

Ethanol Extract of *Elaeocarpus petiolatus* Inhibits Lipopolysaccharide-Induced Inflammation in Macrophage Cells

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Abstract—*Elaeocarpus petiolatus* is known to exert active oxygen scavenging, anti-aging, and whitening actions. However, the biological effects of *E. petiolatus* on inflammation and the underlying mechanisms are yet to be established. In the present study, we investigated the anti-inflammatory effects of the ethanol extract from *E. petiolatus* (EPE) bark in murine Raw264.7 macrophages stimulated with lipopolysaccharide (LPS). EPE inhibited the production of PGE₂, TNF- α , and IL-1 β in a dose-dependent manner in Raw264.7 cells stimulated with LPS. The decrease in PGE₂ production was correlated with reduced COX-2 expression. Furthermore, EPE suppressed the phosphorylation of extracellular signal-related kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 as well as translocation of the NF- κ B p65 subunit from the cytosol to nucleus. Our results suggest that EPE exerts anti-inflammatory activity through inhibition of inflammatory mediators, such as PGE₂, TNF- α , and IL-1 β , and downregulation of COX-2 via suppression of NF- κ B translocation and phosphorylation of ERK, JNK, and p38 in LPS-stimulated Raw264.7 cells.

KEY WORDS: *Elaeocarpus petiolatus*; inflammation; COX-2; NF-kappaB; MAPK.

INTRODUCTION

Elaeocarpus is a genus of tropical and subtropical evergreen trees and shrubs that includes approximately 350 species widely distributed in tropical and subtropical regions from Madagascar in the west through India, Southeast Asia, southern China, Japan, Australia, New Zealand, Fiji, and Hawaii in the east [1]. The islands of Borneo and New Guinea have the greatest concentration of *Elaeocarpus* species [2].

Previously, Osumi and Sakaida in 2003 reported that a skincare preparation comprising an extract from plants of the genus *Elaeocarpus* in the Elaeocarpaceae family, such as *Elaeocarpus sphaericus*, *Elaeocarpus serratus*, *Elaeocarpus petiolatus*, or *Elaeocarpus floribundus*, exerts active oxygen scavenging, hyaluronidase inhibitory, elastase inhibitory, collagenase inhibitory, and tyrosinase inhibitory actions, in addition to high safety and excellent anti-aging and whitening properties.

Lipopolysaccharide (LPS) stimulates the production of inflammatory mediators, such as prostaglandin (PGE₂), tumor necrosis factor- α (TNF- α), and interleukins [3]. Overproduction of inflammatory mediators by activated macrophages is associated with the pathophysiology of many inflammatory diseases, including cardiovascular disease, cancer, and rheumatoid arthritis [4, 5]. PGE₂, a key inflammatory mediator produced following the sequential oxidation of arachidonic acid, dihomo gamma linolenic acid, or eicosapentaenoic acid by cyclooxygenases (COX-1 and COX-2) and terminal

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prostaglandin synthases, stimulates cytokine generation [6] and vasodilation, and mediates fever and pain [7]. These prostaglandin levels are regulated by expression of inducible COX-2 in inflammation [8].

Production of proinflammatory mediators and cytokines by LPS depends on inducible gene expression mediated by the activation of different transcription factors, such as nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPKs) [9–11]. NF- κ B plays critical roles in the regulation of cell survival genes and coordination of the expression of proinflammatory mediators and cytokines, including COX-2, TNF- α , and IL-1 β [12]. MAPK proteins, such as extracellular signal-related kinases (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK), are activated by LPS in macrophages and other cell types [3, 13]. Accordingly, NF- κ B and MAPKs have been investigated as potential targets for anti-inflammatory drug candidates. Some effective plant-derived medicines have been found to suppress the production of proinflammatory cytokines and PGE₂ in macrophages [14]. Data obtained from our screening program designed to identify plants with anti-inflammatory potential showed that the ethanol extract of *E. petiolatus* (EPE) inhibits PGE₂ production. In the present study, we investigated the biological effects of EPE on LPS-induced inflammation of Raw264.7 cells and its underlying mechanisms of action.

To establish the anti-inflammatory actions of EPE in macrophage cells under LPS exposure and the related mechanisms, we specifically investigated its influence on COX-2 expression, translocation of NF- κ B, and activation of MAPK pathways.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS), concanavalin A (Con A), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Amresco (Solon, OH). Antibodies specific for COX-2, β -actin, and MAPKs (ERK, p38, JNK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We obtained p-p38 and pJNK from Assay Designs (Ann Arbor, MI), and pI κ B- α , pERK, and I κ B- α from Cell Signaling Technology (Danvers, MA).

Preparation of the *E. petiolatus* Ethanol Extract

E. petiolatus (Jack) Wall. ex Steud. was collected in chang bing du ying, China, in 2008. The ethanol extract from barks of *E. petiolatus* (FBM004-030) was obtained from International Biological Material Research Center in Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. Stock solution (20 mg/ml) of the extract was prepared in DMSO and stored at -20°C before use.

Cell Culture

RAW 264.7 mouse macrophage cells purchased from the American Tissue Culture Center (Rockville, MD) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% CO₂. After adhesion of Raw264.7 cells for 4 h, various concentrations of EPE were added.

Cell Viability Assay

Cell proliferation was evaluated using the MTT assay involving mitochondria-dependent reduction of MTT to formazan. Briefly, 10 μ l of 5 mg/ml MTT solution was added to the supernatant and incubated for 4 h at 37°C. Media were discarded, followed by the addition of DMSO. The optical density of formazan was measured at 570 nm using a microplate reader (Benchmark, Bio-Rad Laboratories, Hercules, CA). Absorbance of formazan formed by untreated cells was taken as 100%.

