

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

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ABSTRACT

Oxidative stress and chronic inflammation are closely linked to various diseases. However, previous studies have demonstrated that plant extracts could prevent and alleviate these adverse outcomes. *Piper betle* Linn. (*Piper betle* L.) is a cosmopolitan plant that belongs to the Piperaceae family, whose leaves are edible and possess several health benefits. This study sought to characterize the anti-inflammatory and antioxidant effects of a methanol extract of *Piper betle* L. leaves and stems (MPBLLS). MPBLLS was found to have a dose-dependent radical scavenging effect, as demonstrated by the 2,2-diphenyl-1-picrylhydrazyl assay. Additionally, MPBLLS inhibited the lipopolysaccharide (LPS)-stimulated production of nitric oxide and prostaglandin E₂ by reducing the expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 macrophages without affecting cell viability. Furthermore, our findings suggested that the inhibitory effects of MPBLLS on pro-inflammatory cytokines such as tumor necrosis factor- α , interleukin-1 β , and interleukin-6 were due to the inhibition of the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways in LPS-treated RAW 264.7 macrophages. MPBLLS and hydroxychavicol, a major constituent of MPBLLS, suppressed LPS-induced translocation of NF- κ B p65 from cytoplasm to nucleus. Interestingly, MPBLLS increased nuclear factor erythroid 2-related factor 2 (Nrf2) protein levels and transcription levels of Nrf2 target genes in a dose-dependent manner. Collectively, our findings suggest that MPBLLS could serve as a basis for the development of novel orally-administered therapies due to its inhibitory effects on oxidative and inflammatory stress.

Data Availability: The data presented in this study are available on request from the corresponding author.

1. Introduction

Cells produce free radicals during normal metabolic processes, and

therefore antioxidants are needed to neutralize them [1]. In general, cells can maintain a balance between free radicals and antioxidants, but oxidative stress or excess free radicals can be produced by several

Abbreviations: LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; MAPKs, mitogen-activated protein kinases; Nrf2, nuclear factor erythroid 2-related factor 2; IkB α , inhibitor NF- κ B α ; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; NO, nitric oxide; PGE₂, prostaglandin E₂; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; AP-1, activator protein-1; ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase 1; HO-1, heme oxygenase-1; GCLC, glutamate-cysteine ligase catalytic subunit; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species.

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factors, such as lifestyle, pollution, radiation, diet, and the body's natural immune response [1]. Long-term, uncontrolled oxidative stress accelerates aging by damaging cells, proteins, and DNA [2]. Aging accelerated by persistent oxidative stress may lead to chronic inflammation [3]. Inflammation is the process by which the body heals itself from damage such as infection, disease, and injury [4]. This process is regulated by the immune system and is classified as chronic inflammation and acute inflammation [5]. Chronic inflammation has not only been linked to the onset of diabetes, cardiovascular disease, and arthritis but is also closely related to neurodegenerative diseases [6]. Furthermore, oxidative stress and chronic inflammation contribute to the development of several diseases, including cancer, inflammatory disorders, asthma, male infertility, and chronic fatigue syndrome [6,7]. Therefore, the development of functional foods or pharmaceutical supplements to control inflammation continues to be an important research topic worldwide.

RAW 264.7 macrophages are a murine macrophage cell line commonly used for antioxidant and anti-inflammatory studies, which are activated by lipopolysaccharide (LPS) [8–11]. Upon stimulation by LPS, RAW 264.7 macrophages produce pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), and promote the production of representative inflammatory factors such as nitric oxide (NO) and prostaglandin E₂ (PGE₂) [12]. NO plays an important role in the inflammatory response and intracellular signal transduction [13]. It is produced by continuous inflammation and becomes toxic in vivo as a radical inflammatory factor [11]. LPS, TNF- α , IL-1 β , and IL-6 reportedly stimulate intracellular inducible nitric oxide synthase (iNOS) to promote NO formation [14]. Additionally, macrophages stimulated by LPS and IL-1 increase the expression of cyclooxygenase-2 (COX-2) to produce PGE₂, a pro-inflammatory lipid mediator [15,16]. Therefore, it is important to determine whether an anti-inflammatory substance can control the levels of iNOS and COX-2 to prevent various diseases caused by inflammatory responses.

When RAW 264.7 macrophages are stimulated by LPS, the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways regulate the expression of inflammatory factors [17, 18]. LPS stimulation induces the phosphorylation of S32/36 on inhibitor NF- κ B α (I κ B α) and S536 on NF- κ B p65 [19]. Phosphorylation of I κ B α at S32/36 results in I κ B α degradation and disrupts binding to NF- κ B [17, 18]. The released free NF- κ B is phosphorylated and translocated to the nucleus to act as a transcription factor promoting the synthesis of iNOS, COX-2, and inflammatory cytokines [17,20]. Extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 are members of the MAPK family and are involved in the increase of the synthesis of inflammatory mediators in macrophages, thereby increasing the inflammatory response [14,21].

On the other hand, nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that increases cellular defense against oxidative damage such as reactive oxygen species (ROS) and inflammation [22]. Nrf2 interferes with IL-1 β and IL-6 in macrophages and binds to the antioxidant response element (ARE) located in the promoter regions of its target genes when cellular oxidative stress occurs, thereby inducing the expression of oxidative stress-related genes [23,24]. Activation of the Nrf2-ARE pathway leads to the expression of cytoprotective enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and glutamate-cysteine ligase catalytic subunit (GCLC) [23,25, 26].

Piper betle Linn. (*Piper betle* L.) is a cosmopolitan plant that belongs to the Piperaceae family [27]. This edible plant is known for its health-promoting and antioxidant properties in India and other South-East Asian countries [27,28]. *Piper betle* L. is also known to have analgesic and anti-inflammatory effects in vitro as well as antimalarial activity in vivo [28–30]. Other studies have shown that *Piper betle* L. inhibits NO production and has potent antioxidant effects [31–34]. Hydroxychavicol, isolated from *Piper betle* L., also suppressed iNOS and

COX-2 via the NF- κ B signaling pathway [35]. However, it is still unclear how this plant regulates antioxidant and anti-inflammatory mechanisms. Therefore, this study sought to elucidate the antioxidant and anti-inflammatory mechanisms of *Piper betle* L. leaves and stems by focusing on intracellular signaling pathways in RAW 264.7 macrophages.

2. Materials and methods

2.1. Preparation of methanol extract of *Piper betle* L. leaves and stems

Piper betle L. leaves and stems were collected from Toro Village, Lore Lindu National Park, Central Sulawesi, Indonesia. The collected *Piper betle* L. leaves and stems were identified at the Center for Pharmaceutical and Medical Technology (PTFM; Tangerang, Indonesia) and verified at the Herbarium Bogoriense (LIPI; Bogor, Indonesia). Voucher specimens recorded as KRIB 0047041 and PMT 1506 were deposited in the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Korea). The collected *Piper betle* L. specimens were cleaned and then dried in an oven at 40 °C until moisture levels decreased below 10%. The dried samples were then roughly ground, after which 1000 ml of food-grade methanol was added to 100 g of ground sample. The samples were then mixed for 10 min at 200 rpm using a magnetic stirrer and stored in a covered container for 18 h at room temperature. After settling for a few minutes, the samples were passed through filter paper to obtain the first liquid extract. Next, 1000 ml of fresh methanol was added to the solid obtained in the previous process, after which the samples were mixed at 200 rpm with a stirrer for 18 h, then filtered. The first and second liquid extracts were mixed and evaporated using a rotary evaporator at 150 mbar pressure and 40–45 °C temperature. The extracts were then stored in bottles and refrigerated. The methanol extract of *Piper betle* L. leaves and stems (MPBLLS) was obtained from the KRIBB. The stock extract was dissolved in dimethyl sulfoxide (DMSO) (200 mg/ml).

