

Methanol extract of *Euphorbia cotinifolia* L. leaf attenuates inflammation and oxidative stress in RAW 264.7 macrophages via TAK1-mediated suppression of NF- κ B/MAPK and activation of Nrf2 pathways

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ABSTRACT

Euphorbia cotinifolia L. (*E. cotinifolia* L.) is distributed in temperate and tropical regions. It exhibits various biological activities, including antioxidant, antimicrobial, and antiviral properties. Although its antioxidant properties have been described, its regulatory molecular mechanisms remain poorly understood and its anti-inflammatory effects have not been studied yet. This study investigated the effects of methanol extract of *E. cotinifolia* L. (MECL) on inflammation and oxidative stress in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. MECL significantly suppressed the production of inflammatory mediators, including nitric oxide (NO) and prostaglandin E₂ (PGE₂), by downregulating inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). In addition, MECL inhibited the production of key pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α (TNF- α), by suppressing of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways. It suppressed TAK1 phosphorylation, thereby inhibiting IKK α / β . Consequently, I κ B α was stabilized, preventing its degradation and thereby suppressing NF- κ B p65 phosphorylation and nuclear translocation. Additionally, MECL attenuated the activation of JNK, ERK, and p38 MAPK signaling. It also activated nuclear factor erythroid 2-related factor 2 (Nrf2), which is a key antioxidant transcription factor, upregulating its target genes, heme oxygenase-1 and NAD(P)H quinone dehydrogenase 1, which are essential for oxidative stress defense. These findings suggested that MECL mitigated inflammation and oxidative stress by regulating TAK1-mediated NF- κ B/MAPK signaling and Nrf2 activation. Therefore, it showed potential as a natural therapeutic candidate for inflammatory diseases.

1. Introduction

The inflammatory response is a fundamental defense mechanism that protects tissues and cells from infections and injuries. It plays a crucial role in the body's healing process. However, an excessive inflammatory response can contribute to the development of various inflammatory diseases, including heart disease, diabetes, and chronic kidney disease. These diseases are the leading cause of death worldwide, and inflammatory and pain disorders remain serious issues affecting individuals [1–3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are

the most commonly used medications for treating inflammation-related diseases. However, their prolonged use is related to adverse effects on the gastric mucosa, kidneys, liver, and other organs [4–6]. Therefore, alternative therapeutic approaches with fewer side effects should be developed.

During the early phase of inflammation, blood vessels around the affected area dilate, consequently increasing fluid release. At this stage, different immune cells are recruited to the inflammation site. Among them, macrophages are involved in innate immune response as they are widely distributed throughout the body and serve as the first line of

Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; PGE₂, prostaglandin E₂; IL-6, interleukin-6; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drugs; NF- κ B, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; TAK1, transforming growth factor β -activated kinase 1; IKK α / β , I κ B kinase α / β ; I κ B α , inhibitor of NF- κ B α ; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NADPH quinone dehydrogenase 1.

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defense against external stimuli, such as bacterial lipopolysaccharides (LPS) [7–10]. Toll-like receptors (TLRs) expressed on macrophages recognize pathogen-associated molecular patterns, which are necessary to initiate innate immune responses [11,12]. When TLR-4 recognizes LPS and activates macrophages, Toll/interleukin-1-receptor-domain-containing adaptor protein recruits myeloid differentiation primary response 88; consequently, it activates downstream signaling pathways and leads to the activation of the transcription factors nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1) [13–15]. As a result of these responses, macrophages produce inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2; they secrete inflammatory cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , and IL-1 [16–19].

NF- κ B is an inducible transcription factor that regulates numerous genes related to inflammation and immune responses. Within a cell, NF- κ B predominantly forms a p50/p65 heterodimer, and phosphorylation at Ser536 on p65 leads to the expression of inflammatory genes [20–22]. NF- κ B is activated through a complex cascade, which involves site-specific phosphorylation mediated by the I κ B kinase (IKK) complex. When stimulated by LPS, TLR-4 engages multiple adaptor proteins and kinases to activate the IKK complex. Under normal conditions, I κ B remains bound to the NF- κ B p50/p65 heterodimer, thereby preventing its activity. However, when IKK phosphorylates I κ B at Ser32/36, I κ B dissociates from NF- κ B and undergoes ubiquitin-dependent degradation via the proteasome [23,24]. Once released, the NF- κ B p50/p65 heterodimer translocates into the nucleus, where it modulates inflammatory gene expression. Apart from NF- κ B, AP-1 is another transcription factor that regulates inflammatory gene expression; it is primarily activated via the mitogen-activated protein kinase (MAPK) signaling pathway [25,26]. Upon LPS stimulation, the MAPK family, which includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK, facilitates the phosphorylation and activation of AP-1 components, such as c-Jun and c-Fos [27,28]. Through this mechanism, MAPK signaling plays a pivotal role in promoting the production of inflammatory mediators and cytokines; thus, it influences the overall inflammatory response. Transforming growth factor- β -activated kinase 1 (TAK1) functions as a crucial upstream regulator of both NF- κ B and MAPK signaling pathways. When TLR-4 is activated, TAK1 becomes phosphorylated, subsequently activating the IKK complex, which in turn regulates NF- κ B signaling. Concurrently, TAK1 phosphorylates MAPK kinases (MKKs); it initiates the activation of ERK, JNK, and p38 MAPK and ultimately results in AP-1 activation [29,30]. Because of its main role in inflammatory signaling, TAK1 is widely regarded as a promising therapeutic target for controlling excessive inflammatory responses.

Reactive oxygen species (ROS) are released when macrophages are activated during an inflammatory response; they are essential for eliminating pathogenic microorganisms. However, these molecules can cause various diseases if they are not properly regulated [31]. Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a well-known major transcription factor of antioxidant responses. Kelch-like ECH-associated protein 1 (Keap1) acts as an adaptor protein for the cullin-dependent E3 ubiquitin ligase; thus, it continuously facilitates the degradation of Nrf2 through the proteasome pathway under basal conditions [32,33]. However, under oxidative stress conditions, Keap1 undergo structural changes; as a result, Nrf2 dissociates and stabilizes. When Nrf2 is stabilized, it enters the nucleus and triggers the expression of genes involved in antioxidant responses, including heme oxygenase 1 (HO-1) and NAD(P)H-quinone oxidoreductase (NQO1). These gene products reduce excessive ROS within cells and thus helping to maintain cellular homeostasis [34,35].

Various natural extracts are effective in developing anti-inflammatory treatments [36,37]. The *Euphorbia* genus (Euphorbiaceae) is found across all temperate and tropical climates; it represents the third-largest group of flowering plants, offering substantial research potential because of its extensive species diversity. It has been utilized in the treatment of various diseases, including respiratory infections,

gastrointestinal disorders, and cancer [38]. The major active constituents of *Euphorbia* include triterpenes, alkanes, tannins, phytosterols, polyphenols, flavonoids, and diterpenoids. These compounds are known for their important pharmacological activities, contributing to the therapeutic potential of this genus [39,40]. *Euphorbia cotinifolia* L., commonly known as the Caribbean copper plant, has been traditionally used in folk medicine as an emetic and laxative [41,42]. Additionally, it exhibits molluscicidal, antiviral, anticancer, and antioxidant properties [42–46].

Despite these reports of traditional medicinal effects, studies have yet to explore the anti-inflammatory and antioxidant properties of *E. cotinifolia* L at the molecular level. Therefore, this study aimed to evaluate the anti-inflammatory and antioxidant effects of the methanol extract of *E. cotinifolia* L (MECL) and to elucidate its role in cellular signaling pathways in LPS-stimulated RAW 264.7 macrophages. This research aimed to address the existing gap in knowledge and explore the potential therapeutic applications of MECL.