PGE₂ Assay

PGE₂ synthesis was assessed using a commercially available PGE₂ enzyme immunometric assay kit (Cayman Co., Ann Arbor, MI). Diluted standard/sample was pipetted into a 96-well plate pre-coated with a goat polyclonal anti-mouse IgG after LPS (1 μ g/ml) stimulation for 24 h. Aliquots of a PGE₂ monoclonal antibody and PGE₂ acetylcholine esterase conjugate were added to each well and allowed to incubate at room temperature for 16 h. Wells were washed five times with wash buffer containing 0.05% Tween-20, followed by the addition of 200 μ l of Ellman's reagent containing acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). After 60 min, absorbance was measured at 405 nm with a microplate reader.

Reverse Transcription-PCR Analysis

Raw264.7 cells (5×10^6 cells) were incubated for 6 h with or without various concentrations of EPE and LPS (1 $\mu\text{g}/\text{ml}$). After washing twice with PBS, total RNA was isolated from the cell pellet using TRIzol (Invitrogen, Carlsbad, CA). The total amount of RNA was determined by absorbance at 260 nm. RNA (1 μg) was reverse-transcribed into cDNA using AccuPower RT PreMix and oligo(dT)₁₈ primer (Bioneer, Daejeon, Korea). PCR samples contained 20 μl of the reaction mixture comprising 30 mM KCl, 1.5 mM MgCl_2 , 0.25 mM dNTP, 1.0 U of Top DNA polymerase (Bioneer, Daejeon, Korea), and primers in 10 mM Tris-HCl (pH 8.3). The sense and antisense primers were 5'-GAA GTC TTT GGT CTG GTG CCT G-3' and 5'-GTC TGC TGG TTT GGA ATA GTT GC-3' for COX-2, 5'-GGA GCG ACT TGT GGA TTG TC-3' and 5'-GTG AGG GCT TGG CTG AGT GAG-3' for iNOS, and 5'-AGG CTG TGC TGT CCC TGT ATG C-3' and 5'-ACC CAA GAA GGA AGG CTG GAA A-3' for β -actin, respectively. The PCR amplification was performed under the following conditions: 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C (iNOS and β -actin) or 57°C (COX-2) for 45 s, and extension at 72°C for 1 min using an automated DNA thermal cycler (Palm cycler Corbett Research, Sydney, Australia). Amplified products were run on a 1.5% agarose gel and visualized by ethidium bromide staining.

Immunoblot Analysis

Cells (5×10^5 cells/ml) were harvested and washed twice with ice-cold PBS. Nuclear and cytoplasmic fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL), according to the manufacturer's instructions. Total cell lysates were prepared using lysis buffer (Sigma, St Louis, MO). Protein concentrations were measured using a Bradford assay kit (Bio-Rad, Hercules, CA). The protein equivalents of samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skim milk in TBST, probed with antibodies against COX-2, ERK, JNK, p38, and β -actin overnight at 4°C, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunocytochemistry Analysis

Raw264.7 cells were preincubated with EPE for 1 h on Permax plastic chamber slides, and subsequently treated with LPS (1 $\mu\text{g}/\text{ml}$) for 16 h (COX-2) or 1 h (p65). Cells were fixed with ethanol for 30 min at 4°C. Slides were washed three times with PBS and blocked with 3% BSA in PBS for 30 min, followed by overnight incubation at 4°C with an antibody to COX-2 (rabbit polyclonal IgG). For p65 staining, chamber slides were incubated with anti-NF- κB p65 subunit (rabbit polyclonal IgG, Assay Designs) antibodies. Slides were washed three times each with PBS and incubated with anti-rabbit Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. Next, slides were washed with PBS and mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Co., Carlsbad, CA) to highlight cell nuclei. Intensity changes were visualized under a confocal laser scanning microscope (LSM510m, Carl Zeiss, Stuttgart, Germany).

Cytokine Assays in Raw264.7 Cells

The inhibitory effects of extracts on TNF- α and IL-1 β production were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Co., Camarillo, CA) in supernatant fractions collected from EPE-treated cells. Samples were analyzed according to the manufacturer's protocols.

Cytokine Assays in Splenocytes

BALB/c mice were sacrificed at 6–8 weeks and the spleens aseptically removed. Splenocytes were mechanically dissociated with two needles. Red blood cells (RBC) were lysed with RBC lysis buffer (Sigma Chemical, St Louis, MO) at 37°C for 10 min. After washing with PBS, cells were cultured for 4 h in RPMI 1640 containing 10% fetal bovine serum, 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (GibcoBRL, NY, USA). Splenocytes were plated onto 96-well plates at a density of 1×10^6 cells/ml and treated with different concentrations of EPE for 1 h. Following incubation with 1 $\mu\text{g}/\text{ml}$ of ConA (Sigma, St Louis, MO) for a further 3 days, IL-4 and IL-13 concentrations in the supernatant were measured using ELISA kits (R&D Systems, Minneapolis, MN) for mouse cytokines, according to the manufacturer's protocols.

Statistics

Data are expressed as means±standard errors (SEM). Results were obtained from at least three independent experiments performed in triplicate. Statistical significance was determined using a two-tailed Student's *t* test for independent means. *P* values of 0.05 or less were considered statistically significant.