2.2. High-performance liquid chromatography (HPLC) analysis

The freeze-dried stock extract, hydroxychavicol (A294710MG, TCI America, Portland OR, USA), and eugenol (35995, Sigma-Aldrich, St. Louis, MI, USA) were dissolved in methanol and sonicated for 10 min. After filtering the solutions over a 0.2 μ m PTFE filter, the separation was conducted using a reversed-phase HPLC system (Waters 996, Waters Corp., Milford, MA, USA) equipped with Phenomenex Kinetex C18 column (4.6 mm $\times$ 250 mm, 5 μ m). In brief, the injected volume was 20 μ l, 25 °C with the flow rate fixed at 1.0 ml/min, and the detection wavelength was set at 280 nm. The elution solvent A (0.5% acetic acid in water) and B (acetonitrile) were used with the following gradient program: 10% B for 0–2 min, 10–50% B for 2–30 min, 50–90% B for 30–42 min, 90% B for 42–50 min.

2.3. Cell culture

RAW 264.7 macrophages were purchased from the Korea cell line bank (KCLB) and passaged in Dulbecco's Modified Eagles Medium (DMEM; LM001–05, WELGENE Inc., Gyeongsangbuk-do, Korea) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), penicillin (50 U/ml), 1% MEM NEAA (Thermo Fisher Scientific, Inc.), and 1% HEPES (Corning, NY, USA). Human embryonic kidney 293 (HEK 293) cells were obtained from ATCC (Manassas, VA, USA). HEK 293 cells were maintained in DMEM supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (50 μ g/ml; Gibco, Grand Island, NY, USA). Cells were subcultured every 48 h and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. Lipopolysaccharide (L6529) was purchased from Sigma-Aldrich. After dissolving MPBLLS and chemicals in DMSO to make stocks, they were diluted in the cell culture media so that the final

concentration of DMSO exposed to the cells was less than 0.35%.

2.4. Determination of nitric oxide (Griess assay)

RAW 264.7 macrophages (4×10^4 cells/well) were seeded in 96-well culture plates (SPL Life Sciences, Gyeonggi-do, Korea) and allowed to adhere overnight at 37 °C in a 5% CO₂ incubator. The cells were pre-treated with MPBLs for 2 h and then stimulated with LPS for an additional 24 h. One hundred microliters of the cell culture supernatants was collected and moved to a fresh clear uncoated 96-well plate. An equal volume of Griess reagent was added to the collected medium. After 5 min, absorbance was measured at 540 nm with a microplate reader (Synergy H1, BioTek Instruments, Inc., Winooski, VT, USA). A standard curve was generated using sodium nitrite to calculate the NO concentration.

2.5. DPPH radical scavenging assay

The antioxidant activity of MPBLs was determined based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described previously [36]. DPPH solutions were mixed with different concentrations of MPBLs diluted in methanol. Quercetin (Q4951, Sigma-Aldrich) was used as a positive control. Decreases in DPPH absorbance in this assay are indicative of DPPH scavenging activity (i.e., antioxidant effect).

2.6. Cytotoxicity assay

RAW 264.7 macrophages (4×10^4 cells/well) were seeded in 96-well culture plates (SPL Life Sciences) and allowed to adhere overnight at 37 °C in a 5% CO₂ incubator. The extract was pre-treated for 2 h before LPS was added. After LPS treatment for 24 h, the media were replaced with fresh media containing 5% EZ-CYTOX (EZ-1000, DOGEN, Seoul, Korea). One hour later, the absorbance (450 nm, 650 nm) was measured with a microplate reader. The cytotoxic effects of MPBLs on HEK 293 cells were also evaluated upon stimulating the cells with phorbol-12-myristate-13-acetate (PMA; P8139, Sigma-Aldrich) and MPBLs for 24 h.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To measure the expression of pro-inflammatory cytokines, RAW 264.7 macrophages (4×10^4 cells/well) were placed in a 96-well plate and cultured overnight. The macrophages were then pre-treated with different concentrations of MPBLs for 2 h and stimulated with LPS for an additional 24 h. PGE₂ levels in culture supernatants were determined using the Prostaglandin E₂ Express ELISA Kit (500141, Cayman Chemicals, Ann Arbor, MI, USA). TNF- α , IL-1 β , and IL-6 ELISA kits were purchased from Thermo Fisher Scientific.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

To measure mRNA levels of inflammatory mediator genes, RAW 264.7 macrophages were treated with MPBLs for 2 h and then stimulated with LPS for 6 h. To measure mRNA levels of antioxidant target genes, RAW 264.7 macrophages were treated with MPBLs or hydroxychavicol for 24 h. Total RNA was extracted using the Labozol RNA isolation reagent according to the manufacturer's instructions (CMRZ001, CosmoGenetech, Seoul, Korea), and then cDNA was synthesized with 1 μ g of total RNA using the TOPscript cDNA synthesis kit (ENzymatics, Daejeon, Korea). PCR amplification was performed with 25–30 cycles of 30 s denaturation at 95 °C, 20 s of annealing at 55–60 °C, and 1 min of extension at 72 °C. Finally, the final extension was performed at 72 °C for 10 min. The oligonucleotide primers used for PCR amplification are listed in Table 1.

Table 1

Oligonucleotide primer sequences used for RT-PCR.

Primer name	5' to 3'
iNOS (F)	TAG GCT GGA AGC CCC GCT AT
iNOS (R)	GAC TGT GGC TCT GAC CCG TG
COX-2 (F)	GAT GCT CTT CCG AGC TGT G
COX-2 (R)	GGA TTG GAA CAG CAA GGA TTT
IL-6 (F)	TCT AAT TCA TAT CTT CAA CCA AGA GG
IL-6 (R)	TGG TCC TTA GCC ACT CCT TC
IL-1 β (F)	TTG ACG GAC CCC AAA AGA T
IL-1 β (R)	GAT GTG CTG CGA GAT T
TNF- α (F)	GTG CCA GCC GAT GGG TTG TAC C
TNF- α (R)	AGG CCC ACA GTC CAG GTC ACT G
HO-1 (F)	CCT TCC CGA ACA TCG ACA GCC
HO-1 (R)	GCA GCT CCT CAA ACA GGT CAA
NQO1 (F)	AGC CAA TCA GCG TTC GGT AT
NQO1 (R)	GCC TCC TTC ATG GCG TAG TT
GCLC (F)	GGA CAA ACC CCA ACC AT
GCLC (R)	GTT GAA CTC AGA CAT CGT T
GAPDH (F)	GTC TTC ACC ATG GAG AAG G
GAPDH (R)	CCT GCT TCA CCA CCT TCT TGA T

2.9. Western blotting

Western blotting analyses were performed as described in previous studies [36,37]. RAW 264.7 macrophages were pre-treated with MPBLs for 2 h and then stimulated with LPS (1 μ g/ml). LPS exposure time was indicated in each figure legend. Antibodies were purchased from Santa Cruz Biotechnology (NF- κ B p65; SC-372, JNK 1/2/3; SC-7345, IKK β ; SC-8014, p38; SC-7972, I κ B α ; SC-1643, COX-2; SC-1745, iNOS; SC-7271, α -tubulin; SC-8035, HO-1; SC-390991, β -actin; SC-47778, CA, USA), Cell Signaling Technology (p-NF- κ B p65 (S536); 3033, p-IKK α/β (S176/180); 2697, p-p38 (T180/Y182); 9211, p-I κ B α (S32/36); 9246, p-JNK 1/2/3 (T183/Y185); 9251, p-ERK 1/2 (T202/Y204); 9106, ERK 1/2; 9102, Nrf2; 12721, MA, USA), and Genetex (GAPDH; GTX100118, USA).