2. Materials and methods

2.1. Plant collection and voucher specimen information

E. cotinifolia L. extract (FBM276–004) was obtained from the International Biological Material Research Center at the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Republic of Korea). The plant was collected from Hathazari District, Chittagong, Bangladesh in June 2015. A voucher specimen (KRIB 0069064) was kept in the herbarium of KRIBB. Dried leaves (75 g) in the shade were powdered, added to 1 L of 99.9 % methanol (HPLC grade), and extracted through 30 cycles (40 kHz, 1500 W, 15 min ultrasonication-120 min standing per cycle) at room temperature (RT) by using an ultrasonic extractor (SDN-900H, SD- Ultrasonic Co., Ltd.). They were filtered (Qualitative Filter No.100, Hyundai Micro Co., Ltd.) and dried under reduced pressure. Thus, *E. cotinifolia* L. extract (2.91 g) was obtained. The obtained *E. cotinifolia* L. extract was dissolved in dimethyl sulfoxide (DMSO) and used for all subsequent experiments. The final concentration of DMSO was adjusted to 0.1 % (v/v) in all experimental groups.

2.2. Cell culture

RAW264.7 macrophages were obtained from the Korea Cell Line Bank (Seoul, Korea), and human embryonic kidney 293 (HEK 293) cells were purchased from ATCC (Manassas, VA, USA). HEK 293 cells and RAW264.7 macrophages were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Welgene Inc., Gyeongsangbuk-do, Korea) supplemented with 10 % fetal bovine serum (Thermo Fisher Scientific Inc., CA, USA) and 1 % penicillin/streptomycin (Life Technologies, NY, USA). The cells were incubated at 37°C with 5 % CO₂ under humidified conditions.

2.3. Antibodies and reagents

The following antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA): rabbit monoclonal anti-phospho(p)-IKK α / β (Ser176/180; Cat. No. 2697), mouse monoclonal anti-p-I κ B α (Ser32/36; Cat. No. 9246S), rabbit monoclonal anti-p-NF- κ B p65 (Ser536; Cat. No. 3033), rabbit monoclonal anti-p-ERK1/2 (Thr202/Tyr204; Cat. No. 9106), rabbit polyclonal ERK1/2 (Cat. No. 9102), rabbit polyclonal anti-p-JNK (Thr183/Tyr185; Cat. No. 9251), rabbit polyclonal anti-JNK (Thr183/Tyr185; Cat. No. 9252), rabbit polyclonal anti-p-p38 (Thr180/Tyr182; Cat. No. 9211), rabbit monoclonal anti-p-TAK1 (T184/187; Cat. No. 4508), rabbit polyclonal anti-TAK1 (Cat. No. 4505), rabbit monoclonal anti-iNOS (Cat. No. 13120), rabbit monoclonal anti-COX-2 (Cat. No. 12282), rabbit monoclonal anti-Nrf2 (Cat. No. 12721), rabbit monoclonal anti-Keap1 (Cat. No. 8047), rabbit monoclonal anti-NQO1 (Cat. No. 62262), mouse monoclonal anti-p-

I κ B α (Ser32/36; Cat. No. 9246). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): mouse monoclonal NF- κ B p65 (Cat. No. sc-8008), mouse monoclonal anti-p38 (Cat. No. sc-7972), mouse monoclonal HO-1 (Cat. No. sc-390991), mouse monoclonal anti-I κ B α (Cat. No. sc-1643), mouse monoclonal IKK β (Cat. No. sc-8014), and β -actin (Cat. No. SC-47778). The following antibodies were purchased from AbFrontier (Seoul, Korea): polyclonal anti-rabbit IgG Fc-HRP (Cat. No. LF-SA8002) and polyclonal anti-mouse IgG Fc-HRP (Cat. No. LF-SA8001). The following antibodies were also used: rabbit polyclonal anti-GAPDH (Cat. No. GTX100118; GeneTex, Irvine, CA, USA) and Goat anti-Mouse IgG-DyLight 594 conjugated secondary antibody (Cat. No. A90-516D4; Bethyl Laboratories, Montgomery, TX, USA). The following kits were used: TNF- α ELISA kit (eBioscience, San Diego, CA, USA); IL-1 β and IL-6 ELISA kits (Invitrogen, Carlsbad, CA, USA); and The PGE $_2$ ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). LPS, DMSO, and U0126 (Cat. No. U120), SP600125 (Cat. No. S5567), and tert-butylhydroquinone (TBHQ; Cat. No. 112941), rutin (Cat. No. PHL89270), Quercetin (Cat. No. Q4951), Takinib (Cat. No. SML2216) and ML385 (Cat. No. SML-1833) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB203580 (Cat. No. S1076) was bought from Selleckchem (Houston, TX, USA). (+)-Catechin (Cat. No. AA0037YO) was obtained from AABlocks (San Diego, CA, USA).

2.4. Cell viability assay

RAW 264.7 macrophages and HEK 293 cells were seeded (3×10^4 cells/well) in 96-well plates and incubated with 5 % CO $_2$ at 37°C overnight. The cells were pretreated with MECL (20, 50, 100, and 200 μ g/ml) or vehicle (DMSO, 0.1 %) for 2 h and added with or without LPS. The cells were incubated with 5 % CO $_2$ at 37°C for 24 h. After 24 h, the cultured medium was replaced with fresh media containing 5 % EZ-CYTOX (DoGenBio, Seoul, Korea) and incubated at 37°C for 30 min. Absorbance was measured at wavelengths of 450 nm and 650 nm ($A_{450} - A_{650}$) by using a Synergy H1 microplate reader (BioTek Instruments Inc., Vermont, USA).

2.5. NO production assay

RAW 264.7 macrophages were seeded into 96-well plates at a density of 3×10^4 cells per well and incubated with 5 % CO $_2$ at 37°C for 24 h to allow for cell attachment and growth. After incubation, the cells were pretreated with MECL (10, 20, and 50 μ g/ml) or vehicle (DMSO, 0.1 %) for 2 h and treated with or without LPS (1 μ g/ml) for an additional 24 h. After the treatment, 100 μ l of the culture supernatant was collected and mixed with 100 μ l of Griess reagent (0.1 % N-(1-naphthyl) ethylenediamine, 1 % sulfanilamide, and 2.5 % phosphoric acid in distilled water) in a new 96-well plate. The mixture was incubated at RT in the dark for 10 min. Absorbance was measured at 540 nm by using a microplate reader (Synergy H1, BioTek Instruments). Sodium nitrite was used to generate a standard curve, and the nitrite concentration in the samples was calculated by comparing with the standard curve. All experiments were performed in triplicate.

2.6. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of the samples was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. A stock solution of DPPH (0.2 mM) was prepared in methanol. The assay was conducted by mixing 100 μ l of the MECL at different concentrations (10, 20, and 50 μ g/ml) or vehicle (DMSO, 0.1 %) with 100 μ l of DPPH solution in a 96-well plate. The reaction mixture was incubated in the dark at RT for 30 min. After incubation, the absorbance was measured at 517 nm using a microplate reader (Synergy H1, BioTek Instruments). Quercetin was used as a positive control, and methanol without DPPH was used as a blank. The percentage of the DPPH radical scavenging activity was calculated using the following formula: DPPH scavenging

activity (%) [(absorbance of the control – absorbance of the sample) / absorbance of the control] $\times$ 100, where absorbance of the control is the absorbance of the DPPH solution without the sample, and absorbance of the sample is the absorbance in the presence of the test sample. All experiments were performed in triplicate.

2.7. Measurement of intracellular ROS levels

Intracellular reactive oxygen species (ROS) levels were measured using the DCF-DA (2',7'-dichlorofluorescein diacetate) assay. RAW 264.7 cells were seeded in 12-well plates at a density of 2×10^5 cells/well and incubated overnight. Cells were treated with vehicle (DMSO, 0.1 %), MECL (10, 20, and 50 μ g/ml), hydrogen peroxide (H $_2$ O $_2$, 100 μ M), or TBHQ (5 μ g/ml) for 24 h. H $_2$ O $_2$ was used as a positive control. TBHQ was used as a reference compound for Nrf2-mediated antioxidant activation. After treatment, cells were washed twice with PBS and incubated with 10 μ M DCF-DA (Sigma-Aldrich, #D6883) in serum-free DMEM at 37 °C for 30 min in the dark. Following incubation, cells were lysed with cell lysis buffer and then transferred to black 96-well plates. Fluorescence intensity was measured using a microplate reader at excitation/emission wavelengths of 485/535 nm.

