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Esculentoside B inhibits inflammatory response through JNK and downstream NF-κB signaling pathway in LPS-triggered murine macrophage RAW 264.7 cells

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ABSTRACT

Natural compound esculentoside B (EsB), (2S,4aR,6aR,6aS,6bR,8aR,9R,10R,11S,12aR,14bS)-11-hydroxy-9-(hydroxymethyl)-2 methoxycarbonyl-2,6a,6b,9,12a-pentamethyl-10-[(2*S*,3*R*,4*S*,5*R*)-3,4,5-trihydroxyoxan-2-yl] oxy-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid with molecular weight of 664.833, isolated from roots of *Phytolacca acinosa* Roxb has been widely used as a constituent of traditional Chinese medicine (TCM). However, the anti-inflammatory capacity of EsB has not been reported yet. Therefore, the objective of this study was to investigate anti-inflammatory activities of EsB in LPS-treated macrophage RAW 264.7 cells. EsB could inhibit nitric oxide (NO) production. EsB also suppressed gene and protein expression levels of inducible isoform of NO synthase (NOS) and cyclooxygenase-2 in a dose-dependent manner. In addition, EsB decreased gene expression and protein secretion levels of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6. EsB remarkably suppressed nuclear translocation of nuclear factor kappa-B (NF-κB) from cytosolic space. Phosphorylation of IκB was also inhibited by EsB. Moreover, EsB specifically down-regulated phospho-c-Jun N-terminal kinase (p-JNK), but not p-p38 or phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2). Taken together, these results suggest that EsB has inhibitory effect on inflammatory response by inactivating NF-κB and p-JNK. It could be used as a new modulatory drug for effective treatment of inflammation-related diseases.

1. Introduction

Esculentoside B (EsB, phytolaccagenin 3-*O*-β-D-xylopyranoside) isolated from roots of *Phytolacca acinosa* Roxb is a monodesmoside triterpenoid saponin [8]. Roots of *Phytolacca acinosa* Roxb have been commonly used as traditional Chinese medicine (TCM) to treat edema, lymphatic congestion, and mastitis [6]. Other types of Esculentosides such as Esculentoside A, Esculentoside C, Esculentoside E, Esculentoside F, Esculentoside H and Esculentoside T have also been included in triterpenoid saponin ([8]; [29,45]; [16]). It has been reported that these esculentosides exhibit several functions, including anti-inflammatory, anti-tumor, anti-fungal, and anti-bacterial activities ([8]; [20,22,29]). However, it has also been reported that some of esculentosides has proinflammatory activity rather than anti-inflammatory activity [45] as

Abbreviations: ESB, esculentoside B; TCM, traditional Chinese medicine; LPS, lipopolysaccharide; COX-2, cyclooxyenase-2; iNOS, inducible nitric oxide synthase; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PEG_2, prostaglandin E_2; TLR4, toll-like receptor 4; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-B; IκB, inhibitory kappa B; ERK, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase

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rabbit conjunctivae-derived macrophages can induce edema and release NO, TNF-α, and IL-1β upon treatment with fractions and esculentosides isolated from *Phytolacca americana*.

Inflammatory response is an important defense mechanism of host against pathogenic infections or injury damages [4]. Inadequate inflammatory response may lead to various human diseases, including rheumatoid arthritis, chronic bronchitis, diabetes, allergies, psoriasis, atherosclerosis, asthma, lung fibrosis, obesity, and cancer [2,3]. Immune cells are involved in inflammatory processes when dendritic cells, macrophages, neutrophils, mast cells, and lymphocytes are activated [1]. Among them, macrophages are substantially responsible for initiation of innate immune responses, inflammatory reaction, antigen presentation, and phagocytosis [14,44]. When macrophages are activated by harmful stimuli, they can secrete inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) generated through inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) as well as proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β [14,43,44]. NO can be catalyzed from L-arginine by nitric oxide synthase (NOS) [31,41]. Among members of NOS family, inducible NOS (iNOS) is a main source of NO production by macrophages after exposure to proinflammatory cytokines or bacterial products [31,39]. PEG_2, a lipid mediator, is synthesized from arachidonic acid (AA) by COX-2 [37,39]. These inflammatory mediators can induce vasodilation, leakage of protein-rich fluid (plasma), and extravasation of immune cells [32,39]. In addition, PEG_2 can change the set point of body temperature during fever by acting on hypothalamic neurons that control thermoregulation [25]. Proinflammatory cytokines can promote pathological pain and fever [32,39,47].

Lipopolysaccharide (LPS), one major element of the outer membrane of Gram-negative bacteria, is a well-known potent stimulator that activates macrophages through toll-like receptor 4 (TLR4) [14,41,44]. LPS-stimulated macrophages have extensively been used to examine the anti-inflammatory effect of drug candidates in experiments [12,42]. TLR4 is also known as a pattern-recognition receptor (PRR) that can recognize damage-associated molecular patterns (DAMPs) in damaged cells and pathogen-associated molecular patterns (PAMPs) in pathogenic bacteria [1]. Pattern recognition of LPS by TLR4 can lead to activation of down-stream pathways, including nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [28,44]. NF-κB is one of key transcription factors that control gene expression of proinflammatory mediators and proinflammatory cytokines [40]. At unstimulated state, NF-κB is typically inhibited by IκB. However, it is activated by inflammatory stimuli through phosphorylation and degradation of IκB [15,27,46,48]. The degradation of IκB induces translocation of NF-κB to the nucleus where transcription of inflammatory genes occurs [24]. In the MAPK signaling pathway, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 are crucial for NF-κB activation to induce its translocation and regulate inflammatory responses ([14,34]; [17,18]).