2.10. Luciferase reporter assay

HEK 293 cells were seeded in 6-well plates and transfected with a luciferase reporter plasmid (500 ng/well) and gWIZ-green fluorescent protein (GFP) plasmid (100 ng/well) using linear polyethyleneimine (PolySciences, Warrington, PA, USA). NF- κ B and activator protein-1 (AP-1) luciferase reporter plasmids were obtained from Agilent Technology (Santa Clara, CA, USA). The pGL2-ARE-NQO1 luciferase plasmid was a kind gift from Prof. Young-Joon Surh (Seoul National University, Seoul, Korea). The gWIZ-GFP plasmid was used as an internal control for normalization. At 24 h after transfection, the cells were treated with MPBLs and/or PMA for an additional 24 h. The cells were harvested with Cell Culture Lysis 5X Reagent (E1531, Promega, Madison, WI) and centrifuged at 4 °C for 30 min. After centrifugation, the supernatants of the cell lysates were transferred to white and black 96-well plates. Luminescence and GFP values were measured using a microplate reader according to the instructions of the Luciferase Assay System (E1501, Promega). Relative luciferase activity was determined and normalized to GFP values.

2.11. Confocal microscopy

RAW 264.7 macrophages were plated in confocal dish (SPL Life Sciences) and grown overnight at 37 °C in a 5% CO₂ incubator. The cultured media were then replaced with MPBLs or hydroxychavicol containing media. Cells were allowed to grow for another 2 h, and then treated with LPS (1 μ g/ml) for 10 min. Cells were gently washed with phosphate buffered saline (PBS, pH 7.4) three times and fixed with 4% paraformaldehyde in PBS. Permeabilization was performed with 0.2% Triton X-100 in PBS. After blocking dish with 2% BSA in PBS, anti-NF- κ B p65 (1:50; SC-372, Santa Cruz Biotechnology) was applied for 2 h at

room temperature. Alexa 488 conjugated anti-rabbit IgG (1:100; A-11008, Thermo Fisher Scientific, Inc.) was then added and incubated for 1 h at room temperature. DAPI (D9542, Sigma-Aldrich) solution was used for nuclei staining. The green and blue fluorescence images were detected by the Zeiss LSM 800 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). The fluorescence value of each image was measured using Image J software [38].

2.12. Determination of intracellular reactive oxygen species (iROS) production

Protocols of previous studies were used to measure iROS production [39,40]. Briefly, RAW 264.7 macrophages (2×10^6 cells/well) were seeded into 6-well plates and incubated overnight at 37 °C in a 5% CO₂ incubator. Cells were treated with MPBLLS for 2 h and then activated with LPS for another 24 h. After removing cultured media, cells were washed twice with PBS. Cells were incubated with 10 μM of 2', 7'-dichlorofluorescein diacetate (DCF-DA; D6883, Sigma-Aldrich) for 30 min, and then gently washed twice with PBS to remove remaining DCF-DA. The cells were harvested and centrifuged at 4 °C for 15 min. The supernatants of the cell lysates were transferred to a black 96-well plate and fluorescence values were measured using a microplate reader (excitation at 485 nm, emission at 535 nm).

2.13. Statistical analysis

All experiments were repeated at least three times and the data were analyzed using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA, USA). The results are presented as mean ± standard deviation (SD). Differences between the sample group and the control group

were determined by one-way ANOVA. A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Morphological changes of RAW 264.7 macrophages and radical scavenging effect by MPBLLS

Since *Piper betle* L. is known to consist of chemicals with antioxidant and anti-inflammatory effects such as hydroxychavicol [41], the content of hydroxychavicol in MPBLLS was investigated through HPLC analysis (Fig. S1). The HPLC chromatogram of MPBLLS showed a major peak on the retention time (19.777 min), which was nearly identical to the retention time of the hydroxychavicol standard (19.740 min). The maximum absorbance of both peaks (220.6 nm) was the same. The hydroxychavicol content of MPBLLS calculated according to the hydroxychavicol calibration curve was 3.85%.

RAW 264.7 macrophages have been described as an appropriate model of macrophages and commonly used to confirm the anti-inflammatory effects of drug and to evaluate relevant signaling pathways [42–44]. Morphology of RAW 264.7 macrophages was observed upon LPS and MPBLLS treatment (Fig. 1A). The results showed that LPS treatment induced a morphological change of RAW 264.7 macrophages into dendritic-like cells and MPBLLS pre-treatment reduced the number of LPS-induced dendritic-like cells. Moreover, when RAW 264.7 macrophages were cultured with MPBLLS alone, most of the cells maintained their small and round shape. This suggests that MPBLLS has no effects on altering morphology of RAW 264.7 macrophages but suppresses LPS-induced morphological changes.