2.8. ELISA

RAW 264.7 macrophages were seeded in a 12-well plate at a density of 4×10^4 cells/well and incubated at 37°C overnight. The cells were treated with MECL (10, 20, and 50 μ g/ml) or vehicle (DMSO, 0.1 %) for 2 h and stimulated with LPS (1 μ g/ml) for 24 h. Cultured medium supernatants were collected and centrifuged at 3,000 rpm for 1 min. The production of IL-6 (#cat 88-7064-88), IL-1 β (#cat 88-7013-88), TNF- α (#cat 88-7324-88) PGE $_2$ (#cat 514010) was assessed in accordance with the manufacturer's instructions. Each sample was diluted at 1:100 and 1:50 to ensure that the production of IL-6 and TNF- α was accurately measured, respectively. The absorbance values of IL-6, IL-1 β , and TNF- α were measured at 450 nm by using a Synergy H1 microplate reader, and cytokine concentrations were calculated on basis of the standard curve. PGE $_2$ production was assessed using a 96-well goat anti-mouse IgG-coated plate. Absorbance was measured at 405 nm, and the values were calculated using a four-parameter logistic fit.

2.9. Immunoblotting analysis

RAW 264.7 macrophages were treated with MECL (10, 20, and 50 μ g/ml) or vehicle (DMSO, 0.1 %) at 37°C for 2 h, and stimulated with or without LPS (1 μ g/ml). For the analysis of IKK α / β , I κ B α , NF- κ B, TAK1, and MAPKs, the cells were stimulated for 15 min, whereas iNOS and COX-2 were assessed after 24 h of stimulation. RAW 264.7 macrophages were treated with MECL (10, 20, and 50 μ g/ml) or vehicle (DMSO, 0.1 %) alone and incubated at 37°C for 24 h to assess the protein levels of Nrf2, Keap1, HO-1, and NQO1. The cells were washed with PBS and lysed in a cell lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, 0.5 % NP-40, 0.5 % Triton X-100, and 1 % glycerol. The lysis buffer was supplemented with an Xpert protease inhibitor cocktail and Xpert phosphatase inhibitor cocktail (genDEPOT, Texas, USA) to prevent protein degradation and dephosphorylation. The cells were centrifuged at 13000 rpm at 4°C for 15 min. Proteins were quantified using the Bradford protein assay (Bio-Rad, California, USA) in accordance with the manufacturer's instructions. Cell lysates were mixed with 5 $\times$ sample buffer (10 % SDS, 250 mM Tris-HCl [pH 6.8], 50 % glycerol, 5 % β -mercaptoethanol, and 0.01 % bromophenol blue) and adjusted to equal protein concentrations. The samples were boiled at 100°C for 5 min, separated on 8–12 % SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (GE Healthcare, Wisconsin, USA). The membranes were blocked with 5 % skim milk RT for 1 h. Each membrane was washed thrice with TBST (Tris-buffered saline containing 0.1 % Tween-20) for 3 min per wash, and incubated with primary

antibodies diluted at 1:3000 in 5 % BSA in TBST overnight at 4°C. Each membrane was washed thrice with TBST for 3 min per wash, and incubated with HRP-conjugated secondary antibodies diluted at 1:5000 in 5 % skim milk on a shaker at RT for 2 h. After the membranes were washed five times with TBST for 3 min each, target protein bands were detected using an enhanced chemiluminescence (ECL) immunoblotting detection reagent (Pierce, Illinois, USA). Protein levels were quantified using LabWorks software (UVP Inc., CA, USA).

2.10. Luciferase reporter assay

HEK 293 cells were seeded in 100 mm culture dishes and incubated overnight. The following day, they were transfected with pNF-κB-Luc, pAP-1-Luc *cis*-reporter, or NQO1/antioxidant response element (ARE)-Luc reporter plasmids along with the gWIZ-green fluorescent protein (GFP) plasmid by using polyethylenimine (PEI). GFP was used as an internal control to normalize luciferase reporter gene values. After transfection, the cells were split into 12-well plates and incubated for 24 h, treated with MECL (10, 20, and 50 µg/ml) or vehicle (DMSO, 0.1 %) for 2 h, and stimulated with PMA for 24 h. Subsequently, they were lysed using cell culture lysis reagent (Promega, Madison, USA), and equal amounts of lysates were transferred to a white 96-well plate. Luciferin substrate was added, and luminescence was measured using a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA). Fluorescence intensity was measured in a black 96-well plate to quantify the GFP expression.

2.11. qRT-PCR

Total RNA was extracted from RAW 264.7 macrophages by using the Labozol reagent (Cosmogentech, Seoul, Korea) in accordance with the manufacturer's instructions. RNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, USA). cDNA was synthesized from 1 µg of total RNA by using a TOPscript cDNA synthesis kit (Enzymomics, Daejeon, Korea) in accordance with the manufacturer's guidelines. cDNA was mixed with Dyne qPCR 2 × Premix (Dynebio, Gyeonggi-do, Korea) following the manufacturer's instructions, and amplification was performed using a CFX Connect real-time thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR 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. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method, and *GAPDH* was used as the internal reference gene. The sequences of the primers used for qRT-PCR are listed in Table 1.

Table 1
Primer sequences used in qRT-PCR.

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

2.12. Confocal immunofluorescence assay

RAW 264.7 macrophages were seeded onto 35 mm confocal dishes and incubated overnight. The following day, the cells were treated with MECL (50 µg/ml) or vehicle (DMSO, 0.1 %) for 2 h, and stimulated with LPS for 15 min. After the treatment, the cells were washed thrice with PBS and fixed with 4 % paraformaldehyde (PFA) at 4°C for 15 min. They were further washed thrice with PBS and permeabilized with Triton X-100 at RT for 15 min. They were blocked with 2 % BSA in PBS at RT for 1 h to minimize nonspecific binding. The primary antibody (NF-κB) was diluted at 1:100 in 0.1 % BSA in PBS and incubated overnight at 4°C. After being washed thrice with PBS, the cells were incubated with goat anti-mouse DyLight 594-conjugated secondary antibody diluted at 1:100 in 0.1 % BSA in PBS at RT for 1 h. For nuclear staining, the cells were incubated with 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml in PBS) at RT for 5 min, and washed thrice with PBS. Fluorescence images were captured using a Zeiss laser scanning confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.13. Statistical analysis

All experiments were performed independently at least thrice, and data were presented as the mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons using GraphPad Prism 3.0 (GraphPad Software, CA, USA). Results with $p < 0.05$ was considered statistically significant. Significance levels are indicated as follows: *** $p < 0.001$ compared with the untreated control group; and ^a $p < 0.05$, ^b $p < 0.01$, and ^c $p < 0.001$ compared with the LPS-treated group.

3. Results

3.1. Effects of MECL on cell viability in RAW 264.7 macrophages and its inhibition of NO and PGE₂ production through the regulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages

Before the anti-inflammatory properties of MECL were investigated, its cytotoxic effects on RAW 264.7 macrophages were assessed through a cell viability assay. In Fig. 1A, the cells were treated with MECL at concentrations up to 200 µg/ml, with or without LPS stimulation. MECL exhibited no cytotoxicity at concentrations up to 50 µg/ml, regardless of LPS treatment; however, at 100 µg/ml or above, cytotoxic effects were observed in LPS-treated and untreated groups. Therefore, concentrations of up to 50 µg/ml were used to investigate the anti-inflammatory and antioxidant effects of MECL in RAW 264.7 macrophages in this study. The anti-inflammatory effects of MECL were evaluated by measuring its inhibitory effects on the production of the inflammatory mediators NO and PGE₂ in LPS-stimulated RAW 264.7 macrophages. NO production was significantly increased by LPS stimulation, whereas it was dose-dependently reduced by MECL compared with the LPS-only group (Fig. 1B).

The MECL treatment significantly reduced PGE₂ production compared with the LPS-only group; however, the reduction was less substantial than that observed in NO production (Fig. 1C). Since the production of NO and PGE₂ is mediated by iNOS and COX-2, respectively, we examined whether MECL downregulated the iNOS and COX-2 expression. MECL treatment reduced the protein expression levels of iNOS and COX-2, and their mRNA levels similarly decreased (Fig. 1D and E). Therefore, the suppression of iNOS and COX-2 expression by MECL reduced NO and PGE₂ production. Additionally, the MECL-induced reduction in the iNOS expression was more pronounced compared with that in COX-2, reflecting a pattern similar to the changes observed in NO and PGE₂ production. These results indicated that the regulatory effect of MECL was stronger on *iNOS* transcription than on *COX-2* in RAW 264.7 macrophages.

