study. These findings underscore the complex interplay between the ability of MMC to activate Nrf2, the induction of its target gene expression, and its observed radical scavenging effects, collectively enriching our understanding of its multifaceted therapeutic potential.

In conclusion, this investigation provides a comprehensive insight into the underlying molecular mechanisms by which MMC exerts anti-inflammatory and anti-oxidative effects within RAW 264.7 macrophages. MMC effectively reduced the production of NO, PGE₂, and pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β by inhibiting TAK1 phosphorylation and subsequently blocking the NF- κ B and MAPK signaling pathways. Moreover, MMC exhibits a suppressive effect on STAT3 phosphorylation. Additionally, it activates Nrf2, leading to the expression of key target genes, notably HO-1 and NQO1, thereby demonstrating its antioxidant properties. Considering the beneficial effects of inhibiting the TLR4-TAK1 signaling pathway and activating Nrf2 in repressing the inflammatory response and oxidative stress respectively, MMC emerges as a promising candidate for the development of targeted therapeutic approaches for severe inflammation-related

disorders.

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CRedit authorship contribution statement

Eunjeong Kang: Conceptualization, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing, Formal analysis. **Junho Lee:** Investigation, Writing – review & editing. **Sumin Seo:** Investigation. **Salah Uddin:** Methodology and Resources. **Sangwoo Lee:** Methodology and Resources. **Sang Beom Han:** Methodology and Resources. **Sayeon Cho:** Conceptualization, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Please declare any financial or personal interests that might be potentially viewed to influence the work presented. Interests could include consultancies, honoraria, patent ownership or other. If there are none state 'there are none'.

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.115746](https://doi.org/10.1016/j.biopha.2023.115746).

References

- [1] L. Chen, H. Deng, H. Cui, J. Fang, Z. Zuo, J. Deng, Y. Li, X. Wang, L. Zhao, Inflammatory responses and inflammation-associated diseases in organs, *Oncotarget* 9 (2018) 7204–7218, <https://doi.org/10.18632/oncotarget.23208>.
- [2] D. Furman, J. Campisi, E. Verdín, P. Carrera-Bastos, S. Targ, C. Franceschi, L. Ferrucci, D.W. Gilroy, A. Fasano, G.W. Miller, A.H. Miller, A. Mantovani, C. M. Weyand, N. Barzilai, J.J. Goronzy, T.A. Rando, R.B. Effros, A. Lucia, N. Kleinsteuber, G.M. Slavich, Chronic inflammation in the etiology of disease across the life span, *Nat. Med.* 25 (2019) 1822–1832, <https://doi.org/10.1038/s41591-019-0675-0>.
- [3] L.S. Li, S.M. Chiroma, T. Hashim, S.K. Adam, M.A. Mohd Moklas, Z. Yusuf, S. A. Rahman, Antioxidant and anti-inflammatory properties of Erythroxylum cuneatum alkaloid leaf extract, *Heliyon* 6 (2020), e04141, <https://doi.org/10.1016/j.heliyon.2020.e04141>.
- [4] J.F. Oliveira Costa, J.M. Barbosa-Filho, G.L. de Azevedo Maia, E.T. Guimarães, C. S. Meira, R. Ribeiro-dos-Santos, L.C. Pontes de Carvalho, M.B.P. Soares, Potent anti-inflammatory activity of betulinic acid treatment in a model of lethal endotoxemia, *Int. Immunopharmacol.* 23 (2014) 469–474, <https://doi.org/10.1016/j.intimp.2014.09.021>.
- [5] B. Beutler, K. Hoebe, X. Du, R.J. Ulevitch, How we detect microbes and respond to them: the toll-like receptors and their transducers, *J. Leukoc. Biol.* 74 (2003) 479–485, <https://doi.org/10.1189/jlb.0203082>.
- [6] C.-H. Lee, E.Y. Choi, Macrophages and Inflammation, *J. Rheum. Dis.* 25 (2018) 11, <https://doi.org/10.4078/jrd.2018.25.1.11>.