Measuring the radical scavenging activity of novel compounds or

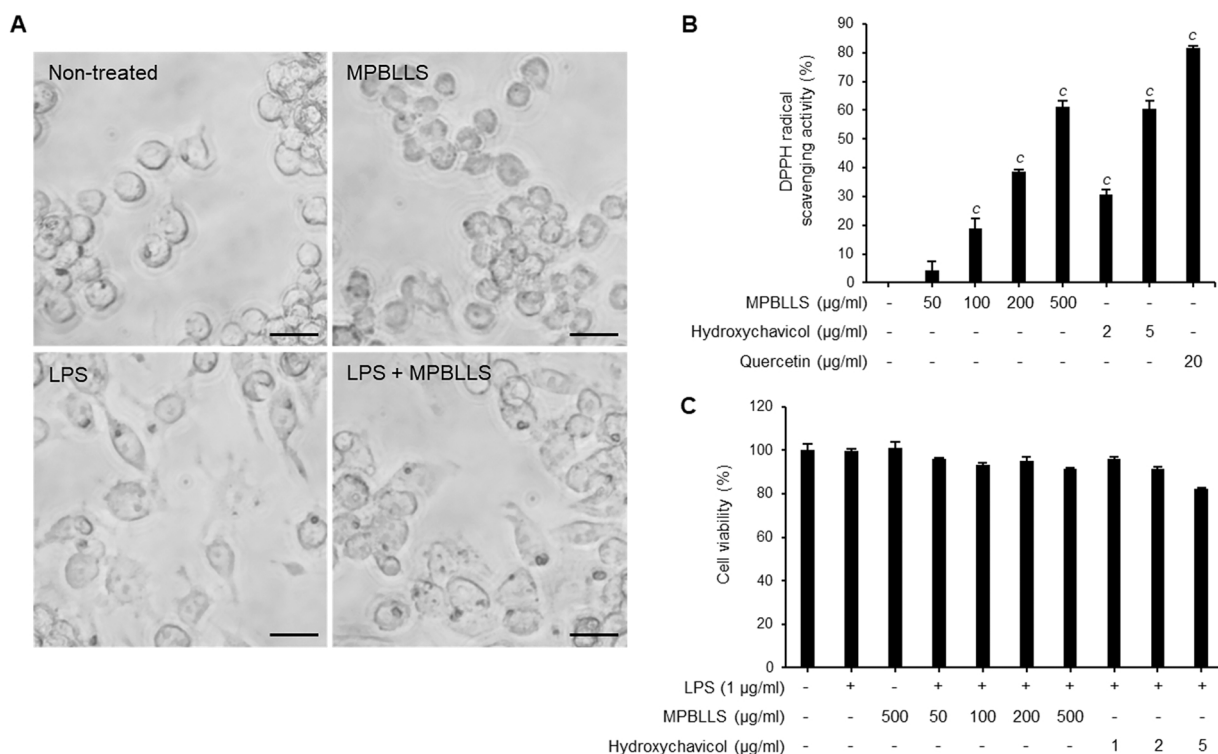


Fig. 1. Morphological changes of RAW 264.7 macrophages, radical scavenging effects, and cytotoxic effects upon treatment with MPBLLS. (A) RAW 264.7 macrophages were pre-treated with MPBLLS (100 μg/ml) for 2 h and then stimulated with LPS (1 μg/ml) for 24 h. After LPS treatment, images were observed using a light microscope at 10 × magnification. Scale bar, 30 μm. (B) The radical scavenging effects of MPBLLS and hydroxychavicol were confirmed by the DPPH assay. Methanol alone and quercetin (20 μg/ml) were used as negative and positive controls, respectively. The mean value of non-treated negative controls was considered 0%. (C) RAW 264.7 macrophages were seeded at a density of 4×10^4 cells/well. The cells were pre-treated with MPBLLS or hydroxychavicol at the indicated concentrations for 2 h. After treatment with or without LPS for 24 h, cell viability was measured as described in Materials and Methods and presented relative to non-treated control. All data were averaged from three independent experiments (n = 3) and presented as the mean ± SD [*p* < 0.001 vs. non-treated control group].

plant extracts is a good way to determine their antioxidant effects. DPPH assay is the most commonly used to evaluate the in vitro antioxidant capacity of plant extract through the scavenging effect of free radicals by reducing the DPPH radicals. The results of our DPPH assays indicated that the radical scavenging effect of MPBLLS was dose-dependent (Fig. 1B), suggesting that MPBLLS has an inherent antioxidant effect. Quercetin, a well-known antioxidant compound [45], was used as a positive control. Hydroxychavicol also showed dose-dependent radical scavenging effects (Fig. 1B).

Prior to studying the antioxidant and anti-inflammatory effects of MPBLLS in RAW 264.7 macrophages, cell viability of the macrophages was measured to confirm that treatment with MPBLLS, hydroxychavicol, or LPS did not cause cellular toxicity (Fig. 1C). When RAW 264.7 macrophages were pre-treated with MPBLLS for 2 h and then treated with LPS for 24 h, the experimental group treated with both LPS and MPBLLS reached a > 95% cell viability at MPBLLS concentration of up to 500 µg/ml. These findings indicated that RAW 264.7 macrophages can be treated with MPBLLS concentrations of 500 µg/ml or less without cytotoxic effects.

3.2. MPBLLS regulates iNOS and COX-2 expression in LPS-induced RAW 264.7 macrophages

To evaluate the anti-inflammatory potential of MPBLLS, Griess and ELISA assays were carried out to measure NO and PGE₂ production, respectively. RAW 264.7 macrophages were pre-treated with various doses of MPBLLS for 2 h and then stimulated with LPS for 24 h. As shown in Fig. 2A, approximately 25 µM of NO was produced in the LPS-stimulated group, whereas NO production decreased sharply as the MPBLLS pre-treatment dose increased. MPBLLS treatment also markedly decreased LPS-induced PGE₂ production (Fig. 2B). Given that NO and PGE₂ production is mediated by iNOS and COX-2, respectively, we investigated whether iNOS and COX-2 protein levels were decreased by MPBLLS treatment in LPS-stimulated RAW 264.7 macrophages. The LPS-induced iNOS protein expression levels were clearly reduced by MPBLLS treatment, whereas a moderate decrease in the COX-2 protein level was observed (Fig. 2C). Similar to the protein reduction pattern, LPS-induced iNOS and COX-2 mRNA levels were decreased upon MPBLLS treatment (Fig. 2D), suggesting that MPBLLS exerts anti-inflammatory effects through transcriptional regulation of iNOS and