A previous HPLC analysis on MECL identified catechin, rutin, and

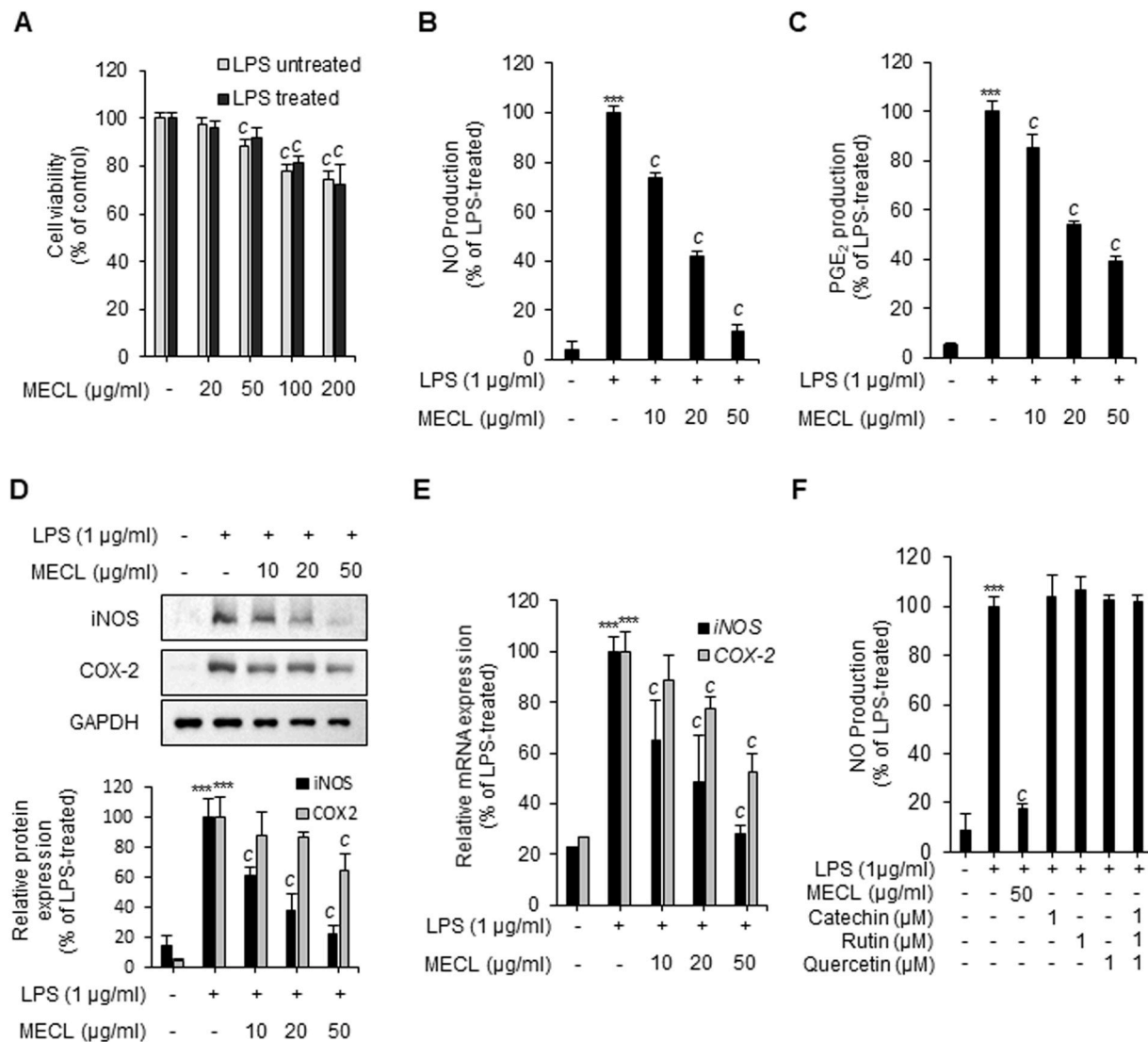


Fig. 1. Effects of MECL on cell viability and the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. (A, B, C, D, and E) RAW 264.7 macrophages were treated with various MECL concentrations for 2 h, and then stimulated with or without LPS (1 μg/ml) for 24 h. (A) Cell viability was evaluated using an EZ-CYTOX assay kit. The values were normalized to the untreated control group. ^c*p* < 0.001 (compared with the MECL untreated group). The values were normalized to the untreated control group. (B) The absorbance at 540 nm was measured using the Griess reagent to quantify the concentration of NO secreted into the cell supernatant. The values were calculated based on a nitrite standard curve. (C) PGE₂ production in the cell supernatant was evaluated using an ELISA assay. (D) Cell lysates were prepared for immunoblotting analysis, and band intensities were quantified using ImageJ. The values were normalized to GAPDH and presented as bar graphs in the lower panel. (E) Total RNA was extracted and used for cDNA synthesis, followed by qRT-PCR analysis. The values were normalized to GAPDH and presented as bar graphs. (F) RAW 264.7 macrophages were pretreated with MECL (50 μg/ml), (+)-catechin (1 μM), rutin (1 μM), or quercetin (1 μM) for 2 h, and stimulated with LPS (1 μg/ml) for an additional 24 h. Data were presented as the mean ± SD. ****p* < 0.001 (compared with the untreated control group); ^c*p* < 0.001 (compared with the LPS-treated group).

quercetin, which are polyphenolic compounds known for their anti-inflammatory effects [42], as major constituents. They also exhibit anti-inflammatory activities in RAW 264.7 macrophages [47–49]. Although their estimated concentrations in MECL (50 μg/ml) were in the nanomolar range, each compound was tested at 1 μM in the NO assay to evaluate their potential anti-inflammatory effects (Fig. 1F). However, none of the compounds showed a significant effect on LPS-induced NO production at this concentration. Therefore, the observed anti-inflammatory effects in this study might be attributed to novel compound(s). LC-MS/MS analysis was conducted to evaluate the presence of major polyphenols in MECL (Fig. S1). Catechin and rutin were detected in MECL. We also confirmed that other known components (chlorogenic acid, gallic acid, and caffeic acid) were present in the extract.

3.2. Inhibitory effects of MECL on the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α in LPS-stimulated RAW 264.7 macrophages

Pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α are released by cells during the inflammatory process to regulate immune responses. In this study, their regulatory effects on pro-inflammatory cytokines were investigated in LPS-stimulated RAW 264.7 macrophages to gain a broader understanding of the anti-inflammatory effects of MECL. MECL treatment dose-dependently reduced the mRNA levels of IL-6, IL-1β, and TNF-α, which were increased by LPS treatment (Fig. 2A). Their cytokine production levels were similarly reduced (Fig. 2B). Notably, the mRNA expression and cytokine production of IL-6 and IL-1β were more strongly suppressed by MECL treatment than by TNF-α in LPS-stimulated RAW 264.7 macrophages. Therefore, MECL suppressed cytokine production in RAW 264.7 macrophages by regulating the