- [7] B.S. Park, J.-O. Lee, Recognition of lipopolysaccharide pattern by TLR4 complexes, e66–e66, *Exp. Mol. Med.* 45 (2013), <https://doi.org/10.1038/emm.2013.97>.
- [8] J.-M. Zhang, J. An, Cytokines, inflammation, and pain, *Int. Anesthesiol. Clin.* 45 (2007) 27–37, <https://doi.org/10.1097/AIA.0b013e318034194e>.
- [9] S. Fujioka, J. Niu, C. Schmidt, G.M. Sclabas, B. Peng, T. Uwagawa, Z. Li, D.B. Evans, J.L. Abbruzzese, P.J. Chiao, NF- κ B and AP-1 connection: mechanism of NF- κ B-dependent regulation of AP-1 activity, *Mol. Cell. Biol.* 24 (2004) 7806–7819, <https://doi.org/10.1128/MCB.24.17.7806-7819.2004>.
- [10] F. Christian, E.L. Smith, R.J. Carmody, The regulation of NF- κ B subunits by phosphorylation, *Cells* 5 (2016), <https://doi.org/10.3390/CELL5010012>.
- [11] J.P. Pradère, C. Hernandez, C. Koppe, R.A. Friedman, T. Luedde, R.F. Schwabe, Negative regulation of NF- κ B p65 activity by serine 536 phosphorylation, *Sci. Signal.* 9 (2016) ra85, <https://doi.org/10.1126/SCISIGNAL.AAB2820>.
- [12] B. Hoesel, J.A. Schmid, The complexity of NF- κ B signaling in inflammation and cancer, *Mol. Cancer* 12 (1) (2013) 15, <https://doi.org/10.1186/1476-4598-12-86>.
- [13] J. Park, J.-S. Min, B. Kim, U.-B. Chae, J.W. Yun, M.-S. Choi, I.-K. Kong, K.-T. Chang, D.-S. Lee, Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF- κ B pathways, *Neurosci. Lett.* 584 (2015) 191–196, <https://doi.org/10.1016/j.neulet.2014.10.016>.
- [14] J.-H. Shi, S.-C. Sun, Tumor necrosis factor receptor-associated factor regulation of nuclear factor κ B and mitogen-activated protein kinase pathways, *Front. Immunol.* 9 (2018), 1849, <https://doi.org/10.3389/fimmu.2018.01849>.
- [15] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on toll-like receptors, *Nat. Immunol.* 11 (2010) 373–384, <https://doi.org/10.1038/ni.1863>.
- [16] W. ZHANG, H.T. LIU, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, *Cell Res.* 12 (2002) 9–18, <https://doi.org/10.1038/sj.cr.7290105>.
- [17] P.P. Tak, G.S. Firestein, NF- κ B: a key role in inflammatory diseases, *J. Clin. Invest.* 107 (2001) 7–11, <https://doi.org/10.1172/JCI11830>.
- [18] W.J. Lee, W.Y. Li, S.W. Lee, S.K. Jung, Anti-inflammatory and antioxidant effects of *Sorosis hirsuta* extract by regulating iNOS/NF- κ B and NRF2/HO-1 pathways in murine macrophage RAW 264.7 cells, *Appl. Sci.* 11 (2021) 4711, <https://doi.org/10.3390/AP11104711>.
- [19] S. Yang, F. Li, S. Lu, L. Ren, S. Bian, M. Liu, D. Zhao, S. Wang, J. Wang, Ginseng root extract attenuates inflammation by inhibiting the MAPK/NF- κ B signaling pathway and activating autophagy and p62-Nrf2-Keap1 signaling in vitro and in vivo, *J. Ethnopharmacol.* 283 (2022), 114739, <https://doi.org/10.1016/J.JEP.2021.114739>.
- [20] P. Deshmukh, S. Unni, G. Krishnappa, B. Padmanabhan, The Keap1–Nrf2 pathway: promising therapeutic target to counteract ROS-mediated damage in cancers and neurodegenerative diseases, *Biophys. Rev.* 9 (2016) 41–56, <https://doi.org/10.1007/S12551-016-0244-4>.