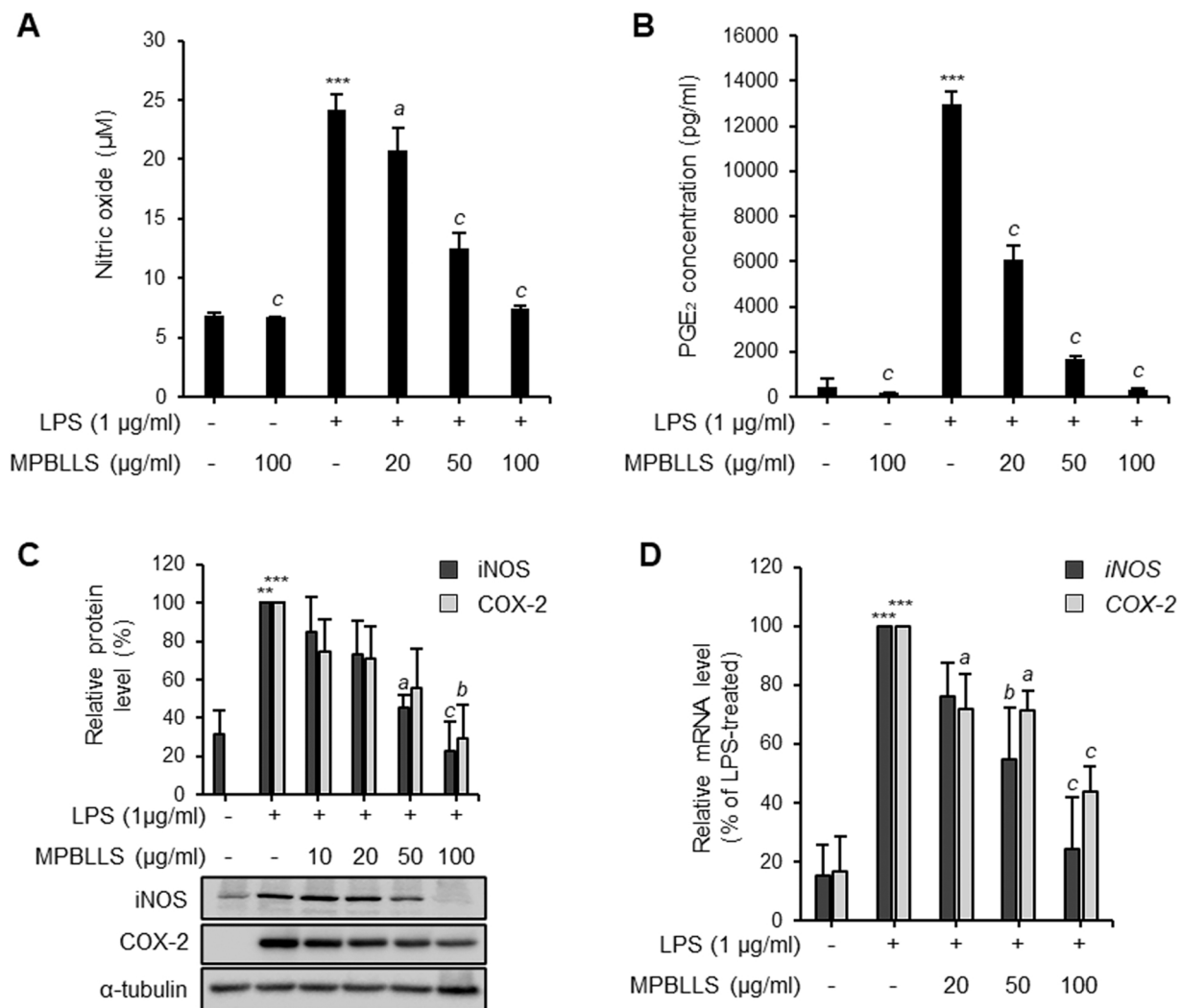


Fig. 2. Reduction of iNOS and COX-2 levels by MPBLLS in LPS-induced RAW 264.7 macrophages. Cells were treated with the indicated concentration of MPBLLS for 2 h and then stimulated with LPS for 24 h. (A) NO and (B) PGE₂ production assessment was evaluated using the cultured media. (C) Cells were seeded in 6-well plates and pre-treated with MPBLLS for 2 h. After LPS treatment for 24 h, the cells were harvested and sampled for western blotting. The α -tubulin protein was detected as an internal control. The protein levels of iNOS and COX-2 were presented relative to the LPS only-treated control group. (D) Cells were seeded in 12-well plates and pre-treated with MPBLLS for 2 h. After 6 h of LPS treatment, the mRNA levels of iNOS and COX-2 were evaluated by RT-PCR. The mRNA levels were presented relative to the LPS only-treated control group. All data were averaged from three independent experiments ($n = 3$) and presented as the mean $\pm$ SD [$^*p < 0.05$, $^b p < 0.01$, and $^c p < 0.001$ vs. LPS only-treated control group; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. non-treated control group].

COX-2.

3.3. MPBLLS down-regulates pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages

RAW 264.7 macrophages normally produce pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α through multiple inflammation signaling pathways upon exposure to LPS. To determine whether MPBLLS pre-treatment before LPS treatment inhibits the production of pro-inflammatory cytokines in RAW 264.7 macrophages, protein level changes of three pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) were measured by ELISA (Fig. 3A–C). The LPS-induced protein levels of IL-1 β , IL-6, and TNF- α were decreased by MPBLLS in a dose-dependent manner. Upon treatment with 20 μ g/ml of MPBLLS, IL-1 β levels were reduced by approximately 60% compared to the LPS only-treated control group (Fig. 3A). In Fig. 3B, the protein level of IL-6 exhibited almost 40% inhibition at a concentration of 50 μ g/ml MPBLLS and more than 95% inhibition at 100 μ g/ml MPBLLS compared to the LPS only-treated control group. When the cells were treated with MPBLLS at the maximum dose in the absence of LPS, there was little difference from the

non-treated control group (Fig. 3A–C). Additionally, the mRNA levels of these pro-inflammatory cytokines were also reduced by MPBLLS treatment in a dose-dependent manner (Fig. 3D). These results suggest that the anti-inflammatory effect of MPBLLS is mediated through transcriptional regulation of pro-inflammatory cytokines.

3.4. MPBLLS attenuates NF- κ B and MAPK signaling pathways

Given that NF- κ B and MAPK signaling are major pathways of inflammatory responses in LPS-stimulated RAW 264.7 macrophages [46], the effect of MPBLLS on these signaling pathways was investigated. The phosphorylation levels of NF- κ B and MAPK signaling proteins were analyzed after treatment of LPS or MPBLLS on RAW 264.7 macrophages. LPS-induced I κ B kinase (IKK) activation leads to the phosphorylation of I κ B [47]. I κ B α undergoes proteolysis after phosphorylation on S32/36 by IKK [19–21]. IKK was activated upon LPS treatment, resulting in the phosphorylation of I κ B α at S32/36 and NF- κ B p65 at S536 (Fig. 4A). The levels of p-I κ B α and p-NF- κ B p65 were significantly increased by LPS treatment, and therefore the total I κ B α protein level decreased due to I κ B α degradation. MPBLLS reduced the LPS-induced levels of p-I κ B α and