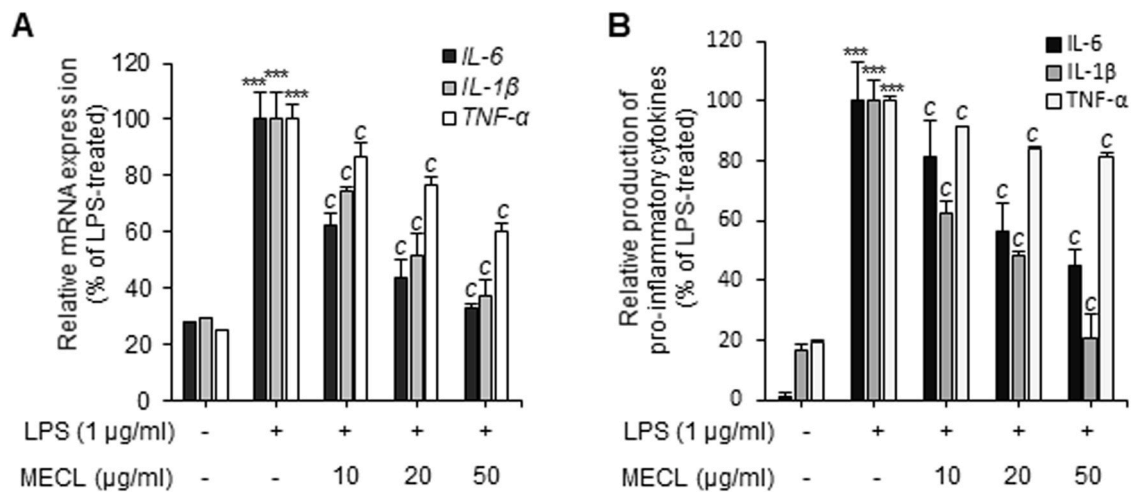


Fig. 2. Inhibitory effects of MECL on the protein production and mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were treated with MECL at various concentrations (10, 20, and 50 μg/ml) for 2 h and stimulated with or without LPS (1 μg/ml) for 24 h. (A) The mRNA levels of *IL-6*, *IL-1β*, and *TNF-α* were analyzed using qRT-PCR. (B) The production of *IL-6*, *IL-1β*, and *TNF-α* in the cell supernatant was examined using ELISA. All bar graphs represent the mean ± SD. *** $p < 0.001$ (compared with the untreated control group); $^{\circ}p < 0.001$ (compared with the LPS-treated group).

transcription of pro-inflammatory cytokines.

3.3. Inhibitory effects of MECL on NF-κB and AP-1 transcriptional activity in PMA-stimulated HEK293 cells

Since NF-κB and AP-1 are key transcription factors involved in the regulation of inflammatory responses, the regulatory effects of MECL on the transcriptional activities of NF-κB and AP-1 were investigated to further elucidate its anti-inflammatory properties. For this purpose, HEK293 cells were transfected with luciferase reporter genes regulated by NF-κB- or AP-1-mediated promoter activity. Because HEK293 cells lack TLR4 receptors, PMA (phorbol 12-myristate 13-acetate) was utilized instead of LPS to stimulate NF-κB and AP-1 transcriptional activation [50]. NF-κB and AP-1 promoter activities were significantly upregulated following PMA stimulation, and MECL treatment reduced their activities (Fig. 3A and B). Moreover, MECL dose-dependently reduced the NF-κB transcriptional activity, whereas the AP-1 activity was weakly suppressed. Therefore, MECL exerted its anti-inflammatory effects by more strongly inhibiting the promoter activity of NF-κB than

that of AP-1.

3.4. MECL modulates MAPK and NF-κB signaling by regulating TAK1 in LPS-stimulated RAW 264.7 macrophages

Given that MECL regulates the transcriptional activities of NF-κB and AP-1 during the inflammatory response, their upstream regulators should be identified. Immunoblotting was performed to elucidate the modulatory effects of MECL on NF-κB and MAPK signaling, which are key regulators of these transcription factors. The influence of MECL on TAK1, an essential upstream mediator of these pathways, was also assessed. Initially, the effects of MECL on NF-κB signaling were assessed in LPS-stimulated RAW 264.7 macrophages. MECL dose-dependently inhibited the phosphorylation of IKKα/β, which subsequently suppressed the phosphorylation of NF-κB and IκB (Fig. 4A). The phosphorylation of IκB at Ser32/36 facilitated its dissociation from NF-κB and subsequent proteasome-mediated degradation. MECL treatment inhibited IκB phosphorylation, resulting in its accumulation within the cells. The phosphorylation of NF-κB at Ser536 induced its nuclear

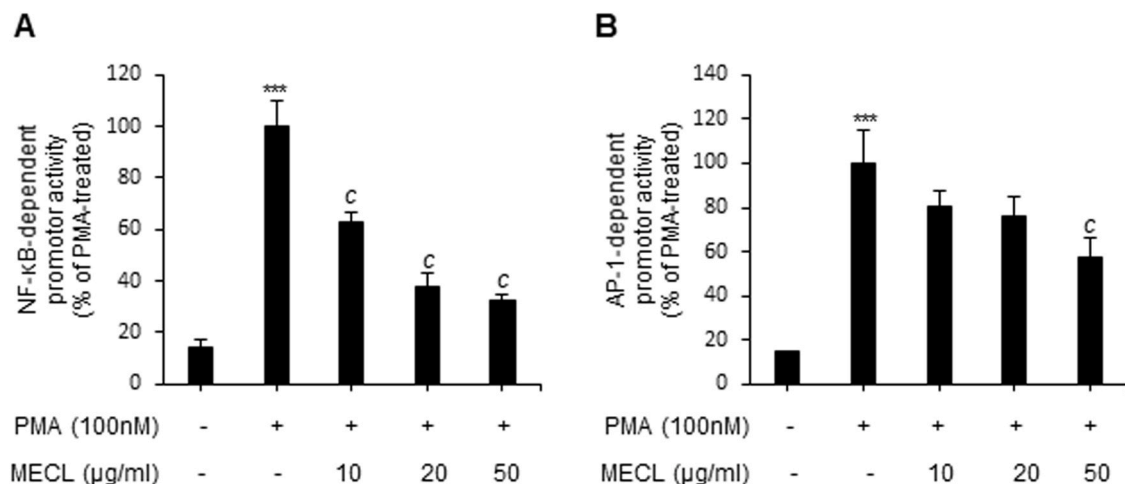


Fig. 3. Inhibitory effects of MECL on the transcriptional activity of NF-κB and AP-1 transcription factors. HEK 293 cells were transfected with (A) a luciferase gene containing the NF-κB binding site or (B) a luciferase gene containing the AP-1 binding site, along with gWiz-GFP. Transfected cells were treated with MECL at various concentrations (10, 20, and 50 μg/ml) for 2 h, stimulated with PMA (100 nM) and incubated for an additional 24 h. Luciferase values were normalized to GFP values. All bar graphs represent the mean ± SD. *** $p < 0.001$ (compared with the untreated control group); $^{\circ}p < 0.001$ (compared with the PMA-treated group).