- [21] H.Y. Seo, S.H. Lee, J.H. Lee, J.S. Hwang, M.K. Kim, B.K. Jang, Kahweol activates the Nrf2/HO-1 pathway by decreasing Keap1 expression independently of p62 and autophagy pathways, *PLoS One* 15 (2020) 1–13, <https://doi.org/10.1371/journal.pone.0240478>.
- [22] M. Hatano, S. Hatamiya, M. Miyara, Y. Kotake, Tributyltin activates the Keap1–Nrf2 pathway via a macroautophagy-independent reduction in Keap1, *J. Toxicol. Sci.* 48 (2023) 161–168, <https://doi.org/10.2131/jts.48.161>.
- [23] A. Loboda, M. Damulewicz, E. Pyza, A. Jozkowicz, J. Dulak, Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism, *Cell. Mol. Life Sci.* 73 (2016) 3221, <https://doi.org/10.1007/S00018-016-2223-0>.
- [24] S. Oteng Mintah, T. Asafo-Agyei, M.-A. Archer, P. Atta-Adjei Junior, D. Boamah, D. Kumadoh, A. Appiah, A. Ocloo, Y. Duah Boakyie, C. Agyare, Medicinal plants for treatment of prevalent diseases, in: *Pharmacogn. - Med. Plants*, IntechOpen, 2019, <https://doi.org/10.5772/intechopen.82049>.
- [25] L.C. Rufatto, A. Gower, J. Schwambach, S. Moura, Genus Mikania: chemical composition and phytotherapeutic activity, *Rev. Bras. Farmacogn.* 22 (2012) 1384–1403, <https://doi.org/10.1590/S0102-695X2012005000099>.
- [26] B. Robinson, B. Mercado, Notes on some growth characteristics of Mikania cordata (BURM. F.) B.L. Robinson, *Biotropia* (Bogor.) (1994), <https://doi.org/10.11598/btb.1994.0.7.110>.
- [27] S.A. Siddiqui, A. Rahman, M. Oliur Rahman, M.A. Akbar, A.S. Shamsur Rouf, M. A. Ali, F.M.A. Al-Hemaid, M.A. Farah, Evaluation of anti-nociceptive, anti-inflammatory and antipyretic potential of Mikania cordata (Burm. f.) Robinson in experimental animal model, *Saudi J. Biol. Sci.* 25 (2018) 1049–1055, <https://doi.org/10.1016/j.sjbs.2018.01.009>.
- [28] M.S. Ali, M.S. Islam, M.M. Rahman, M.R. Islam, M.A. Sayeed, M.R. Islam, Antibacterial and cytotoxic activity of ethanol extract of Mikania cordata (burm.f.) B.L. Robinson leaves, *J. Basic Clin. Pharm.* 2 (2011) 103–107, (<http://www.ncbi.nlm.nih.gov/pubmed/24826008>) (accessed February 3, 2022).
- [29] F. Nasrin, M.L. Hakim, *In vivo* antidiarrheal study of ethanolic extracts of Mikania cordata and Litsea monopetala leaves, *Bangladesh J. Pharmacol.* 10 (2015) 562, <https://doi.org/10.3329/bjp.v10i3.23402>.
- [30] R.K. Paul, A. Jabbar, M. Rashid, Antiulcer activity of Mikania cordata, *Fitoterapia* 71 (2000) 701–703, [https://doi.org/10.1016/S0367-326X\(00\)00216-1](https://doi.org/10.1016/S0367-326X(00)00216-1).
- [31] T.G.C. Uy, A.M. Licuanan, G.E.D. Angeles, M.L.C.C. Bote, E.A.B. Macaayag, C. C. Hernandez, S.D. Jacinto, R.M. Guzman-Gen, Anti-cancer effect and mechanisms of action of Mikania cordata plant extract on MCF-7 human breast adenocarcinoma cells, *Int. J. Cancer Res.* 11 (2015) 80–92, <https://doi.org/10.3923/ijcr.2015.80.92>.
- [32] S. Bhattacharya, S. Pal, A.K.N. Chaudhuri, Pharmacological studies of the antiinflammatory profile of Mikania cordata (Burm) B. L. robinson root extract in rodents, *Phyther. Res.* 6 (1992) 255–260, <https://doi.org/10.1002/ptr.2650060507>.