Several monodesmoside triterpenoid saponins such as EsA, EsB, EsC, EsE, EsF, EsH, and EsT have been isolated from *Phytolacca acinosa* Roxb. However, anti-inflammatory activities of these compounds have not been reported yet. Since the anti-inflammatory effect of EsB has not been reported yet, the objective of this study was to isolate EsB and examine the anti-inflammatory activity of EsB for the first time in macrophage RAW 264.7 cells.

2. Materials and methods

2.1. Reagents

LPS (*Escherichia coli* 0111:B4), Griess reagent, Hoechst staining solution, antibodies against β-actin, p-p38, p38, COX-2, iNOS, p-ERK1/2, NF-κB p65, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies specific for p-JNK and JNK were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-ERK1/2 antibody was obtained from Upstate (Atlanta, GA, USA). Anti-lamin B antibody was obtained from Abcam (Cambridge, UK). ELISA kits specific for mouse IL-6, IL-1β, and TNF-α were obtained from eBioscience, Inc. (San Diego, CA, USA).

2.2. EsB preparation

EsB has been isolated as a white powder with the molecular formula $C_{36}H_{56}O_{11}$ and the structure of 3-*O*-β-D-xylopyranosy phytolaccagenin, as supplied by our collaborator Dr. Hyeun-Wook Chang, College of Pharmacy, YeungNam University, Gyungsan, Korea. From the n-BuOH fraction of the aqueous alcoholic extract from the roots of *Phytolacca acinosa* Roxb, a triterpene saponin esculentoside B has initially been isolated and identified. The structure of EsB is based on nuclear magnetic resonance (NMR) spectral data (1D and 2D NMR as well as Mass) and comparison with the earlier NMR and Mass spectral data. In brief, the aqueous extract of the leaves of *Phytolacca acinosa* Roxb (100 g) in 2 L water solution has been extracted with n-hexane (5 × 2 L), CH_2Cl_2 (3 × 2 L) and n-BuOH (3 × 2 Ll). The n-BuOH layered residue (20 g) has further been subjected to the C-18 column with 40% MeOH-100% MeOH gradient and 90% MeOH-100% MeOH, giving an esculentoside B (15 mg).

2.3. Cell culture and cell viability assay

RAW264.7 murine macrophage cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 ng/mL streptomycin, and 100 units/mL penicillin. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2. To determine cell viability, macrophage RAW264.7 cells were seeded into a 96-well plate at density of 2×10^4 cells/well followed by treatment with EsB at 0, 10, 30, and 50 μM. Cell viability was then determined using MTT assay.

2.4. Production of nitric oxide

Macrophage RAW264.7 cells were simultaneously treated with 100 ng/mL of LPS and various concentrations (0–50 μM) of EsB. After 24 h of treatment, nitrite concentration levels in cell culture medium were analyzed by Griess reaction. To measure nitrite levels, Griess reagent (1% sulfanilamide/0.1% naphtylethyenediamine dihydrochloride in 2.5% H_3PO_4) was mixed with cell culture medium for 10 min at room temperature. Nitrite concentrations were then determined by measuring the optical density at wavelength of 540 nm on an ELISA plate/microplate reader. For relative calculation of $NaNO_2$ concentration, a standard curve was obtained and used.

2.5. Determination of TNF-α, IL-1β, and IL-6 levels.

Levels of pro-inflammatory cytokines including IL-6, TNF-α, and IL-1β in supernatants were measured with ELISA assay kit (eBioscience) according to the manufacturer's instructions. The experimental assays are previously described [33].

2.6. RT-PCR analysis

Total RNAs extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After total RNAs extraction, cDNA was synthesized from total RNA (1 μg). Target gene was then amplified by RT-PCR with specific oligo dT primers. The following primers were used: COX-2, forward (5′-GGAGAGACTATCAA GATAGT-3′) and reverse (5′-ATGGTCAGTAGACTTTTACA-3′); iNOS, forward (5′-ATGTCCGAAGCAAACATCAC-3′) and reverse (5′-TAATGT