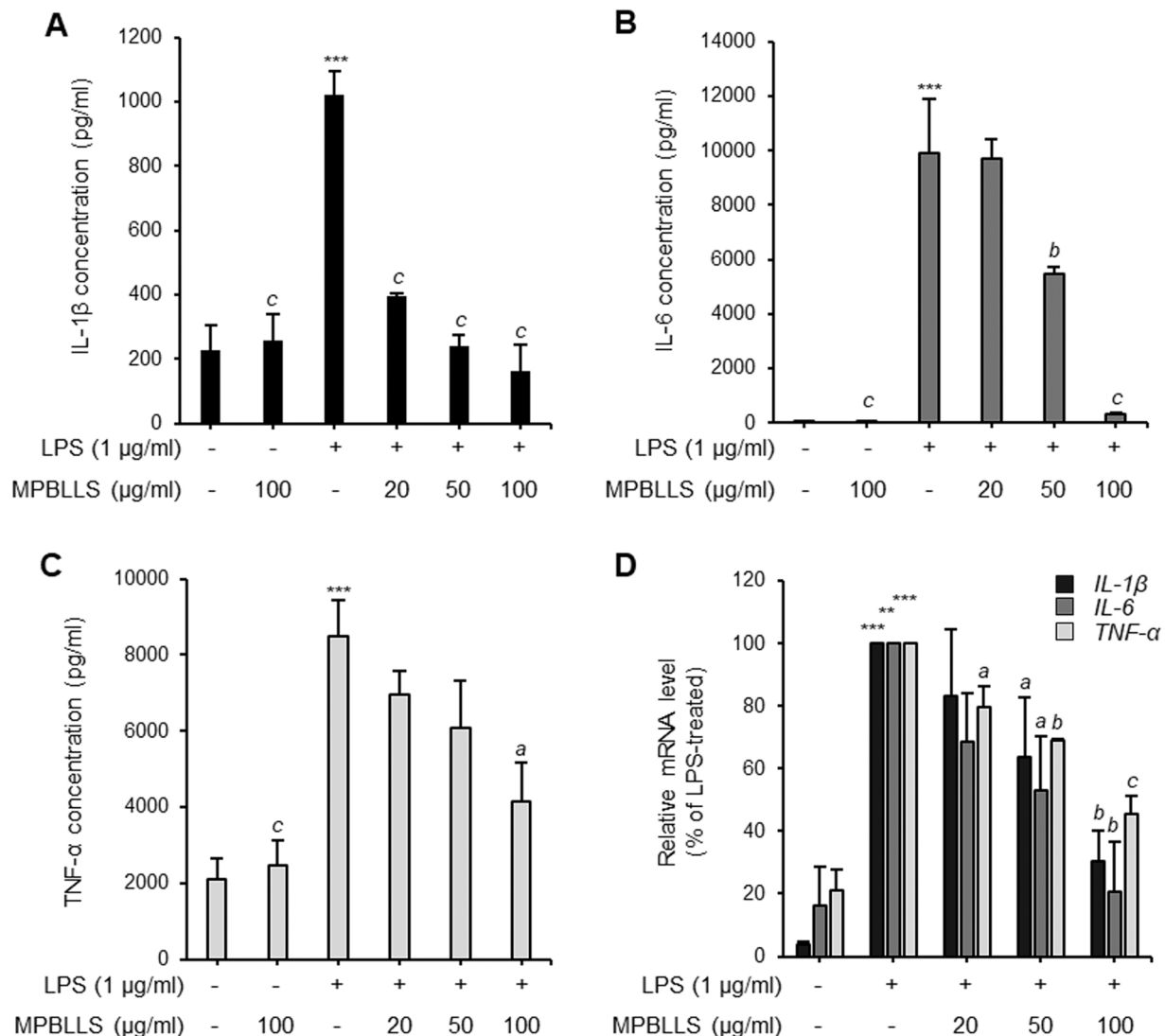


Fig. 3. Effects of MPBLLS on the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages. Cells were seeded in 96-well plates and pre-treated with MPBLLS at the indicated concentration. After 24 h of stimulation with LPS, the levels of (A) IL-1 β , (B) IL-6, and (C) TNF- α in the supernatants were analyzed by ELISA. (D) Cells seeded in 6-well plates were pre-treated with MPBLLS for 2 h and stimulated with LPS for 6 h. The mRNA levels of IL-1 β , IL-6, and TNF- α were analyzed by RT-PCR and presented relative to the LPS only-treated control group. All data were averaged from three independent experiments (n = 3) and presented as the mean $\pm$ SD [^ap < 0.05, ^bp < 0.01, and ^cp < 0.001 vs. LPS only-treated control group; ^{**}p < 0.01 and ^{***}p < 0.001 vs. non-treated control group].

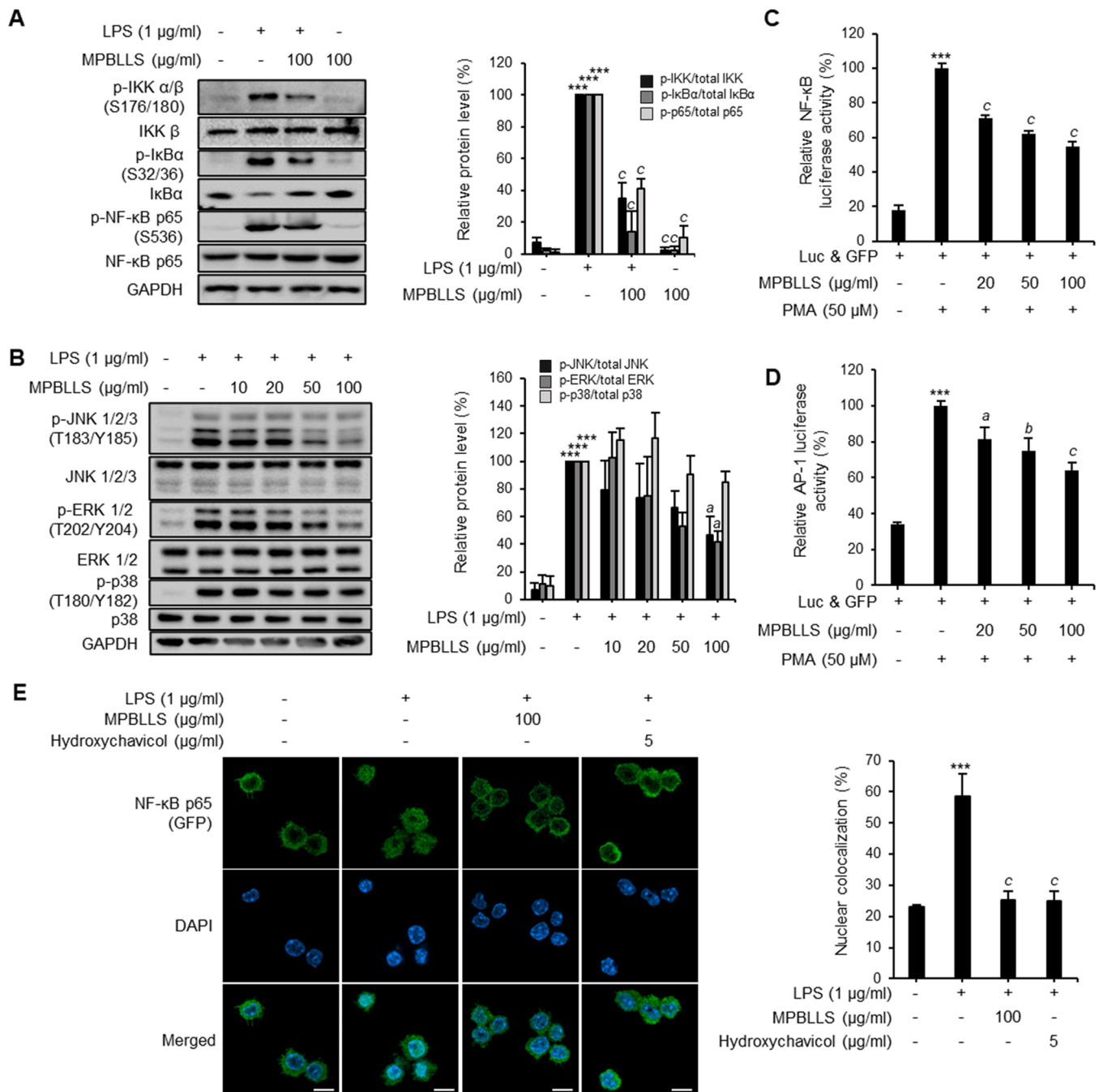


Fig. 4. Effects of MPBLLS on NF-κB and MAPK signaling pathways. (A, B) RAW 264.7 macrophages were seeded in 6-well plates and pre-treated with MPBLLS for 2 h prior to LPS treatment. After LPS treatment for 3 min for (A) and 15 min for (B), the cells were harvested for western blotting. GAPDH was used as an internal control. The relative phosphorylation levels of proteins were compared with the LPS only-treated control group (100%). All experiments were conducted in triplicate (n = 3) and presented as the mean ± SD [^ap < 0.05, and ^cp < 0.001 vs. LPS only-treated control group; ^{***}p < 0.001 vs. non-treated control group]. (C, D) HEK 293 cells were transiently co-transfected with reporter plasmids and gWIZ-GFP plasmid. After 24 h, the cells were treated with the indicated concentrations of MPBLLS and/or PMA for an additional 24 h. Luciferase activity values were measured and normalized to GFP values. The relative luciferase activity was compared with the PMA only-treated control group (100%). All data were averaged from three independent experiments (n = 3) and presented as the mean ± SD [^ap < 0.05, ^bp < 0.01, and ^cp < 0.001 vs. PMA only-treated control group; ^{**}p < 0.01 vs. non-treated control group; ^{***}p < 0.001 vs. non-treated control group]. (E) RAW 264.7 macrophages were seeded in confocal dish and pre-treated with MPBLLS or hydroxychavicol for 2 h prior to LPS treatment. After 10 min of LPS treatment, cells were observed using confocal microscope. The Image J software were used for quantifying NF-κB p65 in the nuclear region. The ratio of the GFP area in the nuclear region to the total DAPI area was expressed as the colocalization (%) of DAPI and NF-κB p65 in the nuclear region. Scale bar, 10 µm. All data were averaged from three independent experiments (n = 3) and presented as the mean ± SD [^cp < 0.001 vs. LPS only-treated control group; ^{***}p < 0.001 vs. non-treated control group].