- [33] S. Bhattacharya, S. Pal, A. Chaudhuri, Neuropharmacological studies on Mikania cordata root extract, *Planta Med.* 54 (1988) 483–487, <https://doi.org/10.1055/s-2006-962524>.

- [34] F. Nasrin, M. Lukman Hakim, Hypoglycemic study of ethanolic extract of *Mikania cordata* leaf, *World J. Pharm. Res.* 4 (1) (2015) 9.
- [35] A.B.H.Y. Ab Patar, The analysis of aqueous and ethanolic extracts of Malaysian *Mikania cordata* leaves towards the potential for medicinal substances, *Eur. J. Sci. Res.* 73 (2012) 434–440.
- [36] J. López-García, M. Lehocý, P. Humpolíček, P. Sába, HaCaT keratinocytes response on antimicrobial atelocollagen substrates: extent of cytotoxicity, cell viability and proliferation, *J. Funct. Biomater.* 5 (2014) 43–57, <https://doi.org/10.3390/jfb5020043>.
- [37] Y.-C. Cho, H.L. Vuong, J. Ha, S. Lee, J. Park, A.E. Wibow, S. Cho, Inhibition of inflammatory responses by *Centella asiatica* via suppression of IRAK1-TAK1 in mouse macrophages, *Am. J. Chin. Med.* 48 (2020) 1103–1120, <https://doi.org/10.1142/S0192415X20500548>.
- [38] J. Seo, U. Lee, S. Seo, A.E. Wibow, O.B. Pongtulan, K.J. Lee, S.B. Han, S. Cho, Anti-inflammatory and antioxidant activities of methanol extract of Piper betle Linn. (Piper betle L.) leaves and stems by inhibiting NF- κ B/MAPK/Nrf2 signaling pathways in RAW 264.7 macrophages, *Biomed. Pharmacother.* 155 (2022), 113734, <https://doi.org/10.1016/j.biopha.2022.113734>.
- [39] S. Lee, E. Kang, U. Lee, S. Cho, Role of pelitinib in the regulation of migration and invasion of hepatocellular carcinoma cells via inhibition of Twist1, *BMC Cancer* 23 (2023) 1–13, <https://doi.org/10.1186/s12885-023-1217-2>.
- [40] Y.-C. Cho, Y.R. Kim, B.R. Kim, T.T. Bach, S. Cho, Thunbergia alata inhibits inflammatory responses through the inactivation of ERK and STAT3 in macrophages, *Int. J. Mol. Med.* 38 (2016) 1596–1604, <https://doi.org/10.3892/ijmm.2016.2746>.
- [41] A. Al Nayeem, A. Khatun, S. Rahman, M. Rahman, Evaluation of phytochemical and pharmacological properties of *Mikania cordata* (Asteraceae) leaves, *J. Pharmacogn. Phytother.* 3 (2011) 118–123. (<http://www.academicjournals.org/JPP>).
- [42] K. Imai, I. Nakanishi, K. Ohkubo, Y. Ohba, T. Arai, M. Mizuno, S. Fukuzumi, K. ichiro Matsumoto, K. Fukuhara, Synthesis of methylated quercetin analogues for enhancement of radical-scavenging activity, *RSC Adv.* 7 (2017) 17968–17979, <https://doi.org/10.1039/C7RA02329D>.
- [43] S.K. Niture, J.W. Kaspar, J. Shen, A.K. Jaiswal, Nrf2 signaling and cell survival, *Toxicol. Appl. Pharmacol.* 244 (2010) 37–42, <https://doi.org/10.1016/J.TAAP.2009.06.009>.
- [44] J. An, B. Chen, X. Kang, R. Zhang, Y. Guo, J. Zhao, H. Yang, Neuroprotective effects of natural compounds on LPS-induced inflammatory responses in microglia, *Am. J. Transl. Res.* 12 (2020) 2353–2378.
- [45] E. Confalone, G. D'Alessio, A. Furia, IL-6 Induction by TNF α and IL-1 β in an osteoblast-like cell line, *Int. J. Biomed. Sci.* 6 (2010) 135–140.