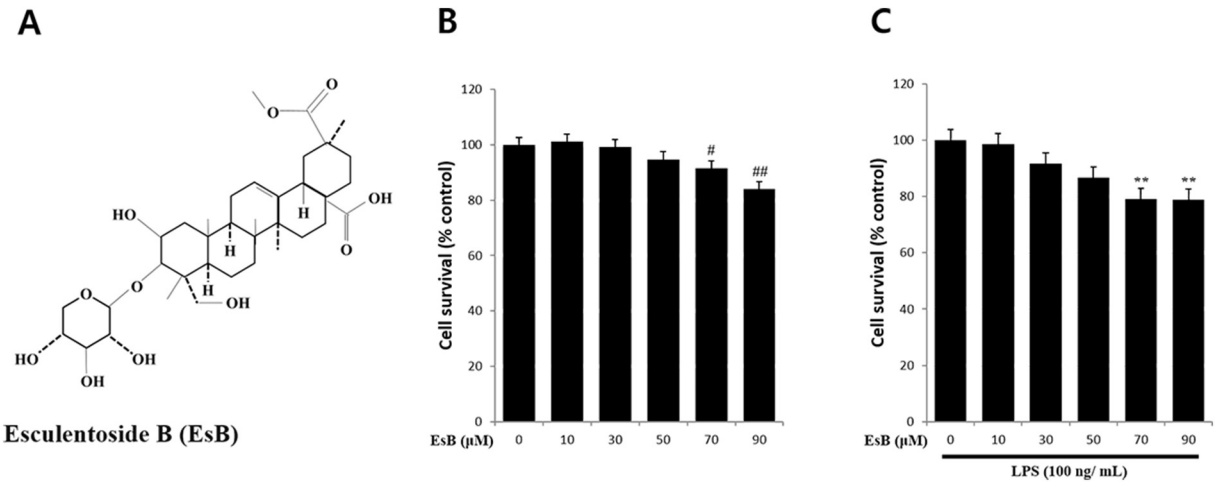

Fig. 1. Chemical structure and effects of EsB on cell viability in macrophage RAW 264.7 cells. (A) Chemical structure of EsB. (B, C) Cells (2×10^4 cell/well) were treated with EsB (0, 10, 30, 50, 70, 90 μM) in the absence or presence of LPS for 24 h and cell viability was then determined by MTT assay. Data are presented as mean ± SEM from three independent experiments. NT, no treatment.

CCAGGAAGTAGGTG-3′); TNF-α, forward (5′-TCAGCCTCTTCTCATTC CTG-3′) and reverse (5′-TGAAGAGAAC-CTGGGAGTAG-3′); IL-6, forward (5′-CCGGAGAGGAGACTTCACAG-3′) and reverse (5′-TCCACGAT TTCCCAG-AGAAC-3′); IL-1β, forward (5′-TGCAGAGTTCCCCAACTGGT ACA-3′) and reverse (5′-GTGCTGCCTAATGTCCCCTT-G-3′); β-actin, forward (5′-GATCCGTGAAGATCAAGATCATTGCT-3′) and reverse (5′-TGATCTTCATTTTTTACGCGTGAATT-3′). After PCR amplification, PCR products were electrophoretically separated on 1.5% agarose gels and DNA bands were visualized after ethidium bromide staining. Intensities of DNA bands were quantified with a Gel-Doc analysis system (Image J, Bethesda, MD, USA).

2.7. Western blot analysis

Macrophage RAW264.7 cells were cultured in 12-well plates (6×10^5 cells/well) with or without 0–50 μM EsB and LPS (100 ng/mL) for 24 h. These cells were then washed at least once with washing buffer, PBS (pH 7.4). Cells were lysed with 1% NP-40 lysis buffer consisted of 1 M HEPES (pH 7.45), 1.5 M NaCl, 100 mM Na-Orthovanadate, 100 mM NaF, 100 mM Na-Pyrophosphate, protease inhibitor cocktail, and NP-40. They were then placed at 4 °C in a refrigerator for 25 min. Cell lysate was then prepared by cold centrifugation for 10 min at 4 °C and 13,000 RPM. Cytosolic and nuclear proteins were prepared by extraction using NE-PERTM cytoplasmic and nuclear extraction reagents (Thermo Scientific) according to the manufacturer's guideline. Protein concentrations were quantified using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (10–50 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis for separation. Separated proteins were then electrotransferred to PVDF membranes followed by blocking with 5% skim milk in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.01% Tween 20 (TBS-T buffer). For the detection of target proteins, those membranes were incubated with primary antibodies specific for COX-2, iNOS, ERK1/2, p38, JNK, p-ERK1/2, p-p38, p-JNK, p-c-Jun, and NF-κB P65 at 4 °C overnight. Each membrane was then washed at least three times with TBS-T buffer (10 mins each wash) and further incubated with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G secondary antibody conjugated with horseradish peroxidase (HRP). Antibodies-based detection was carried out using enhanced chemiluminescence (ECL) reagent. Detection density images were captured with a Bio-western imaging analyzing system Chemi-DOC (Davinch-K, Davinch-In vivo tm, Seoul, Korea).

2.7.1. Immunofluorescence

Macrophage RAW 264.7 cells were plated at sub-confluent density onto 12 mm-diameter sterile coverslips in 24-well plates set for cell cultures. After pre-treatment with EsSB (50 μM) for 30 min, they on coverslips were further treated with or without LPS for 15 min. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min. After cells were washed at least three times with PBS solution, they were permeabilized with 0.2% Triton-100 in PBS at room temperature for 20 min. To block non-specifically bound forms, 1% BSA in PBS was used at room temperature for 1 h. To investigate cellular localization of NF-κB, cells were incubated with NF-κB-specific antibody (1:500 in 1% BSA in PBS) at 4 °C overnight. Cells were then washed with 0.1% Tween-20 in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody at 4 °C for 3 h. For nuclear-specific staining, Hoechst reactive solution at a final concentration of 0.5 μg/mL was incubated with cells for 10 min in the dark. After final washing with PBS-T solution, mounted slides were treated with anti-fade reagents (Molecular Probes) [5,13]. Images were captured with a fluorescence microscope (LSM 700, AxioObserver, C-Apochromat 63×/ 1.20WKorr M27).

2.8. Statistical analysis

All experiments were performed in triplicates. One representative set was used to demonstrate results in figures. Experimental results of multiple groups were analyzed by one-way ANOVA test. Statistically significant differences were indicated by $*p < 0.05$, $**p < 0.01$ vs cells treat LPS only, $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs no treatment cells.