p-NF-κB p65, whereas the MPBLLS only-treated group had no significant effect on the total and phospho-protein levels of IKK, IκBα, and NF-κB, suggesting that MPBLLS regulates LPS-induced IKK, IκBα, and NF-κB proteins. Given that increased phosphorylation levels of the MAPK

signaling factors are mediated by LPS treatment [48], the phosphorylation of each MAPK was also investigated. As shown in Fig. 4B, the phosphorylation levels of JNK and ERK were decreased by MPBLLS in a dose-dependent manner, whereas p38 levels remained largely

unchanged. When the effects of hydroxychavicol on NF- κ B and MAPK signaling pathways were measured, hydroxychavicol inhibited NF- κ B but not MAPK signaling pathway (Fig. S2).

Luciferase reporter assays with NF- κ B and AP-1 promoters were performed to investigate whether MPBLLS regulates the expression of genes that are located downstream of NF- κ B and JNK signaling pathways (Fig. 4C and D). HEK 293 cells were used instead of RAW 264.7 macrophages for luciferase reporter assays, as HEK 293 cells have much higher transfection efficiency than RAW 264.7 macrophages. PMA, an

activator of protein kinase C, was used to induce the activation of NF- κ B and JNK signaling because HEK 293 cells do not express toll-like receptor 4 that recognizes LPS. Upon treatment with PMA, the transcriptional activities of NF- κ B and AP-1 were increased. As the concentration of MPBLLS increased, the NF- κ B and AP-1 transcriptional activities were inhibited in a concentration-dependent manner. Since MPBLLS and PMA did not induce cytotoxic effects on HEK 293 cells (Fig. S3A), these results suggest that MPBLLS suppresses the transcription of target genes downstream of the NF- κ B and MAPK signaling pathways. However, it is

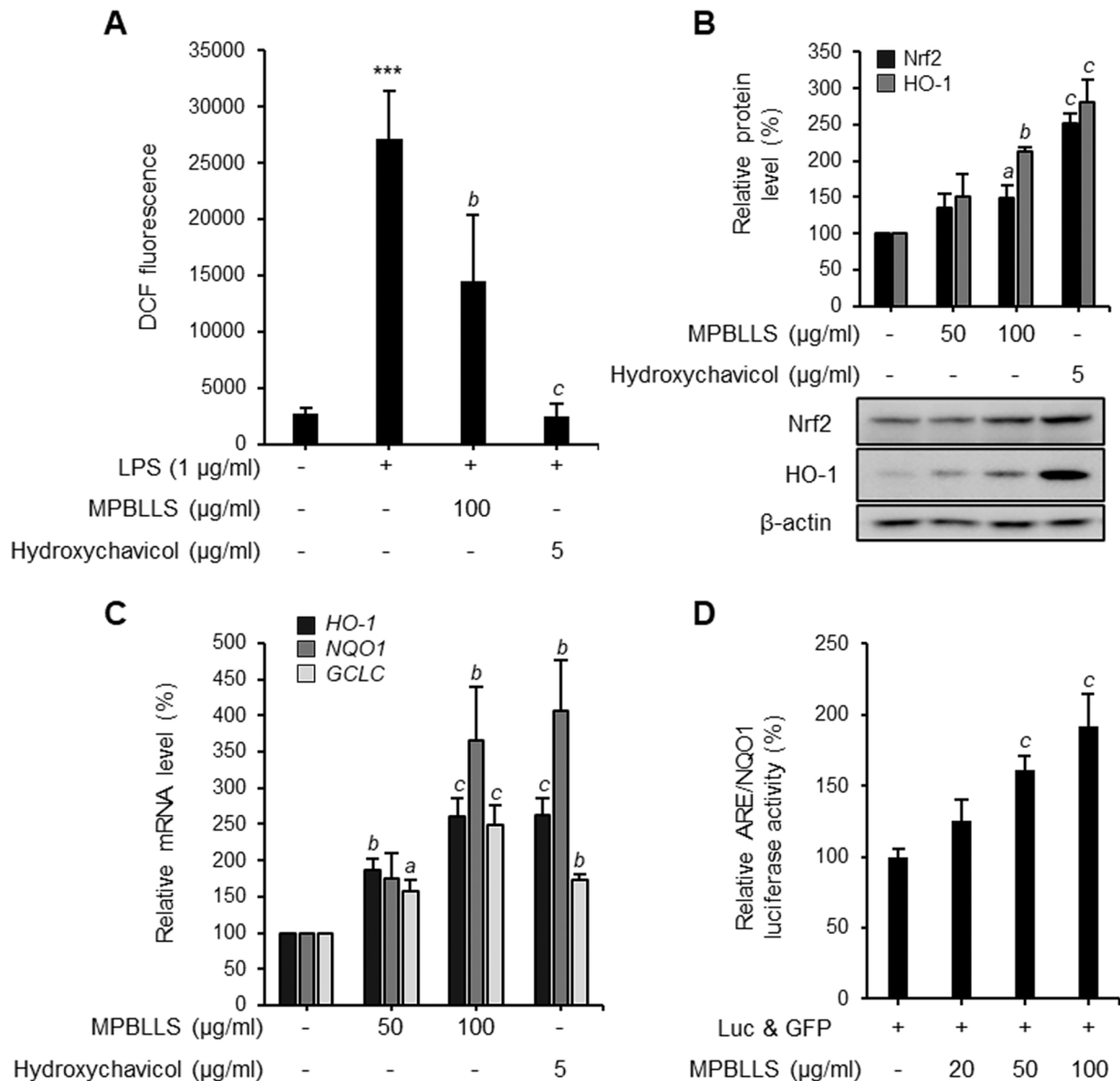


Fig. 5. Antioxidant effects of MPBLLS by regulating the Nrf2 signaling pathway in RAW 264.7 macrophages. (A) RAW 264.7 macrophages were seeded in 6-well plates and pre-treated with MPBLLS or hydroxychavicol for 2 h. After LPS treatment for 24 h, intracellular ROS levels were measured using DCF-DA as a substrate as described in Materials and Methods. All data were averaged from three independent experiments ($n = 3$) and presented as the mean $\pm$ SD [$^b p < 0.01$ and $^c p < 0.001$ vs. LPS only-treated control group; $^{***} p < 0.001$ vs. non-treated control group]. (B) RAW 264.7 macrophages were seeded in 6-well plates. Cells were harvested after treatment with MPBLLS or hydroxychavicol for 24 h at the indicated concentration without LPS treatment. Relative protein levels were compared with the non-treated control group (100%). All experiments were conducted in triplicate ($n = 3$) and presented as the mean $\pm$ SD [$^a p < 0.05$, $^b p < 0.01$, and $^c p < 0.001$ vs. non-treated control group]. (C) RAW 264.7 macrophages were seeded in 12-well plates and treated with MPBLLS or hydroxychavicol for 24 h. The mRNA levels of HO-1, NQO1, and GCLC were evaluated by RT-PCR and were presented relative to the non-treated control group (100%). All data were averaged from three independent experiments ($n = 3$) and presented as the mean $\pm$ SD [$^a p < 0.05$, $^b p < 0.01$, and $^c p < 0.001$ vs. non-treated control group]. (D) HEK 293 cells were transiently co-transfected with ARE/NQO1 reporter plasmids and gWIZ-GFP. At 24 h after transfection, the cells were treated with the indicated concentrations of MPBLLS for an additional 24 h. Luciferase values were measured and normalized to GFP values. The relative luciferase activity was compared with the non-treated control group (100%). All data were averaged from three independent experiments ($n = 3$) and presented as the mean $\pm$ SD [$^c p < 0.001$ vs. non-treated control group].