RESULTS

EPE Inhibits PGE₂ Production by Suppressing COX-2 Expression in LPS-Induced Raw264.7 Cells

To determine whether EPE affects COX-2 expression induced by LPS, the PGE₂ concentrations in culture media and COX-2 expression were examined in Raw264.7 cells stimulated with LPS. Incubation of Raw264.7 cells with LPS for 24 h resulted in a 38-fold increase in the PGE₂ level. Notably, EPE (50 μg/ml) inhibited PGE₂ release by 39.0±8.2% (Fig. 1a), but had no effects on cell viability (Fig. 1b).

Preincubation of cells with EPE prevented COX-2 protein (Fig. 2a) and mRNA (Fig. 2b) expression in a dose-dependent manner. To further examine the expression of COX-2 protein in cell, immunocytometric analysis was performed. As shown as Fig. 2c, treatment with LPS resulted in a marked increase of COX-2

protein levels, which was inhibited upon pretreatment with EPE (50 μg/ml). Our results suggest that EPE inhibits the release of PGE₂ via suppression of COX-2 mRNA and protein expression.

EPE Suppresses Phosphorylation of MAPK Molecules in LPS-Induced Raw264.7 Cells

To examine the specific cell signaling pathway involved in EPE-mediated COX-2 suppression, we measured the phosphorylation level of MAPK in EPE-treated Raw264.7 cells via Western blotting using anti-phospho-MAPK and anti-MAPK antibodies. As shown as Fig. 3, LPS induced phosphorylation of ERK, p38, and JNK, which was reduced in a dose-dependent manner upon pretreatment with EPE. These results imply that EPE inhibits LPS-induced COX-2 expression by suppressing the MAPK signaling pathway.

EPE Blocks the Degradation of IκB-α and NF-κB Activation in LPS-Induced Raw264.7 Cells

To determine the effects of EPE on activation and translocation of the p65 subunit of NF-κB, we examined IκB-α and NF-κB activation using immunoblot analysis. PARP or β-actin was used as an internal control and for confirmation of no contamination during extraction of nuclear or cytosolic fraction. After LPS stimulation, we observed p65 accumulation in the nucleus. However, the p65 level in the nuclear fraction was significantly

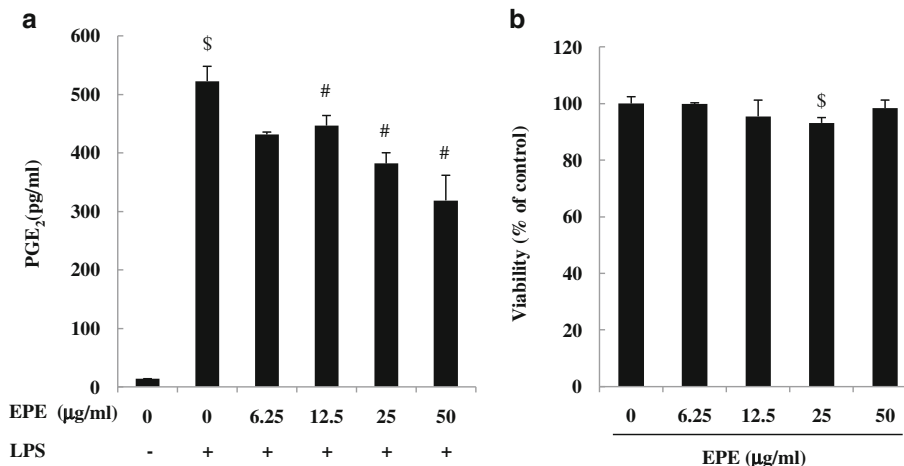


Fig. 1. Differential effects of EPE on LPS-induced PGE₂ in Raw264.7 cells. **a** PGE₂ EIA assay; cells were preincubated with EPE for 1 h, followed by treatment with LPS (1 μg/ml) for 24 h. The supernatant was collected and the PGE₂ level measured as described in "MATERIALS AND METHODS." "Basal" represents the amount of PGE₂ produced from cells not treated with LPS. ^{\$}*P*<0.005, significant difference from untreated cells and [#]*P*<0.05, significant difference from LPS-treated cells. **b** Cell viability; effects of EPE on cell growth were determined using the MTT assay. ^{\$}*P*<0.005 significant difference from untreated cells.