3. Results

3.1. Chemical structure and cytotoxicity of EsB in macrophage RAW 264.7 cells

Esculentoside (EsB), a monodesmoside triterpenoid glycoside, was isolated from roots of *Phytolacca acinosa* Roxb. The molecular weight of EsB was 664.82. Its chemical structure is illustrated in Fig. 1A. We firstly evaluated cytotoxicity of EsB to macrophage RAW 264.7 cells. Cells were treated with different concentrations (0, 10, 30, 50, 70, 90 μM) of EsB or co-treated with EsB and LPS. After 24 h of treatment, cell viability as a cytotoxic parameter was periodically assessed with MTT reagent-based assay. As shown in Fig. 1B and C, EsB treatment at concentrations of 0–50 μM showed no cytotoxicity to macrophage RAW 264.7 cells. These results suggest up to 50 μM EsB has no cytotoxicity as the means of the viability on RAW 264.7 cells.

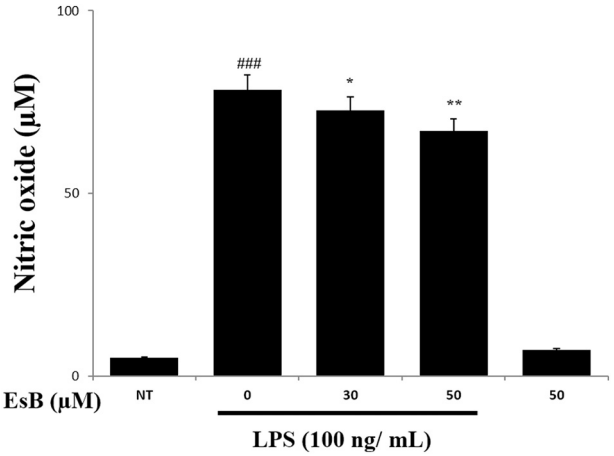

Fig. 2. Effects of EsB on LPS-induced NO production in macrophage RAW 264.7 cells. Cells (5×10^5 cell/well in 12-well pate) were treated with EsB (0, 30, 50 μM) in the absence or presence of LPS (100 ng/mL) for 24 h. The amount of nitrite oxide in the medium was measured using Griess assays. Data are presented as mean ± SEM from three independent experiments. NT = no treatment, $*p < 0.05$, $**p < 0.01$, $###p < 0.001$.

3.2. EsB treatment reduces the level of LPS-triggered NO synthesis in RAW 264.7 cells

As EsB exerts anti-inflammatory effect, it might have effect on LPS-stimulated NO synthesis in RAW 264.7 cells. To test this possibility, cells were incubated with different concentrations (0, 30, 50 μM) of EsB in the presence or absence of LPS (100 ng/mL) for 24 h. After 24 h of incubation, NO production levels in culture supernatants were measured with Griess reagent. As shown in Fig. 2, EsB dose-dependently reduced NO production level in LPS-induced macrophage RAW 264.7 cells. These results clearly suggest that EsB might have anti-inflammatory potential by decreasing NO production in LPS-induced RAW 264.7 cells.

3.3. EsB decreases LPS-stimulated iNOS and COX-2 expression levels in RAW 264.7 cells

To precisely observe the anti-inflammatory potential of EsB, effects of EsB on protein and mRNA expression levels of iNOS and COX-2 elevated by endotoxin LPS were examined using immunoblotting analysis and RT-PCR. Macrophage RAW 264.7 cells were incubated with isolated EsB (0, 30, 50 μM) with or without 100 ng/mL of LPS for 24 h. As shown in Fig. 3A, protein expression levels of iNOS and COX-2 were remarkably decreased by EsB treatment in a dose-dependent manner. Similarly, mRNA expression levels of iNOS and COX-2 were significantly decreased by EsB treatment in a dose-dependent manner (Fig. 3B). These results suggest that EsB can exert anti-inflammatory effects by downregulating iNOS and COX-2 expression at protein and mRNA levels.

3.4. EsB inhibits LPS-stimulated expression of TNF-α, IL-6, and IL-1β in mRNA and cytokines levels in RAW 264.7 cells

During inflammatory process, macrophages are known to release and secrete several proinflammatory cytokines such as IL-6, TNF-α, and IL-1β [9]. These proinflammatory cytokines can mediate several key events in inflammation response, including pathogen-sensing and activation of macrophages [10,19]. As shown in Fig. 4A–D, upregulated expressions of such cytokines upon LPS-stimulation were significantly decreased after EsB treatment. For example, expression levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α were significantly downregulated by EsB treatment. Since EsB suppressed mRNA expression levels of LPS-induced pro-inflammatory cytokines, we performed ELISA to assess extracellular production of LPS-stimulated pro-inflammatory cytokines TNF-α, IL-6, and IL-1β. As shown in Fig. 4E–F, production levels of TNF-α, IL-6, and IL-1β in culture supernatants of EsB treated groups after LPS stimulation were significantly decreased compared to those in LPS-stimulated group.