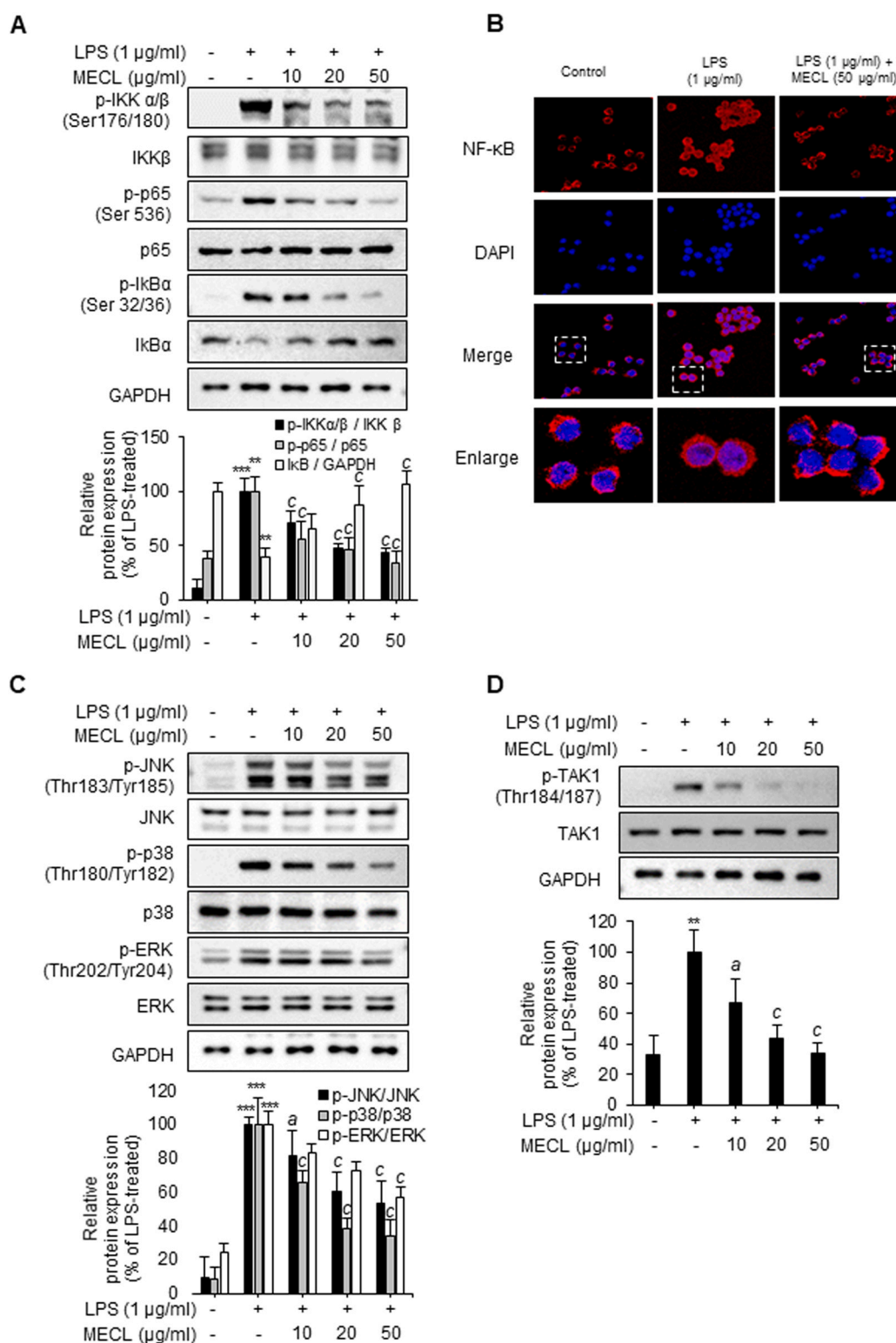


Fig. 4. Inhibitory effects of MECL on NF-κB and MAPK signaling through the suppression of TAK1 phosphorylation in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 macrophages were treated with various MECL concentrations (10, 20, and 50 µg/ml) for 2 h and stimulated with LPS for 15 min. (A, C, and D) Protein levels were quantified by immunoblotting analysis, and the corresponding values were presented as bar graphs in the lower panels. The phosphorylated proteins were normalized to their respective total protein levels. (B) NF-κB was probed using an anti-NF-κB polyclonal primary antibody and fluorescently labeled with a goat anti-Mouse IgG-DyLight 594 conjugated secondary antibody. The nucleus was stained with DAPI, and images were acquired through confocal microscopy. All bar graphs represent the mean ± SD. ***p* < 0.01, ****p* < 0.001 (compared with the untreated control group); ^a*p* < 0.05, ^c*p* < 0.001 (compared with the LPS-treated group).

translocation, which was visualized using confocal microscopy to determine whether MECL inhibited this process (Fig. 4B). Immunofluorescence analysis demonstrated that NF- κ B was primarily localized in the cytoplasm in untreated RAW 264.7 macrophages. LPS stimulation induced the nuclear translocation of NF- κ B, whereas MECL treatment

significantly inhibited this translocation.

The effects of MECL on MAPK signaling related to the AP-1 transcription factor were analyzed. MECL reduced the activation loop phosphorylation of JNK, p38, and ERK in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner (Fig. 4C). TAK1, a critical

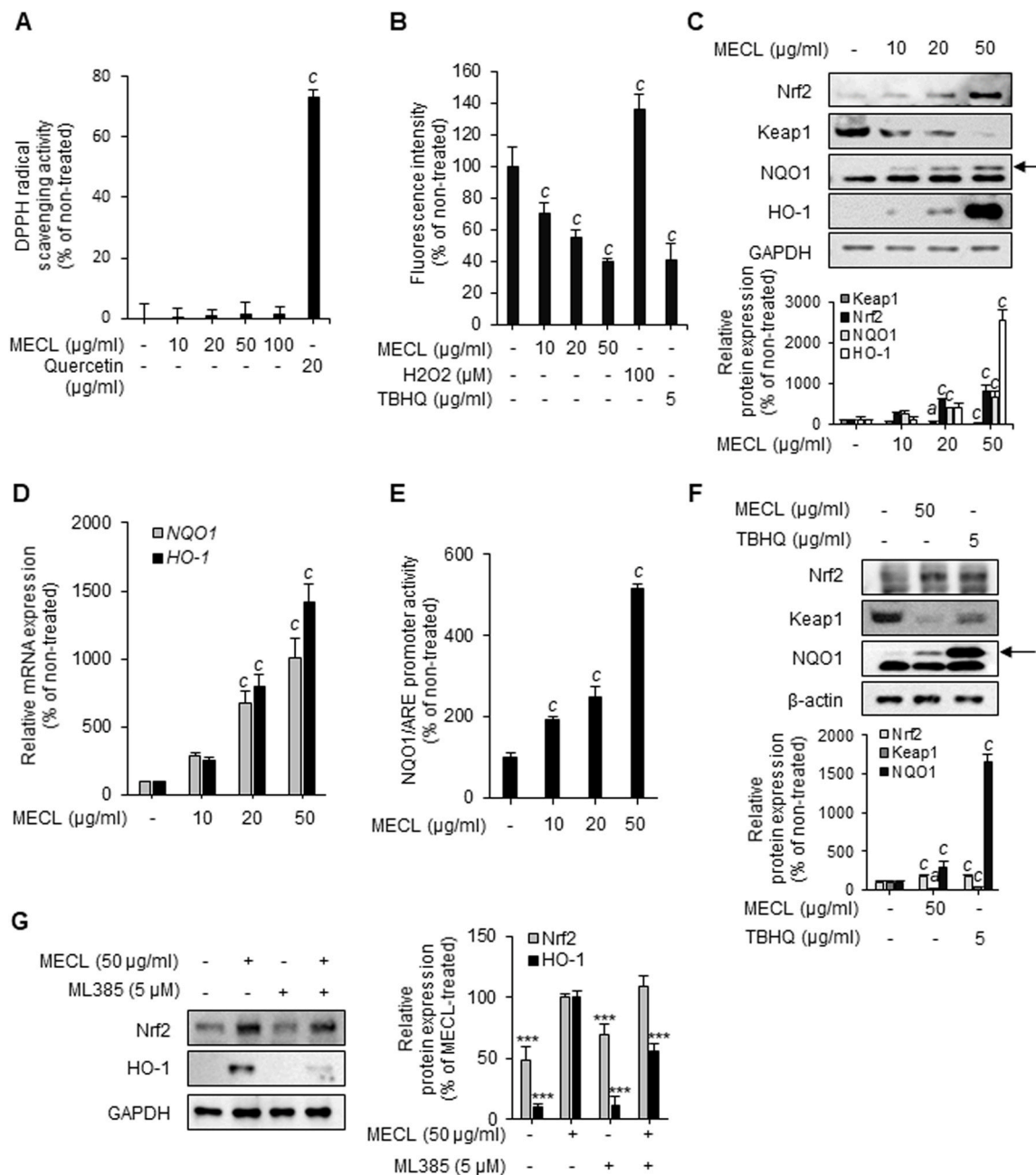


Fig. 5. Antioxidant effects of MECL through Nrf2 activation in RAW 264.7 macrophages. (A) The radical scavenging effects various MECL concentrations (10, 20, 50, and 100 μ g/ml) were evaluated using the DPPH assay. Quercetin was used as a positive control. (B, C, and D) RAW 264.7 macrophages were treated with various MECL concentrations (10, 20, and 50 μ g/ml) for 24 h. (B) Intracellular ROS levels were measured using the DCF-DA assay. H₂O₂ and TBHQ were used as positive controls and administered for 24 h to assess antioxidant responses. Fluorescence intensity was quantified using a microplate reader. The relative ROS levels were expressed as a percentage of the untreated control group. (C) Cell lysates were prepared, and protein levels were analyzed by immunoblotting. Each value was quantified relative to GAPDH and presented as bar graphs in the lower panels. (D) The RNA levels of NQO1 and HO-1 were measured via qRT-PCR and normalized to that of GAPDH. (E) HEK293 cells were transfected with a luciferase vector containing the ARE site recognized by NQO1 along with the gWiz-GFP vector. Transfected cells were treated with various MECL concentrations (10, 20, and 50 μ g/ml) for 24 h. Luciferase activity luminescence values were normalized to GFP fluorescence values. (F) RAW 264.7 macrophages were treated with MECL (50 μ g/ml) or TBHQ (5 μ g/ml) for 24 h. TBHQ was used as a positive control for Nrf2 activation. Each protein expression level was quantified relative to β -actin and presented as a bar graph in the lower panel. (G) RAW 264.7 macrophages were treated with MECL (50 μ g/ml) or ML385 (5 μ M) for 24 h. For the co-treatment, cells were pretreated with ML385 for 2 h, followed by the addition of MECL for the remaining duration. Each protein expression level was quantified relative to GAPDH and presented as a bar graph in the lower panel. All bar graphs represent the mean $\pm$ SD. ^a p < 0.05 and ^p < 0.001 (compared with the untreated group). ^{***} p < 0.001 (compared with the MECL-treated group).

upstream kinase for both signaling pathways, might be a potential target of MECL since NF- κ B and MAPK signaling pathways were inhibited by MECL. The phosphorylation levels of TAK1 Thr184/187 in LPS-stimulated RAW 264.7 macrophages were analyzed using immunoblotting to investigate this finding. The results demonstrated that MECL inhibited TAK1 phosphorylation in a dose-dependent manner (Fig. 4D). Therefore, MECL exerted its anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages by inhibiting TAK1 phosphorylation, thereby suppressing NF- κ B and MAPK signaling pathways.