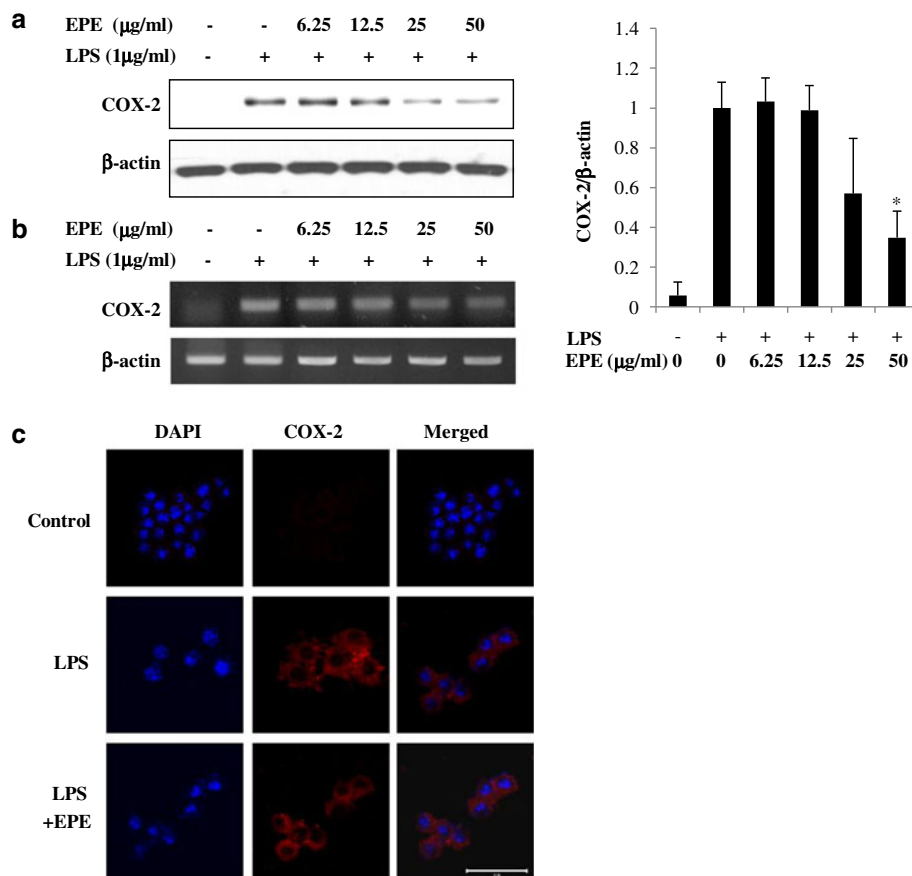


Fig. 2. Differential effects of EPE on LPS-induced COX-2 expression in Raw264.7 cells. **a** Cells were preincubated with EPE for 1 h and treated with LPS ($1\mu\text{g/ml}$) for 16 h. The COX-2 protein level was determined with the immunoblot assay. **b** Cells were preincubated with EPE for 1 h, followed by treatment with LPS ($1\mu\text{g/ml}$) for 6 h. COX-2 gene expression was determined using reverse transcription-PCR as described in “**MATERIALS AND METHODS**.” Densitometric data are normalized by β -actin and presented as the relative ratio of COX-2 levels in only LPS-treated cells. Values are expressed as the mean \pm SEM of three independent experiments each performed in triplicate. * p <0.05. **c** Immunocytochemistry analysis of COX-2 expression was performed as described in “**MATERIALS AND METHODS**” (magnification, $\times 400$). Control: DMSO (0.1%), LPS: LPS only ($1\mu\text{g/ml}$), LPS+EPE: EPE ($50\mu\text{g/ml}$) and LPS treatment.

reduced by pretreatment with EPE, compared with that in the presence of LPS only. Moreover, phosphorylation and degradation of $\text{I}\kappa\text{B-}\alpha$ were dose-dependently reduced by EPE (Fig. 4a).

To establish the effect of EPE on p65 translocation to the nucleus, we performed further immunocytochemical analysis. As shown in Fig. 4b, p65 was mainly localized to the cytosol in control cells. However, after LPS treatment, the majority of intracellular p65 translocated from the cytoplasm to the nucleus, as evident from strong NF- κB p65 nuclear staining. Pretreatment with EPE resulted in weaker intensity of p65 staining in the nucleus. Based on these results, we suggest that EPE

exerts inhibitory action on LPS-induced translocation of p65 via suppressing phosphorylation of $\text{I}\kappa\text{B-}\alpha$.

EPE Inhibits Proinflammatory Cytokine Production in LPS-Induced Raw264.7 Cells

To determine the effects of EPE on release of cytokines, we investigated the TNF- α and IL-1 β levels using a specific ELISA kit. As shown in Fig. 5, incubation of Raw264.7 cells with LPS for 24 h induced an increase in the TNF- α ($419.84\pm 1.70\text{ pg/ml}$) and IL-1 β ($68.83\pm 6.40\text{ pg/ml}$) levels. Pretreatment with EPE ($50\mu\text{g/ml}$)