worth noting that the MPBLLS-mediated inhibition of NF- κ B promoter activity is stronger than that of AP-1, which is consistent with the results shown in Fig. 4A and B. When immunofluorescence staining analysis was performed to observe nuclear translocation of NF- κ B p65, the cytoplasmic-to-nuclear translocation of NF- κ B p65 induced by LPS was inhibited by MPBLLS or hydroxychavicol (Fig. 4E). Taken together, these results indicate that MPBLLS induces anti-inflammatory effects by regulating the NF- κ B and MAPK signaling pathways.

3.5. MPBLLS activates the Nrf2 signaling pathway

The inhibitory effect of MPBLLS on iROS production in RAW 264.7 macrophages was assessed using DCF-DA (Fig. 5A). DCF-DA is non-fluorescent in cells, which is later oxidized to fluorescent DCF when cells produce ROS. In RAW 264.7 macrophages, the fluorescence value was significantly increased by LPS but decreased upon pre-treatment with MPBLLS or hydroxychavicol.

Given that *Piper betle* L. possesses antioxidant activity [28,29,34,41,49,50], it was investigated whether MPBLLS induces the activation of the Nrf2 signaling pathway in RAW 264.7 macrophages. When RAW 264.7 macrophages were treated with MPBLLS or hydroxychavicol, the expression of Nrf2 and HO-1 was induced (Fig. 5B). These results suggest that the antioxidant potency of MPBLLS correlates with the activation of the Nrf2 signaling pathway as well as radical scavenging activity.

When the transcription levels of *HO-1*, *NQO1*, and *GCLC*, which are the target genes of Nrf2, were measured with RT-PCR, MPBLLS and hydroxychavicol induced transcription of Nrf2 target genes (Fig. 5C). Reporter assays in HEK 293 cells using the luciferase reporter containing ARE in the promoter confirmed the antioxidant effect of MPBLLS (Fig. 5D). As expected, the Nrf2 protein levels increased in HEK 293 cells treated with MPBLLS (Fig. S3B). These results suggest that the increased Nrf2 protein by MPBLLS induces the transcription of the target genes.

4. Discussion

The pathogenesis of many diseases often begins with inappropriate responses to oxidative stress and inflammatory responses [51]. Therefore, controlling oxidative and inflammatory responses constitutes the first line of defense against diseases. Several compounds derived from edible plants have been found to be directly or indirectly responsible for changes in the structure and expression of genes that play important roles in human chronic diseases [52]. Therefore, characterizing the effects of edible plants and their mechanisms is crucial. Our findings demonstrated the intracellular mechanisms of the anti-inflammatory and antioxidant effects of MPBLLS in RAW 264.7 macrophages.

The anti-inflammatory effect of MPBLLS has been shown to be mediated by the down-regulation of the NF- κ B and MAPK pathways. As shown in Fig. 4A and B, NF- κ B, ERK, and JNK were inhibited by MPBLLS, whereas p38 expression remained largely unchanged. In inflammatory reactions, both NF- κ B and MAPKs activate the production of pro-inflammatory proteins such as IL-1 β , IL-6, TNF- α , COX-2, and iNOS [53–57]. However, the expression of pro-inflammatory genes appears to be differentially regulated by NF- κ B and MAPKs. Our previous studies showed that the expression of IL-1 β , iNOS, and IL-6 was mainly mediated by NF- κ B, whereas that of TNF- α and COX-2 was mainly regulated by MAPKs [58,59]. In this study, MPBLLS showed stronger inhibition of IL-1 β , iNOS, and IL-6 production than TNF- α and COX-2 production. Furthermore, MPBLLS inhibited NF- κ B more strongly than the MAPK signaling pathway, which supports our previous results. The differential regulation of pro-inflammatory genes by NF- κ B and MAPK may be due to the different binding affinities of transcription factors to the promoter region or differences in the number of binding sites in the promoters of pro-inflammatory genes.

Hydroxychavicol identified in MPBLLS has been reported to possess antioxidant, anti-mutagenic, anti-carcinogenic, antimicrobial, and anti-fungal properties [60–63]. In addition, eugenol is another active

compound of *Piper betle* L. that is often used as a food or flavoring agent and is extracted from other plants including clove, nutmeg, cinnamon bark and leaves, pepper, and ginger [64,65]. Even though eugenol has antioxidant effects and a similar chemical structure to hydroxychavicol [66,67], it was not identified in MPBLLS (Fig. S1). Several attempts have been made to identify eugenol in MPBLLS by HPLC, but no retention time peak corresponding to eugenol was obtained, which suggests that eugenol might not be a major constituent in MPBLLS.

Since hydroxychavicol is abundant in MPBLLS and showed transcriptional induction of Nrf2 target genes; *HO-1*, *NQO1*, and *GCLC*, the significant antioxidant effect of MPBLLS might be due to hydroxychavicol. Although MPBLLS inhibited the MAPK signaling pathway, hydroxychavicol failed to inhibit the MAPK signaling pathway in LPS-induced RAW 264.7 macrophages (Figs. 4B and S2B). This result suggests that the MAPK signaling pathway is regulated by other compounds rather than hydroxychavicol in MPBLLS.

Other studies have shown that the inflammatory response and antioxidant response are closely related [68–70]. NQO1 and HO-1 could inhibit the transcription of pro-inflammatory mediators by blocking the activation of NF- κ B [71,72]. Additionally, IKK could promote Nrf2 ubiquitination and degradation [73]. Similarly, NF- κ B interferes Nrf2 activation [74]. Therefore, the effects of MPBLLS on NF- κ B activity and Nrf2 may suggest to some extent that MPBLLS induces cross-regulation between NF- κ B and Nrf2.

In conclusion, the effect of MPBLLS can be attributed to the synergistic combination between Nrf2, NF- κ B, and MAPK signaling pathways by MPBLLS. Therefore, our findings suggest that the antioxidant and anti-inflammatory effects of MPBLLS are mediated by a complex association between the Nrf2, NF- κ B, and MAPK signaling pathways in RAW 264.7 macrophages.

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CRediT authorship contribution statement

Jihye Seo: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Unju Lee:** Investigation, Writing – review & editing. **Sumin Seo:** Investigation. **Agung Eru Wibowo:** Methodology, Resources. **Olivia Bunga Pongtuluran:** Methodology, Resources. **KyuJong Lee:** Methodology, Resources. **Sang Beom Han:** Methodology, Resources. **Sayeon Cho:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

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.2022.113734.

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