3.5. Induction of antioxidant gene expression via Nrf2 activation by MECL in RAW 264.7 macrophages

During pathological inflammatory processes, macrophages are recruited to the inflammation site, where they produce large amounts of ROS as part of the anti-inflammatory response. Since excessive ROS accumulation can induce DNA damage, the primary strategy to

counteract ROS accumulation in macrophages involves the activation of Nrf2 signaling.

MECL exhibits antioxidant properties [42,51–53]. Thus, MECL was examined for its potential antioxidant activity in RAW 264.7 macrophages. Before the intracellular antioxidant effects of MECL were investigated, its intrinsic radical scavenging activity was evaluated using the DPPH assay (Fig. 5A). However, MECL showed only minimal radical scavenging activity, even at high concentrations, compared with that of quercetin, which is a well-established antioxidant [54]. Since MECL may exert its antioxidant effects via cell signaling pathways but not through intrinsic activity, it was assessed for its antioxidant potential in RAW 264.7 macrophages. As shown in Fig. 5B, MECL significantly reduced ROS levels in a dose-dependent manner, as assessed by the DCF-DA assay. TBHQ, a well-established Nrf2 activator, similarly attenuated ROS accumulation, thereby supporting the involvement of Nrf2 signaling in the antioxidant activity of MECL.

To elucidate the molecular mechanism underlying this antioxidant

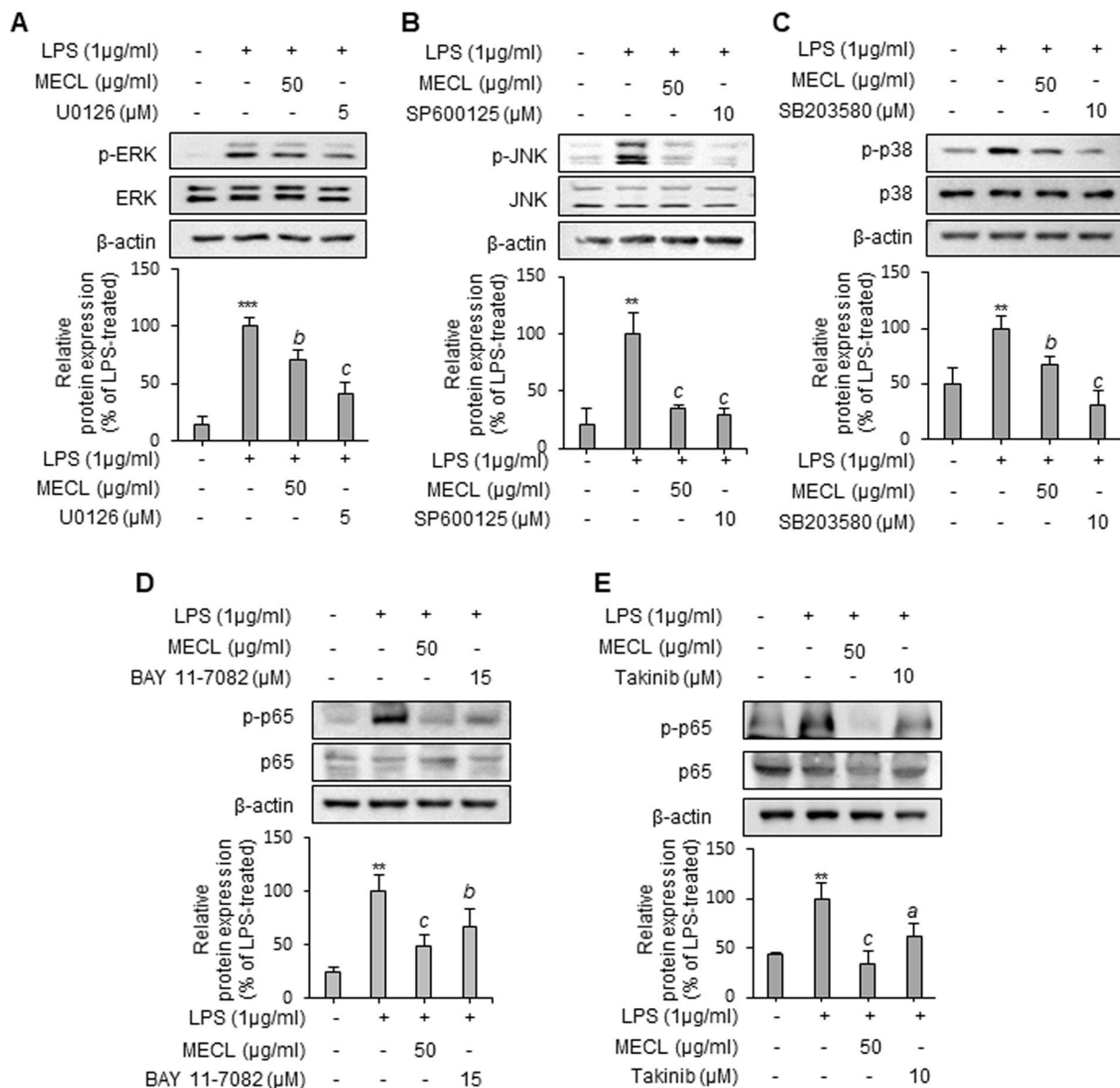


Fig. 6. Inhibitory effects of MECL compared with MAPK and NF- κ B inhibitors in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with MECL (50 μg/ml) or with the following selective inhibitors for 2 h: U0126 (5 μM) for ERK (A), SP600125 (10 μM) for JNK (B), SB203580 (10 μM) for p38 (C), BAY 11-7082 (15 μM) for NF- κ B (D), and Takinib (10 μM) for TAK1 (E). The phosphorylated protein levels were normalized to their respective total protein levels and presented as bar graphs in the lower panels. β-Actin was used as a loading control. All bar graphs represent the mean $\pm$ SD. ** p < 0.01, *** p < 0.001 (compared with the untreated control group); ^b p < 0.01, ^c p < 0.001 (compared with the LPS-treated group).

effect, we further examined the Nrf2 signaling pathway by immunoblotting. It induced a dose-dependent increase in Nrf2 protein levels and a decrease in Keap1 in RAW 264.7 macrophages (Fig. 5C). Additionally, the protein and mRNA expression levels of Nrf2 target genes *NQO1* and *HO-1* were significantly upregulated (Fig. 5C and D). An NQO1/ARE reporter assay was performed to investigate whether MECL-induced Nrf2 activation led to the increased promoter activity of its target genes (Fig. 5E). The results showed that MECL enhanced the NQO1/ARE promoter activity in a dose-dependent manner, with a more pronounced effect at higher concentrations. The antioxidant capacity of MECL was compared with that of TBHQ, a known Nrf2 activator (Fig. 5F). MECL and TBHQ increased the Nrf2 protein expression at comparable levels, and induced Keap1 degradation. Although the NQO1 expression was increased by MECL and TBHQ, the effect was more pronounced with the latter. The regulatory relationship between Nrf2 and HO-1 was examined using ML385, a selective Nrf2 inhibitor (Fig. 5G) [55]. Co-treatment with MECL and ML385 did not noticeably alter Nrf2 protein levels compared to MECL treatment alone; however, HO-1 expression was significantly reduced. These findings suggest that MECL-induced HO-1 expression is at least partially mediated through Nrf2 activity.