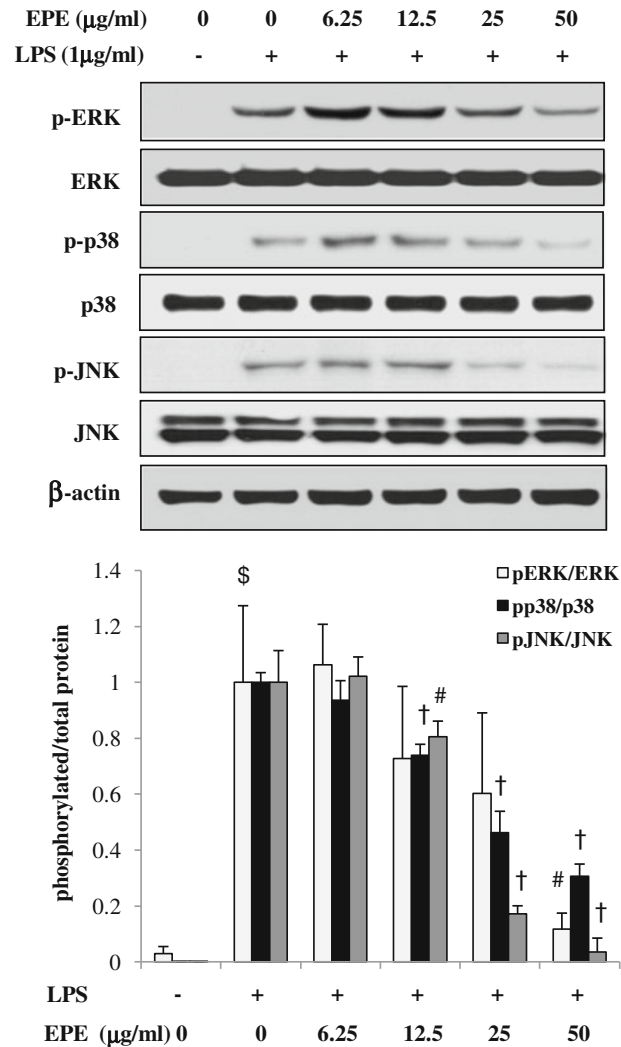


Fig. 3. Differential effects of EPE on phosphorylation of ERK, p38, and JNK in LPS-stimulated Raw264.7 cells. Cells were preincubated with EPE for 1 h, followed by stimulation with LPS for 15 min. Total cell lysates were prepared using lysis buffer (Sigma, St Louis, MO). Protein concentrations were measured using the Bradford assay kit (Bio-Rad, Hercules, CA). Similar amounts of protein were separated by 10% SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with antibodies against ERK, JNK, p38, and β -actin overnight at 4°C , and exposed to HRP-conjugated secondary antibodies for 1 h. Antibody-specific proteins were visualized using an enhanced chemiluminescence detection system. One of similar results is represented and relative ratio is also indicated, normalizing to total ERK, p38 or JNK signal. Values are expressed as the mean \pm SEM of three independent experiments each performed in triplicate. $^{\$}P < 0.05$, significant difference from unstimulated cells; $^{\#}P < 0.05$ and $^{\dagger}P < 0.005$, significant difference from LPS-treated cells.

resulted in significant inhibition of TNF- α (by $59.9 \pm 6.4\%$) and IL-1 β (by $51.7 \pm 4.7\%$) production.

EPE Inhibits IL-4 and IL-13 Cytokine Production in ConA-Induced Splenocytes

To examine the effects of EPE on production of cytokines (IL-4 and IL-13), the cytokine levels in

splenocytes were investigated using an ELISA kit. As shown as Fig. 6, ConA treatment markedly enhanced the production of IL-4 (446.56 ± 13.99 pg/ml) and IL-13 (345.00 ± 2.83 pg/ml) in splenocytes. The IL-4 and IL-13 levels in Raw264.7 cells pretreated with 50 $\mu\text{g/ml}$ EPE were markedly suppressed by $96.77 \pm 0.28\%$ and $85.47 \pm 11.85\%$, respectively.