- [46] Y.-C. Cho, J. Park, S. Cho, Anti-inflammatory and anti-oxidative effects of luteolin-7-O-glucuronide in LPS-stimulated murine macrophages through TAK1 inhibition and Nrf2 activation, *Int. J. Mol. Sci.* 21 (2020) 2007, <https://doi.org/10.3390/ijms21062007>.
- [47] V. Vila-del Sol, M. Fresno, Involvement of TNF and NF- κ B in the transcriptional control of cyclooxygenase-2 expression by IFN- γ in macrophages, *J. Immunol.* 174 (2005) 2825–2833, <https://doi.org/10.4049/jimmunol.174.5.2825>.
- [48] B.R. Kim, Y.-C. Cho, H.T.T. Le, H.L. Vuong, S. Lee, S. Cho, Suppression of inflammation by the rhizome of *Anemarrhena asphodeloides* via regulation of nuclear factor- κ B and p38 signal transduction pathways in macrophages, *Biomed. Rep.* 6 (2017) 691–697, <https://doi.org/10.3892/br.2017.895>.
- [49] Y.-C. Cho, T.T. Bach, B.R. Kim, H.L. Vuong, S. Cho, *Spilanthes acmella* inhibits inflammatory responses via inhibition of NF- κ B and MAPK signaling pathways in RAW 264.7 macrophages, *Mol. Med. Rep.* 16 (2017) 339–346, <https://doi.org/10.3892/mmr.2017.6555>.
- [50] N.I. Ibrahim, I.N. Mohamed, Interdependence of anti-inflammatory and antioxidant properties of squalene-implication for cardiovascular health, *Life* 11 (2021) 1–19, <https://doi.org/10.3390/life11020103>.
- [51] R. Reifen, A. Karlinsky, A.H. Stark, Z. Berkovich, A. Nyska, α -Linolenic acid (ALA) is an anti-inflammatory agent in inflammatory bowel disease, *J. Nutr. Biochem.* 26 (2015) 1632–1640, <https://doi.org/10.1016/j.jnutbio.2015.08.006>.
- [52] R.O. Silva, F.B.M. Sousa, S.R.B. Damasceno, N.S. Carvalho, V.G. Silva, F.R.M. A. Oliveira, D.P. Sousa, K.S. Aragão, A.L.R. Barbosa, R.M. Freitas, J.V.R. Medeiros, Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress, *Fundam. Clin. Pharmacol.* 28 (2014) 455–464, <https://doi.org/10.1111/fcp.12049>.
- [53] A. Cárdeno, M. Aparicio-Soto, S. Montserrat-de la Paz, B. Bermudez, F.J. G. Muriana, C. Alarcón-de-la-Lastra, Squalene targets pro- and anti-inflammatory mediators and pathways to modulate over-activation of neutrophils, monocytes and macrophages, *J. Funct. Foods* 14 (2015) 779–790, <https://doi.org/10.1016/j.jff.2015.03.009>.
- [54] J. Ren, S.H. Chung, Anti-inflammatory effect of alpha-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF- κ B and mitogen-activated protein kinase pathways, *J. Agric. Food Chem.* 55 (2007) 5073–5080, <https://doi.org/10.1021/jf0702693>.
- [55] C. Fan, Q. Li, Y. Zhang, X. Liu, M. Luo, D. Abbott, W. Zhou, J.F. Engelhardt, I κ B α and I κ B β possess injury context-specific functions that uniquely influence hepatic NF- κ B induction and inflammation, *J. Clin. Invest.* 113 (2004) 746–755, <https://doi.org/10.1172/JCI17337>.
- [56] Y.-R. Xu, C.-Q. Lei, TAK1-TABs complex: a central signalosome in inflammatory responses, *Front. Immunol.* 11 (2021), 608976, <https://doi.org/10.3389/fimmu.2020.608976>.
- [57] L. Ling, Z. Cao, D.V. Goeddel, NF- κ B-inducing kinase activates IKK- α by phosphorylation of Ser-176, *PNAS* 95 (1998) 3792–3797, <https://doi.org/10.1073/PNAS.95.7.3792>.