Therefore, MECL exerted potent antioxidant effects by activating Nrf2 within cells, thereby inducing the expression of key antioxidant target genes.

3.6. Comparative anti-inflammatory effects of MECL and TAK1/MAPK/NF- κ B inhibitors

Selective chemical inhibitors were used as reference controls to compare the inhibitory effects of MECL on MAPK and NF- κ B signaling pathways (Fig. 6). The following inhibitors were used: U0126 (5 μ M) as an ERK inhibitor (Fig. 6A), SP600125 (10 μ M) as a JNK inhibitor (Fig. 6B), SB203580 (10 μ M) as a p38 inhibitor (Fig. 6C), BAY 11–7082 (15 μ M) as an NF- κ B inhibitor (Fig. 6D), and Takinib (10 μ M) as a TAK1 inhibitor (Fig. 6E). Each inhibitor specifically reduced the phosphorylation of its respective target in LPS-stimulated RAW 264.7 cells. Furthermore, MECL attenuated the activation of these pathways, exhibiting an overall inhibitory pattern comparable with that of classical pathway-specific inhibitors. Therefore, MECL possibly modulated multiple inflammatory signaling pathways in a coordinated manner.

4. Discussion

Inflammation is a critical immune response and its dysregulation can cause chronic diseases [56]. Natural extracts have been widely explored in drug development because of their low toxicity and multi-target effects [57,58]. Although *E. cotinifolia* L. has been traditionally used in folk medicine, its anti-inflammatory and antioxidant properties have yet to be investigated. Therefore, this study aimed to investigate the anti-inflammatory and antioxidant effects of MECL and elucidate its underlying mechanisms in LPS-stimulated RAW 264.7 macrophages.

Catechin, rutin, and quercetin are major polyphenolic constituents of *E. cotinifolia* leaf extract; they exhibit anti-inflammatory properties in RAW 264.7 macrophages [47–49]. However, our results showed that none of these compounds effectively inhibited NO production. This discrepancy may stem from differences in extraction solvents and conditions compared to those used in previous studies. Despite the limited effect of the synthetic standards at the tested concentrations, it is still possible that plant-derived forms of these compounds—potentially existing in more bioactive, glycosylated, or conjugated forms within MECL—may contribute to the observed pharmacological effects. Therefore, further experiments using purified compounds directly isolated from MECL are warranted to clarify this possibility. In addition, bioactivity-guided fractionation studies should be performed to identify other active constituents and to assess potential synergistic interactions underlying the overall anti-inflammatory activity of MECL.

In contrast to the individual compounds, MECL as a whole extract significantly suppressed inflammatory responses in LPS-stimulated RAW 264.7 cells. It effectively suppressed iNOS and COX-2 expression, thereby reducing the production of the NO and PGE₂ in LPS-stimulated RAW 264.7 macrophages. It also inhibited the expression of pro-inflammatory cytokines, including IL-6, IL-1 β , and TNF- α . Notably, it elicited a stronger inhibitory effect on NF- κ B transcriptional activity than on AP-1. Our previous study indicated that AP-1 primarily regulates TNF- α and COX-2, whereas NF- κ B activation is more closely associated with iNOS, IL-6, and IL-1 β [59–62]. Consistent with these findings, our results showed that MECL also exhibited a stronger inhibitory effect on the expression of iNOS, IL-6, and IL-1 β than on TNF- α and COX-2. Although NF- κ B and AP-1 are both key regulators of inflammatory gene expression, their relative contributions may vary depending on promoter structure and transcription factor binding affinities [50,63,64]. Factors such as post-transcriptional regulation, differences in signal transduction kinetics, and interactions with co-regulatory proteins may also contribute to the selective inhibitory effects of MECL. Further studies should elucidate the precise molecular mechanisms involved. The diverse bioactive compounds in MECL may selectively regulate the transcription of inflammatory genes. Because of the critical role of inflammatory mediators and cytokines in the inflammatory response, their inhibition represents a fundamental approach in anti-inflammatory drug development. For example, widely used NSAIDs such as ibuprofen and naproxen primarily target COX-1 and COX-2 to suppress PGE₂ production [4,65]. In this context because MECL can suppress inflammatory mediators by inhibiting iNOS and COX-2 expression, and elicit inhibitory effects on pro-inflammatory cytokines, it shows potential as a promising candidate for anti-inflammatory drug development.

MECL inhibited the TAK1 phosphorylation, which subsequently suppressed downstream signaling components, including NF- κ B and MAPKs. Through this modulation, NF- κ B- and AP-1-dependent promoter activities were downregulated. Furthermore, confocal microscopy analysis demonstrated that MECL inhibited the nuclear translocation of NF- κ B p65.

The effect of MECL on NF- κ B and MAPK signaling was compared with that of the established pathway-specific chemical inhibitors to better contextualize its inhibitory effects. MECL treatment clearly suppressed each signal transduction pathway, exhibiting a pattern of inhibition consistent with that observed for chemical inhibitors. However, direct comparisons should be cautiously interpreted because of differences in concentration, target specificity, and mechanisms of action. Collectively, these findings suggested that the anti-inflammatory effects of MECL were mediated partly through TAK1 inhibition, highlighting its potential as a natural anti-inflammatory agent.

Given that the excessive production of ROS during inflammation can lead to cellular damage and contribute to chronic inflammatory diseases, the Nrf2/Keap1 signaling pathway participates in cellular defense mechanisms by inducing the expression of downstream antioxidant target genes; consequently, it reduces ROS levels [66]. In a previous study, MECL exhibited approximately 50 % DPPH radical scavenging activity at approximately 19 μ g/ml [44]. However, in our study, the extract showed negligible scavenging activity even at concentrations up to 100 μ g/ml. This discrepancy might be attributed to differences in plant origin and extraction methods. Although the direct free radical scavenging activity of MECL appeared limited, its antioxidant potential might instead be mediated through cellular signaling pathways. The MECL-induced increase in the Nrf2 expression might contribute to the enhanced cellular antioxidant capacity by promoting the transcription of protective genes such as HO-1 and NQO1. TBHQ induces autophagy by triggering conformational alterations in Keap1, thereby affecting its interaction with Nrf2 [67,68]. MECL and TBHQ activated Nrf2 by promoting Keap1 degradation; this finding suggested that MECL might directly or indirectly regulate Keap1 stability, similar to the mechanism of TBHQ. In addition, since p62 also mediates the autophagic

degradation of Keap1, MECL may be involved in the regulation of the p62–Keap1 signaling axis. Further studies should clarify the regulatory roles of Keap1 and p62 in this context.

Although this study demonstrated that the anti-inflammatory and antioxidant effects of MECL involve TAK1, NF- κ B/MAPK, and Nrf2 pathways, the specific bioactive compounds responsible for these effects remain unknown. Future studies should aim to identify individual active constituents and elucidate potential synergistic interactions that contribute to the overall pharmacological activity of MECL. In addition, *in vivo* studies are warranted to validate the therapeutic relevance of MECL under physiologically relevant conditions. Other species within the *Euphorbia* genus, such as *E. supina* and *E. hirta*, have demonstrated anti-inflammatory effects in animal models of colitis and arthritis, respectively, further supporting the potential of this genus in *in vivo* settings [69–71]. These findings suggest that *Euphorbia* plants may possess *in vivo* therapeutic potential. However, no such *in vivo* evidence currently exists for *E. cotinifolia*, which remains a limitation of this study. Nonetheless, our findings provide the first molecular evidence that MECL exerts anti-inflammatory and antioxidant effects via TAK1-mediated suppression of NF- κ B/MAPK signaling and activation of the Nrf2 pathway, supporting its promise as a natural therapeutic agent for inflammatory diseases.

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CRediT authorship contribution statement

Sang Beom Han: Methodology, Resources. **Choe Jeong:** Investigation. **Sayeon Cho:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Salah Uddin:** Resources, Methodology. **Junho Lee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare no competing interests.

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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.2025.118372](https://doi.org/10.1016/j.biopha.2025.118372).

Data availability

Data will be made available on request.

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