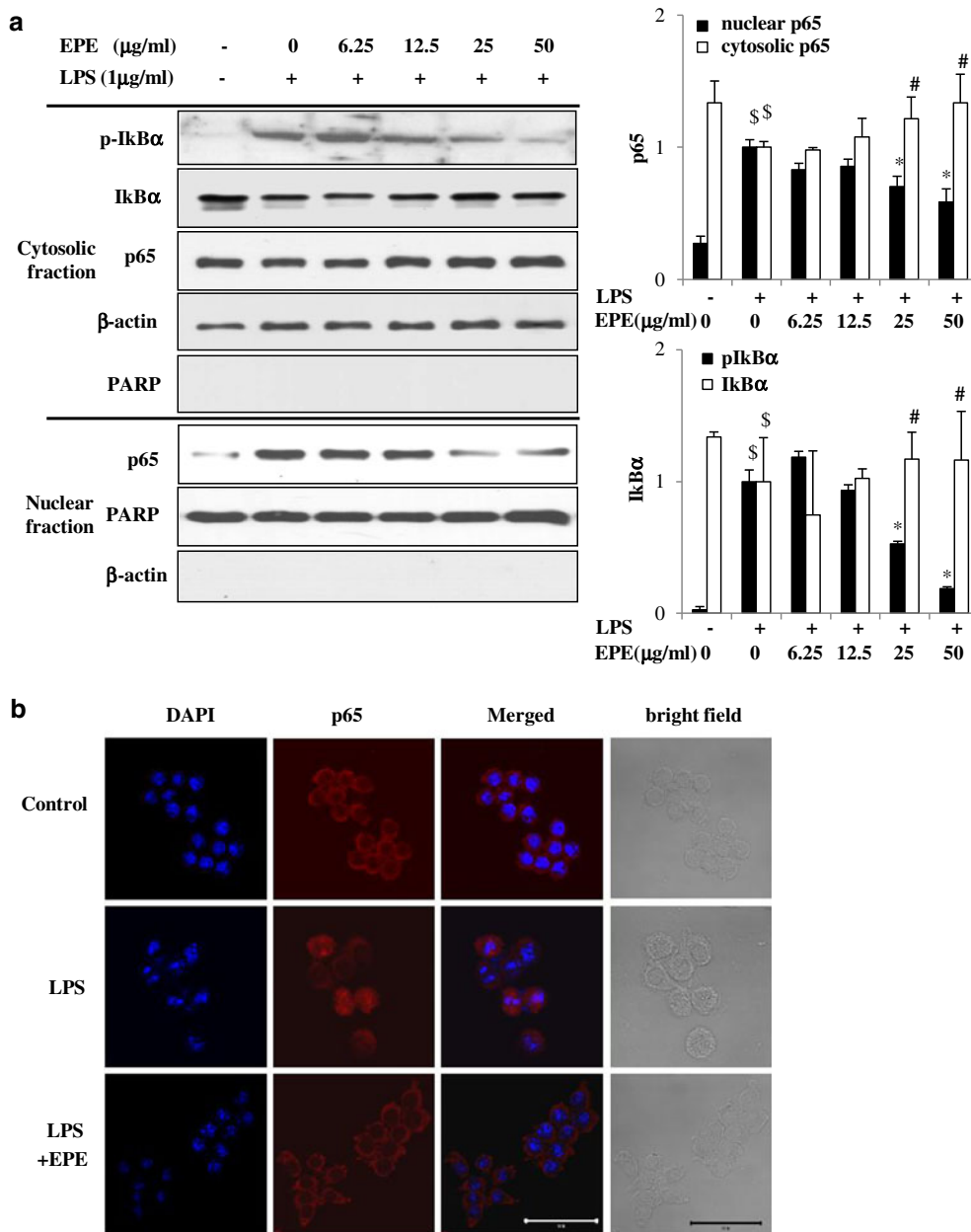


Fig. 4. Differential effects of EPE on activation of IκB-α and translocation of p65 in LPS-stimulated Raw264.7 cells. **a** Cells were treated with EPE for 1 h, followed by stimulation with LPS for 10 min. Equal amounts of protein were analyzed using specific antibodies for IκB-α and p65. Values are expressed as the mean±SEM of three independent experiments each performed in triplicate. ^{\$}*P*<0.05 significant difference from unstimulated cells; [#]*P*<0.05 and ^{*}*P*<0.005, significant difference from LPS-treated cells. **b** Raw264.7 cells were pretreated with different concentrations of EPE for 1 h and stimulated with LPS (1 µg/mL) for another 1 h. Cells were fixed, permeabilized, and incubated with a specific antibody for NF-κB (p65), followed by Texas Red-conjugated anti-rabbit Ig (red). Nuclei of the corresponding cells were visualized using DAPI staining (magnification, ×400). Control: DMSO (0.1%), LPS: LPS only (1 µg/ml), LPS+EPE: EPE (50 µg/ml) and LPS treatment.

DISCUSSION

In the present study, we demonstrate that EPE suppresses PGE₂ production in LPS-stimulated Raw

264.7 cells in a dose-dependent manner but has no effect on cell viability. Macrophages play an important role in different immunopathological phenomena during inflammation, fever, and septic shock. Primary macrophages

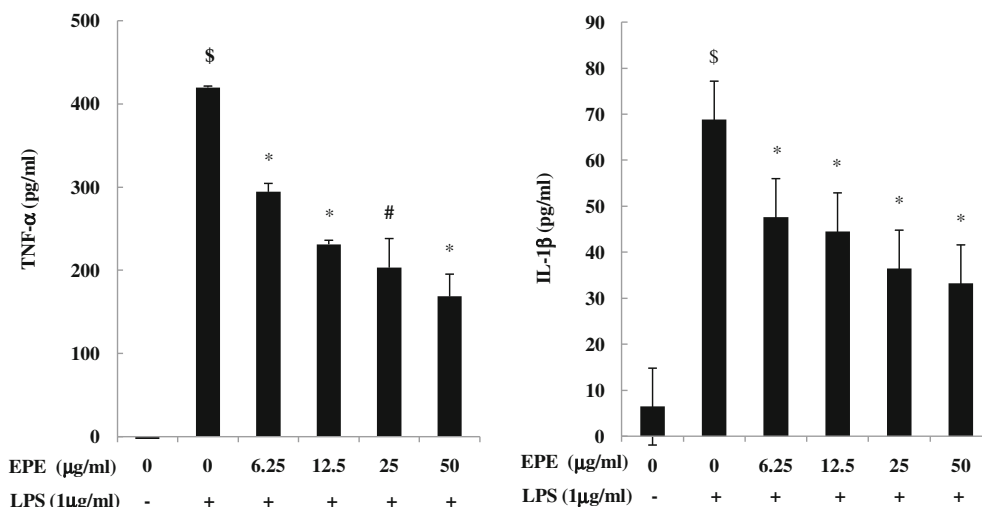


Fig. 5. Differential effects of EPE on TNF- α and IL-1 β production in LPS-stimulated Raw264.7 cells. Cells were pretreated with EPE for 1 h and stimulated with LPS (1 μ g/ml) for another 24 h. Cytokine levels in the supernatant were measured with the ELISA. Data are presented as means \pm SEM from three independent experiments. $^{\$}P < 0.005$, significant difference from unstimulated cells; $^{\#}P < 0.05$ and $^*P < 0.005$, significant difference from LPS-treated cells.

(Raw264.7 cells) and cancerous macrophage-like (J774) cells induced by LPS are regarded as a useful *in vitro* model for evaluating the potency of anti-inflammatory drugs and exploring their mechanisms of action [15]. Inflammation is related to the overproduction of inflammatory mediators (PGE₂ and NO) and proinflammatory cytokines (TNF- α and IL-1 β) and is caused to the unbalance of Th1 type cytokines (IL-1 and IL-6) and Th2 type cytokines (IL-4 and IL-13). Th1 type cytokines was detected in LPS-treated macrophages, and Th2 type

cytokines was released in ConA-treated splenocytes. PGE₂ is generated by activated inducible cyclooxygenase (COX)-2 [16–18].