3.5. EsB inhibits LPS-activated nuclear translocation of NF-κB in macrophage RAW 264.7 cells

LPS is one of the inflammatory inducers. Pathologically abnormal stimuli can activate macrophages. Upon stimulation, signaling molecule IκB is intracellularly phosphorylated, leading to nuclear translocation of NF-κB. We examined whether the anti-inflammatory effect of EsB might be associated with suppression of LPS-triggered nuclear translocation of NF-κB. As shown in Fig. 5A, NF-κB was translocated to the nucleus from the cytosol. However, the nuclear translocation behavior of NF-κB was significantly suppressed by EsB treatment in a dose-dependent manner. Treatment with EsB at concentrations of 30–50 μM clearly inhibited nuclear translocation of NF-κB compared to treatment with LPS only. The expression level of p-IκB was decreased while that of IκB was increased by EsB treatment (Fig. 5B). These results indicate that EsB can inhibit the phosphorylation of IκB-α, consequently decreasing nuclear translocation of NF-κB in LPS-induced macrophage RAW 264.7 cells. To observe the inhibitory effect of EsB on NF-κB translocation to nuclear area, we performed immunofluorescence confocal microscopy analysis. As shown in Fig. 5C, nuclear translocation of NF-κB in LPS-treated cells was suppressed by EsB treatment. These results suggest that EsB is a down regulator of LPS-stimulated NF-κB translocation to the nucleus in macrophage RAW 264.7 cells.

3.6. EsB inhibits phosphorylation of JNK upon LPS stimulation, but not phosphorylation of ERK1/2 or p38 in macrophage RAW 264.7 cells

MAPK signaling pathways play significant roles in biological functions such as activation of transcription factor NF-κB and gene expression of proinflammatory second messenger-producing enzymes such as iNOS and COX-2 [21,23,30,38]. Therefore, inhibitory effects of EsB on LPS-stimulated phosphorylation of MAPKs including ERK1/2, JNK, and p38 in macrophage RAW 264.7 cells were determined using immune blot analysis to determine molecular signaling pathways (Fig. 6A–D). LPS stimulation elevated phosphorylation levels of downstream signaling proteins including ERK1/2, JNK, and p38. Although EsB did not alter total expression levels of signaling molecules such as ERK1/2, p38, or JNK, it decreased the expression level of p-JNK. In other words, EsB specifically inhibited p-JNK. These data indicate that EsB can inhibit inflammatory signaling pathway to exert its effect by keeping decreased regulation status of MAPK pathway while only targeting p-JNK in LPS-stimulated macrophage RAW 264.7 cells.

4. Discussion

Esculentoside B (EsB) isolated from roots of *Phytolacca acinosa* Roxb is a monodesmoside triterpenoid glycoside with molecular weight of 664,833. It has a molecular structure of (2S,4aR,6aR,6aS,6bR,8aR,9R,10R,11S,12aR,14bS)-11-hydroxy-9-(hydroxymethyl)-2 methoxycarbonyl-2,6a,6b,9,12a-pentamethyl-10-[(2*S*,3*R*,4*S*,5*R*)-3,4,5-trihydroxyoxan-2-yl]oxy-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid [8]. EsB exhibits anti-fungal, down-regulatory activity against agrobacterial plant transformation in previous reports ([8]; [22]). However, no study has reported the anti-inflammatory potential of EsB in endotoxin LPS-induced macrophage RAW 264.7 cells or any other equivalent cells. Inflammation-related antitumor activity of EsB has not been reported either. Here, for the first time, we investigated anti-inflammatory potentials and effects of EsB as well as mechanisms

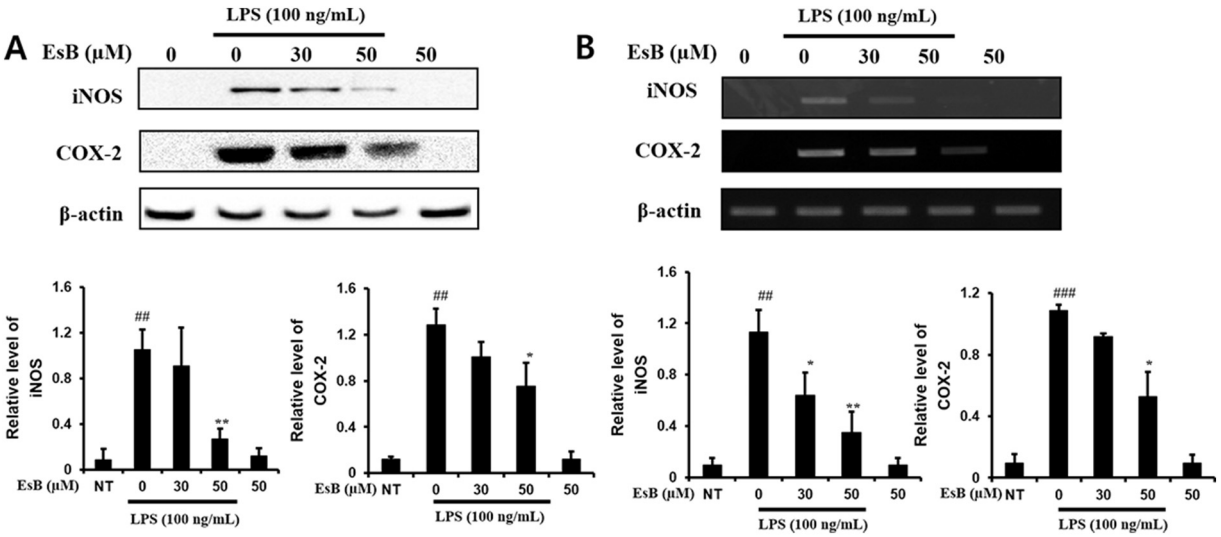

Fig. 3. Effects of EsB on LPS-induced iNOS and COX-2 expression in macrophage RAW 264.7 cells. Cells (5×10^5 cell/well in 12-well pate) were treated with EsB (0, 30, 50 μM) in the absence or the presence of LPS (100 ng/mL) for 24 h. (A) Protein levels of COX-2 and iNOS were determined by Western blot analysis. (B) RT-PCR results of iNOS and COX-2 mRNA levels. Data are presented as mean ± SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ##$p < 0.01$, ###$p < 0.001$ indicate significant differences compared to results from cells treated with LPS alone.

involved as a basic step of anti-tumor pharmacology.