- [58] J. Wang, K. Shi, S. Li, L. Chen, W. Liu, X. Wu, Y. Shen, Y. Sun, J. Cheng, X. Wu, Q. Xu, Meisoidin attenuates dextran sulfate sodium-induced experimental colitis via its inhibition of TAK1 in macrophages, *Int. Immunopharmacol.* 101 (2021), 108239, <https://doi.org/10.1016/J.INTIMP.2021.108239>.
- [59] C. Song, M.Y. Kim, J.Y. Cho, Olea europaea suppresses inflammation by targeting TAK1-mediated MAP kinase activation, *Molecules* 26 (2021) 1540, <https://doi.org/10.3390/MOLECULES26061540>.
- [60] Prather Zuo, Garrison Stetskiv, Peace Meade, Zhou, Inflammation and oxidative stress in human diseases: from molecular mechanisms to novel treatments, *Int. J. Mol. Sci.* 20 (2019) 4472, <https://doi.org/10.3390/ijms20184472>.
- [61] S.Y. Ji, H.J. Cha, I.M.N. Molagoda, M.Y. Kim, S.Y. Kim, H. Hwangbo, H. Lee, G. Y. Kim, D.H. Kim, J.W. Hyun, H.S. Kim, S. Kim, C.Y. Jin, Y.H. Choi, Suppression of lipopolysaccharide-induced inflammatory and oxidative response by 5-aminolevulinic acid in RAW 264.7 macrophages and Zebrafish larvae, *Biomol. Ther.* 29 (2021) 685, <https://doi.org/10.4062/BIMOLTHER.2021.030>.
- [62] Y. Lin, G. Tang, Y. Jiao, Y. Yuan, Y. Zheng, Y. Chen, J. Xiao, C. Li, Z. Chen, P. Cao, Propionibacterium acnes induces intervertebral disc degeneration by promoting iNOS/NO and COX-2/PGE 2 activation via the ROS-dependent NF- κ B pathway, *Oxid. Med. Cell. Longev.* 2018 (2018), <https://doi.org/10.1155/2018/3692752>.
- [63] D. Yang, S.G. Elner, Z.M. Bian, G.O. Till, H.R. Petty, V.M. Elner, Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells, *Exp. Eye Res.* 85 (2007) 462–472, <https://doi.org/10.1016/J.EXER.2007.06.013>.
- [64] S. Saha, B. Buttari, E. Panieri, E. Profumo, L. Saso, An overview of Nrf2 signaling pathway and its role in inflammation, *Molecules* 25 (2020) 5474, <https://doi.org/10.3390/molecules25225474>.
- [65] S. Hossain, J. Islam, F. Ahmed, M. Amjad Hossain, M.A.K. Siddiki, S.M. Moazzem Hossen, Free radical scavenging activity of six medicinal plants of Bangladesh: a potential source of natural antioxidant, *J. Appl. Pharm.* 7 (2015) 96–104, <https://doi.org/10.21065/19204159.7.1.78>.
- [66] J. Costa, M. Islam, P. Santos, P. Ferreira, G. Oliveira, M. Alencar, M. Paz, É. Ferreira, C. Feitosa, A. Citó, D. Sousa, A. Melo-Cavalcante, Evaluation of antioxidant activity of phytol using non- and pre-clinical models, *Curr. Pharm. Biotechnol.* 17 (2016) 1278–1284, <https://doi.org/10.2174/1389201017666161019155715>.
- [67] T.-F. Ko, Y.-M. Weng, Y.-Y. Chiou, Squalene content and antioxidant activity of Terminalia catappa leaves and seeds, *J. Agric. Food Chem.* 50 (2002) 5343–5348, <https://doi.org/10.1021/jf0203500>.
- [68] P. Raj, M. Singh, J.K. Rawat, S. Gautam, S.A. Saraf, G. Kaithwas, Effect of enteral administration of α -linolenic acid and linoleic acid against methotrexate induced intestinal toxicity in albino rats, *RSC Adv.* 4 (2014) 60397–60403, <https://doi.org/10.1039/C4RA01213D>.