The inhibitory effects of EPE against COX-2 expression (Fig. 2) and PGE₂ production (Fig. 1a) suggest that anti-inflammatory activity is exerted via reduction of cytokines (Figs. 5 and 6), based on the knowledge that COX-2 expression coincides with PGE₂ secretion in the pathogenesis of various inflammatory diseases. Several groups have recently demonstrated that

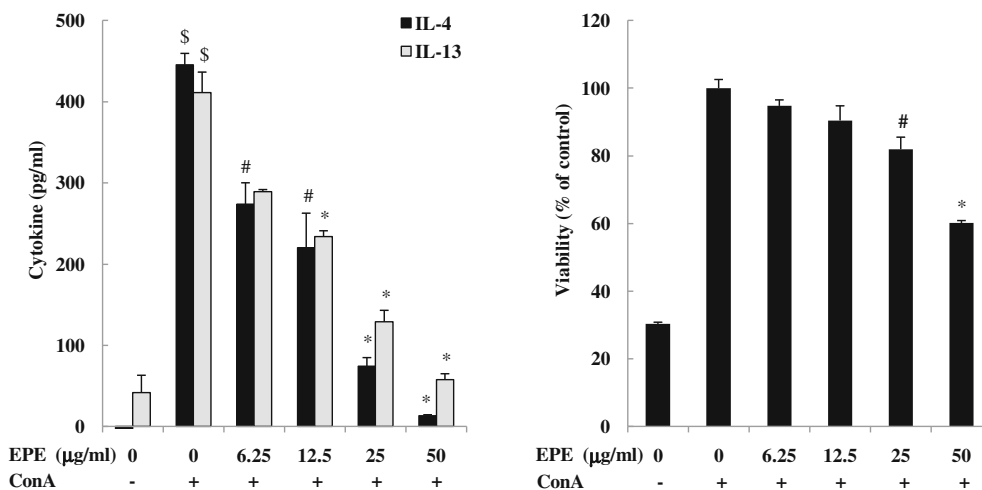


Fig. 6. Differential effects of EPE on IL-4 and IL-13 production in ConA-stimulated splenocytes. Splenocytes were plated onto 96-well plates and treated with different concentrations of EPE for 1 h. After the addition of 1 μ g/ml of concanavalin A for a further 3 days, concentrations of IL-4 and IL-13 from the supernatant fractions were measured using ELISA kits (R&D systems). Data are presented as mean values of three experiments \pm SEM. $^{\$}P < 0.005$, significant difference from untreated cells; $^{\#}P < 0.05$ and $^*P < 0.005$, significant difference from ConA-treated cells.

the effects of plant extracts on the expression of inflammation-related genes [19–21] are strongly related to inhibitory effects on COX-2 protein expression in LPS-stimulated macrophages.

COX-2 expression is regulated at both the transcriptional and post-transcriptional levels [22, 23], and the specific signaling pathways involved are dependent on the stimulus type [22]. Induction of COX-2 has been shown to require activation of the ERK signaling pathway [24], while other researchers have demonstrated that constitutively active MEKK1 induces COX-2 expression by activating the SEK1/MKK4-p38 kinase pathway [25]. The c-Jun N-terminal kinases (JNKs), known as stress-activated protein kinases (SAPKs), are also associated with induction of COX-2 [19]. Here, we have shown that EPE inhibits LPS-induced COX-2 expression via suppressing ERK, p38, and JNK phosphorylation (Fig. 3).

NF- κ B, a known pleiotropic regulator of several genes involved in immune and inflammatory responses, is activated in macrophages by various stimuli, including LPS and TNF- α [26]. Our data showed that EPE is involved in downregulation of the NF- κ B signaling pathway in LPS-stimulated Raw264.7 cells, in support of our hypothesis. Various stimuli trigger signaling pathways that induce phosphorylation, ubiquitination, and subsequent degradation of I κ B proteins, resulting in translocation of NF- κ B from the cytoplasm to nucleus and binding to its motif [12, 26, 27]. However, in the cytoplasm of unstimulated cells, the I κ B family of proteins prevents translocation of NF- κ B to the nucleus by masking its nuclear localization sequence. Here, we found that EPE inhibits the translocation of NF- κ B via dephosphorylation of I κ B- α (Fig. 4).

LPS-stimulated NF- κ B activation is a cellular event mainly managed by surface receptors, such as Toll-like receptor (TLR), and several cytosolic adapters, such as MyD88 [28–30]. TLR-MyD88 modulates NF- κ B activation via several signal transducers associated with the function of the IKK complex and MEKK [31, 32]. The possible involvement of TLR-mediated signaling, including IKK complex and MEKKs, or other proteins requiring for LPS-TLR binding, such as MD-2, CD14, or LBP, in EPE-mediated inhibition of NF- κ B activation should be further investigated.

In summary, we demonstrate that EPE inhibits the PGE₂ production in LPS-stimulated Raw264.7 cells by blocking the phosphorylation of ERK, JNK, p38 kinase, and I κ B- α and translocation of NF- κ B which positively

regulates transcription of TNF, IL-1 β , and COX-2. In view of these findings, we propose that EPE is a useful potential anti-inflammatory agent. Further studies are required to identify the active compounds within the extract.