The present data indicate that EsB can suppress NO production in a concentration-dependent manner (Fig. 2). Furthermore, EsB can suppress expression levels of second messenger-producing inflammatory enzymes including iNOS and COX-2 in LPS-treated murine macrophage RAW 264.7 cells at both protein and mRNA levels (Fig. 3A and B). During inflammatory response, macrophages are known to release several extracellular proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [44]. Transcription and gene expression levels of these inflammatory cytokines (TNF-α, IL-6 and IL-1β) were effectively inhibited by a low dose of EsB in rodent macrophage RAW 264.7 cells (Fig. 4A–G). NF-κB is known to regulate the production of proinflammatory cytokines, cell survival, and leukocyte recruitment that play pivotal roles in inflammation [26]. EsB decreased total amount of NF-κB in the nucleus and phosphorylation level of IκB in the cytosol (Fig. 5A and B). However, EsB increased IκB expression levels in the cytosol (Fig. 5B). These results (Fig. 5A, B and C) clearly demonstrated that EsB suppressed NF-κB translocation from cytosol to the nucleus of macrophage RAW 264.7 cells under LPS-treated condition.

On the other hand, EsB suppressed the phosphorylation of JNK, but not the phosphorylation of signaling kinases ERK1/2 and p38 in LPS-stimulated macrophage RAW 264.7 cells (Fig. 6A–D). Meanwhile, a

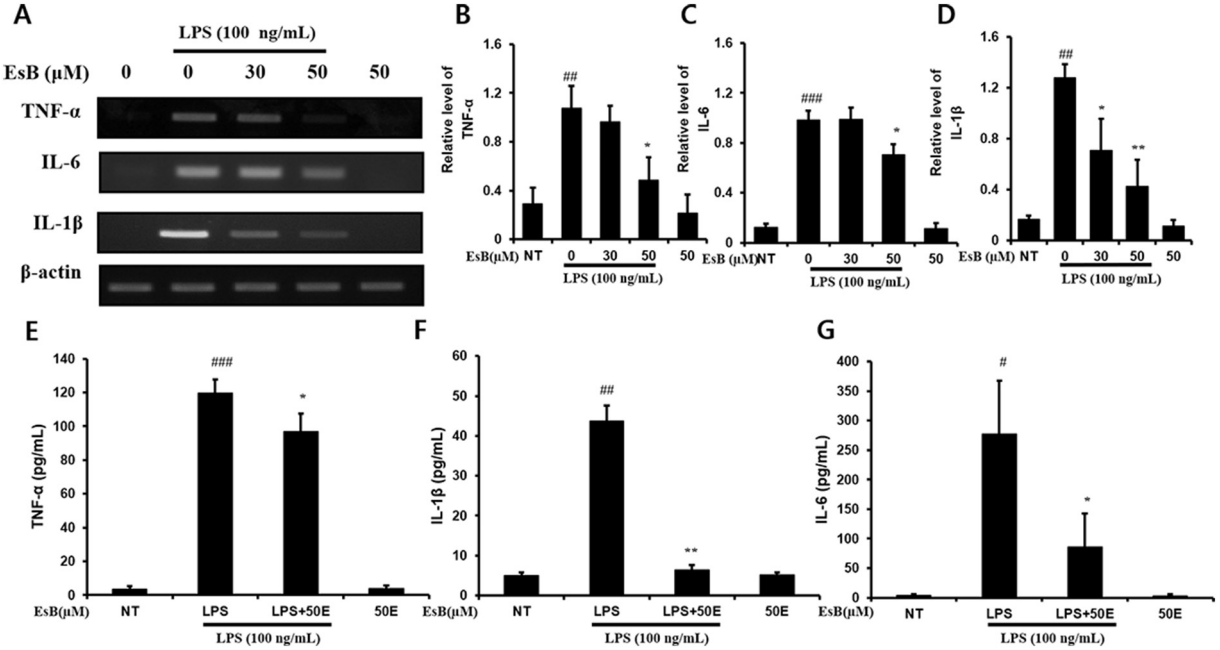

Fig. 4. Effects of EsB on LPS-induced mRNA and cytokines levels of TNF-α, IL-6, and IL-1β in macrophage RAW 264.7 cells. (A, B, C, D) Cells (5×10^5 cell/well in 12-well pate) were treated with EsB (0, 30, 50 μM) in the absence or presence of LPS (100 ng/mL) for 24 h and mRNA levels of TNF-α, IL-6 and IL-1β were determined by RT-PCR. Band intensity (versus β-actin) is indicated as mean ± SEM from three independent experiments. (E, F, G) Cells (5×10^5 cell/well in 12-well pate) were treated with EsB (0, 50 μM) in the absence or the presence of LPS (100 ng/mL) for 24 h. The cytokines levels of TNF-α, IL-6 and IL-1β in the supernatant were measured using the ELISA assay kit. The data shown are representative of three independent and indicate mean ± SEM. *$p < 0.05$, **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ indicate significant differences compared to results from cells treated with LPS alone.

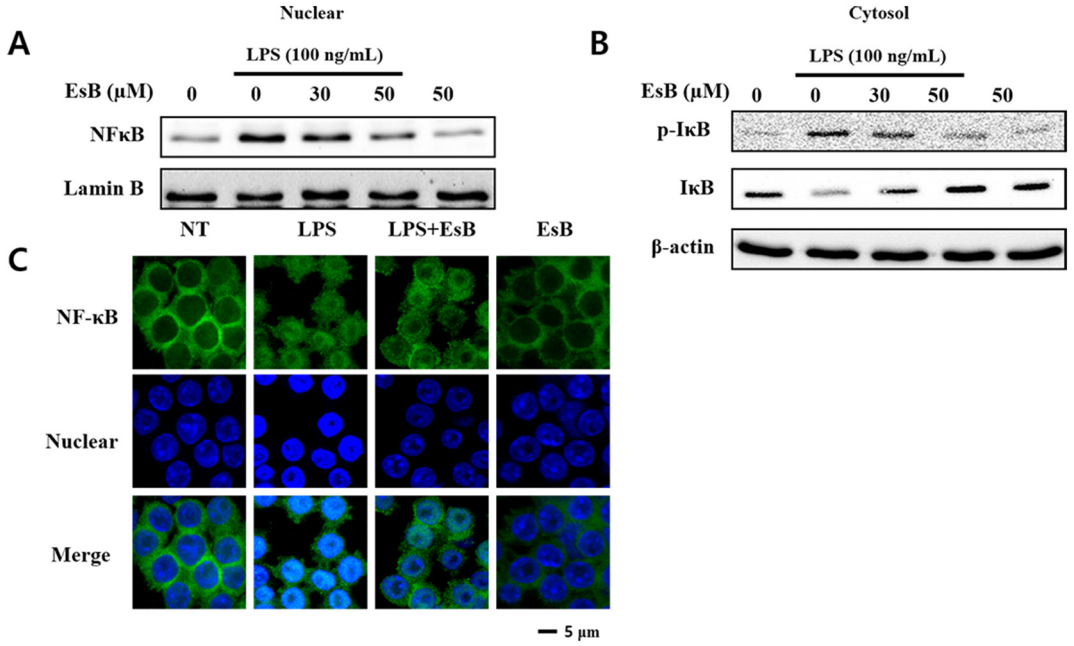

Fig. 5. Effects of EsB on LPS-stimulated nuclear translocation of NF-κB in macrophage RAW 264.7 cells. Cells were pre-treated with various concentrations of EsB (0, 30 and 50 μM) for 30 min and further incubated in the absence or presence of LPS (100 ng/mL) for 10 min. Cells were harvested to prepare nuclear and cytosolic extracts. (A, B) Nuclear and cytosolic extracts were separated by SDS-PAGE for Western blot analysis. Lamin B and β-actin were used as internal controls. Nuclear translocation of NF-κB was analyzed using confocal microscopy. For immunostaining of cells, FITC (for NF-κB) and Hoechst (for nuclei) were used. Scale bars, 5 μm.

previous report has shown that EsA can reduce phosphorylation of p38 and JNK, but not that of ERK1/2 in LPS-treated RAW 264.7 macrophages [27]. These results might be due to minor differences in chemical structures between EsA and EsB that might have different inhibitory modes in the phosphorylation of MAPKs. This indicates that action mechanisms involved in the suppressive capacity of these two compounds are different. And also another previous study has shown that some esculentosides, especially EsC has proinflammatory effects [45]. These interesting results also derived from slight differences in chemical structure between EsB and EsC. EsB has one hydroxy group at 11′ carbon, whereas EsC has one glucose at xylose at 10′ carbon [45].

As an LPS-binding protein on several innate immune cells, Toll-like receptor 4 (TLR4) is known to be a transmembrane protein receptor that plays significant roles in inflammatory response through recognition of various microbial patterns such as PAMP and DAMP [36]. The two patterns of PAMP and DAMP handle invasive potentials of