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REFERENCES

1. Wu, Z.Y., P.H. Raven, and D.Y. Hong. 2007. *Flora of China*. Beijing/St. Louis: Science Press/Missouri Botanical Garden Press.
2. Coode, M.J.E. 2001. *Elaeocarpus* in New Guinea—new taxa in the Debruyinii subgroup of the Monocera group. Contributions to the Flora of Mt Jaya, V. *Kew Bulletin*, United Kingdom.
3. Guha, M., and N. Mackman. 2001. LPS induction of gene expression in human monocytes. *Cellular Signalling* 13: 85–94.
4. Turini, M.E., and R.N. DuBois. 2002. Cyclooxygenase-2: a therapeutic target. *Annual Reviews Medicine* 53: 35–57.
5. Rocca, B., and G.A. FitzGerald. 2002. Cyclooxygenases and prostaglandins: shaping up the immune response. *International Immunopharmacology* 2: 603–630.
6. Williams, J.A., and E. Shacter. 1997. Regulation of macrophage cytokine production by prostaglandin E₂. Distinct roles of cyclooxygenase-1 and -2. *The Journal of Biological Chemistry* 272: 25693–25699.
7. Vane, J.R. 1976. Prostaglandins as mediators of inflammation. *Advances in Prostaglandin and Thromboxane Research* 2: 791–801.
8. Vane, J.R., J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxtall, and D.A. Willoughby. 1994. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 91: 2046–2050.
9. Johnson, G.L., and R. Lapadat. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911–1912.
10. Kim, Y.H., S.H. Lee, J.Y. Lee, S.W. Choi, J.W. Park, and T.K. Kwon. 2004. Triptolide inhibits murine-inducible nitric oxide synthase expression by down-regulating lipopolysaccharide-induced activity of nuclear factor-kappa B and c-Jun NH2-terminal kinase. *European Journal of Pharmacology* 494: 1–9.
11. Olsson, S., and R. Sundler. 2006. The role of lipid rafts in LPS-induced signaling in a macrophage cell line. *Molecular Immunology* 43: 607–612.
12. Baeuerle, P.A., and D. Baltimore. 1996. NF-kappa B: ten years after. *Cell* 87: 13–20.
13. Vanden Berghe, W., S. Plaisance, E. Boone, K. De Bosscher, M.L. Schmitz, W. Fiers, and G. Haegeman. 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappa B p65 transactivation mediated by tumor necrosis factor. *The Journal of Biological Chemistry* 273: 3285–3290.

14. Genovese, M.C. 2005. Biologic therapies in clinical development for the treatment of rheumatoid arthritis. *Journal of Clinical Rheumatology* 11: S45–S54.
15. Kobori, M., M. Yoshida, M. Ohnishi-Kameyama, and H. Shinmoto. 2007. Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. *British Journal of Pharmacology* 150: 209–219.
16. Qureshi, N., S.N. Vogel, C. Van Way 3rd, C.J. Papsian, A.A. Qureshi, and D.C. Morrison. 2005. The proteasome: a central regulator of inflammation and macrophage function. *Immunologic Research* 31: 243–260.
17. Malyshev, I.Y., and A. Shnyra. 2003. Controlled modulation of inflammatory, stress and apoptotic responses in macrophages. *Current Drug Targets. Immune, Endocrine and Metabolic Disorders* 3: 1–22.
18. Jiang, Q., I. Elson-Schwab, C. Courtemanche, and B.N. Ames. 2000. gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 97: 11494–11499.
19. Kwon, O.K., M.Y. Lee, J.E. Yuk, S.R. Oh, Y.W. Chin, H.K. Lee, and K.S. Ahn. Anti-inflammatory effects of methanol extracts of the root of *Lilium lancifolium* on LPS-stimulated Raw264.7 cells. *Journal of Ethnopharmacology* 130:28–34.
20. Lii, C.K., H.W. Chen, W.T. Yun, and K.L. Liu. 2009. Suppressive effects of wild bitter melon (*Momordica charantia* Linn. var. *abbreviata* ser.) fruit extracts on inflammatory responses in RAW264.7 macrophages. *Journal of Ethnopharmacology* 122: 227–233.
21. Sheeba, M.S., and V.V. Asha. 2009. *Cardiospermum halicacabum* ethanol extract inhibits LPS induced COX-2, TNF-alpha and iNOS expression, which is mediated by NF-kappaB regulation, in RAW264.7 cells. *Journal of Ethnopharmacology* 124: 39–44.
22. Mestre, J.R., K. Subbaramaiah, P.G. Sacks, S.P. Schantz, T. Tanabe, H. Inoue, and A.J. Dannenberg. 1997. Retinoids suppress phorbol ester-mediated induction of cyclooxygenase-2. *Cancer Research* 57: 1081–1085.
23. Dean, J.L., M. Brook, A.R. Clark, and J. Saklatvala. 1999. p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *The Journal of Biological Chemistry* 274: 264–269.
24. Xie, W., and H.R. Herschman. 1996. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. *The Journal of Biological Chemistry* 271: 31742–31748.
25. Guan, Z., S.Y. Buckman, A.P. Pentland, D.J. Templeton, and A.R. Morrison. 1998. Induction of cyclooxygenase-2 by the activated MEKK1 → SEK1/MKK4 → p38 mitogen-activated protein kinase pathway. *The Journal of Biological Chemistry* 273: 12901–12908.
26. Rajapakse, N., M.M. Kim, E. Mendis, and S.K. Kim. 2008. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells by carboxybutyrylated glucosamine takes place via down-regulation of mitogen-activated protein kinase-mediated nuclear factor-kappaB signaling. *Immunology* 123: 348–357.
27. Kretz-Remy, C., P. Mehlen, M.E. Mirault, and A.P. Arrigo. 1996. Inhibition of I kappa B-alpha phosphorylation and degradation and subsequent NF-kappa B activation by glutathione peroxidase overexpression. *The Journal of Cell Biology* 133: 1083–1093.
28. Palaniyar, N., J. Nadesalingam, and K.B. Reid. 2002. Pulmonary innate immune proteins and receptors that interact with gram-positive bacterial ligands. *Immunobiology* 205: 575–594.
29. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 11: 115–122.
30. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annual Review of Immunology* 21: 335–376.
31. Hayden, M.S., and S. Ghosh. 2004. Signaling to NF-kappa B. *Genes & Development* 18: 2195–2224.
32. Huang, Q., J. Yang, Y. Lin, C. Walker, J. Cheng, Z.G. Liu, and B. Su. 2004. Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3. *Nature Immunology* 5: 98–103.