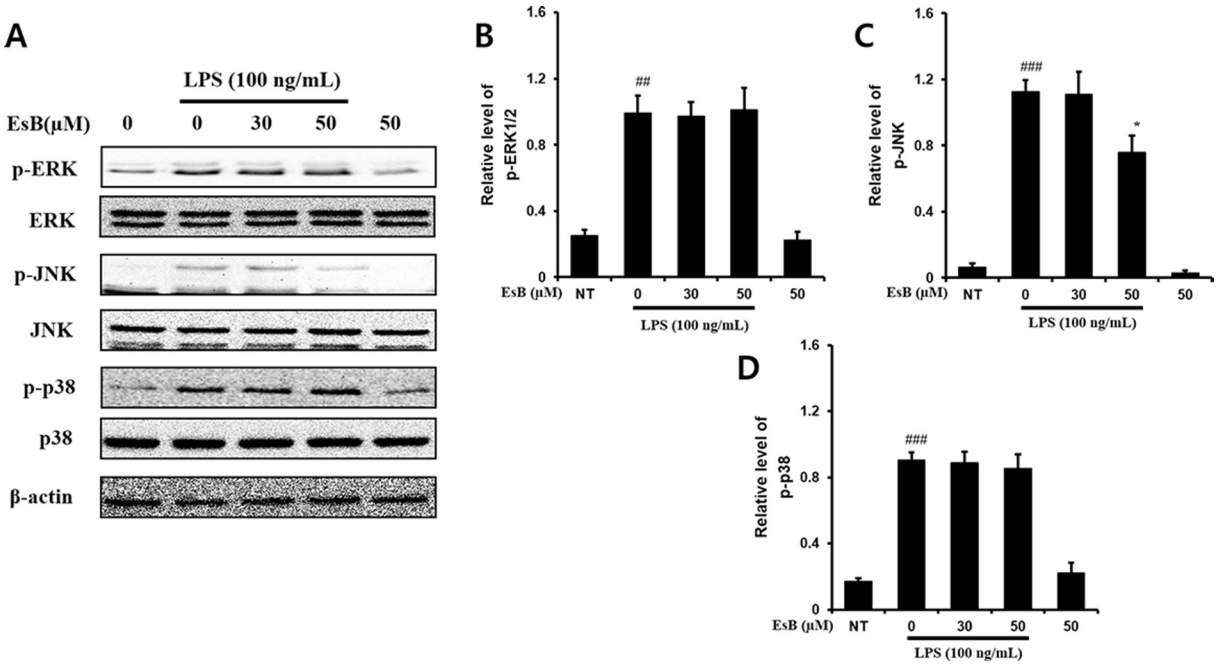

Fig. 6. Inhibitory effects of EsB on MAPK phosphorylation in macrophage RAW 264.7 cells. (A, B, C, D) Cells were pre-treated with various concentrations (0, 30 and 50 μM) of EsB for 30 min followed by incubation with or without LPS (100 ng/mL) for 10 min. Phosphorylation levels of ERK, p38, and JNK in protein samples were analyzed by Western blotting. ERK, JNK, and p38 were used as loading controls. Data are presented as mean ± SEM from three independent experiments. $^{*}p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ indicate significant differences from the LPS alone-treated cells.

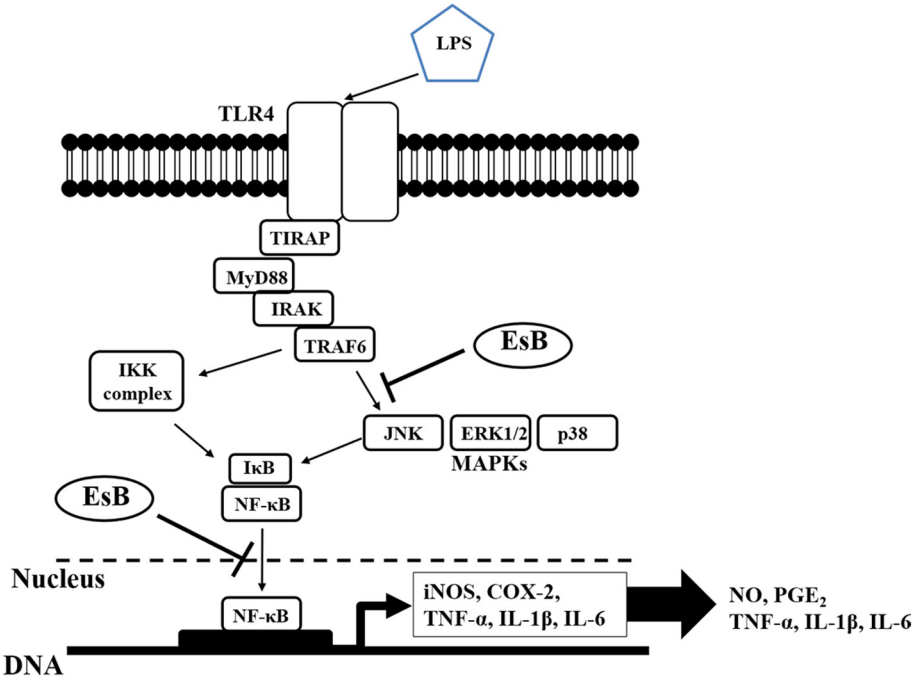

Fig. 7. A schematic diagram showing mechanism involved in the anti-inflammatory activity of EsB in macrophage RAW 264.7 cells. EsB suppressed inflammatory response through inhibiting NF-κB, p-JNK, TNF-α, IL-6, and IL-1β in macrophage RAW 264.7 cells.

pathogens and cell damage or tissue injury [36]. To transduce TLR4 intracellular signaling to the downstream pathway, dimerization of TLR4 as a homo type is essential for its interaction with microbial patterns [17,18]. After dimerization, TLR4 recruits plasma membrane proteins such as toll-interleukin receptor (TIR) domain-containing adaptors. Reported adaptor proteins include TRAM, TRIF, TIRAP, IRAK, TRAF6 and MyD88 [35,36]. TLR then activates downstream signaling kinases for chain signaling reaction to consequently activate NF-κB through these adaptors [11,35].

Although many natural compounds have been reported to possess anti-inflammatory activities, their activity levels are not satisfactory yet. Non-steroidal anti-inflammatory drugs (NSAIDs) have been recently revisited to alleviate inflammation and related disorders [7,37,42]. However, continuous usage of NSAIDs can enhance the risk of severe side effects such as bone marrow depression, kidney and gastrointestinal-duodenal damage, adverse cardiovascular thrombosis, hypertension, retention of water and salts, and so on [7,37,42]. As one candidate for solving this problem, EsB might become an alternatively and therapeutically available agent for treating inflammatory diseases and related disorders with largely reduced side effects.

In conclusion, the present study demonstrated that EsB could inhibit inflammation by inhibiting intracellular translocation of NF-κB and phosphorylation of JNK signal in macrophage RAW 264.7 cells as illustrated in Fig. 7. Results of the current study also suggest that EsB is potentially effective as an anti-inflammatory agent to prevent and treat inflammatory responses.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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