

# Ethyl acetate extract from *Asparagus cochinchinensis* exerts anti-inflammatory effects in LPS-stimulated RAW264.7 macrophage cells by regulating COX-2/iNOS, inflammatory cytokine expression, MAP kinase pathways, the cell cycle and anti-oxidant activity

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**Abstract.** *Asparagus cochinchinensis* (*A. cochinchinensis*) is a medicine traditionally used to treat fever, cough, kidney disease, breast cancer, inflammatory disease and brain disease in northeast Asian countries. Although numerous studies of the anti-inflammatory effects of *A. cochinchinensis* have been conducted, the underlying mechanisms of such effects in macrophages remain to be demonstrated. To investigate the mechanism of suppressive effects on the inflammatory response in macrophages, alterations of the nitric oxide (NO) level, the cell viability, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression levels, inflammatory cytokine expression, the mitogen-activated protein kinase (MAPK) signaling pathway, cell cycle arrest and reactive oxygen species (ROS) levels were measured in lipopolysaccharide (LPS)-activated RAW264.7 cells following treatment with ethyl acetate extract from *A. cochinchinensis* root (EaEAC). RAW264.7 cells pretreated two different concentrations of EaEAC prior to LPS treatment exhibited no significant toxicity. The concentration of NO was significantly decreased in the EaEAC + LPS treated group compared

with the vehicle + LPS treated group. A similar decrease in mRNA transcript level of COX-2, iNOS, pro-inflammatory cytokines [tumor necrosis factor- $\alpha$  and interleukin (IL)-1 $\beta$ ] and anti-inflammatory cytokines (IL-6 and IL-10) was detected in the EaEAC + LPS treated group compared with the vehicle + LPS treated group, although the decrease rate varied. Enhancement of the phosphorylation of MAPK family members following LPS treatment was partially rescued in the EaEAC pretreated group, and the cell cycle was arrested at the G2/M phase. Furthermore, the EaEAC pretreated group exhibited a reduced level of ROS generation compared with the vehicle + LPS treated group. Taken together, these results suggest that EaEAC suppresses inflammatory responses through inhibition of NO production, COX-2 expression and ROS production, as well as differential regulation of inflammatory cytokines and cell cycle in RAW264.7 cells. In addition, these results provide strong evidence to suggest that EaEAC may be considered as an important candidate for the treatment of particular inflammatory diseases.

## Introduction

*Asparagus cochinchinensis* (*A. cochinchinensis*), a perennial herb belonging to the Liliaceae family, is widely distributed in China, Japan and Korea, and has been used as a traditional medicine in those countries for thousands of years (1). The root of *A. cochinchinensis* has long been considered a therapeutic drug due to its anti-inflammatory, diuretic, antiseptic, antitussive, antibacterial, nervine, sialogogue, antipyretic and stomachic effects, and is also administered in combination with other herbs as a medicine to treat lung disease, immune system-associated diseases and aging (1,2).

The root of *A. cochinchinensis* contains 19 amino acids, polysaccharides, and >20 multi-functional compounds. These functional compounds include  $\beta$ -sitosterol (3), daucosterol (4),

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n-ethatriacontanoic acid (5), palmitic acid (6), 9-heptacosylene (7), smilagenin (8), diosgenin (9), sarsasapogenin-3-O- $\beta$ -D-glucoside imidacloprid (10), 5-methoxy methyl furfural, yame sapogenin, diosgenin-3-O- $\beta$ -D imidacloprid glycosides (11,12), aspacochioside D (13), iso-agatharesinoside (14) and seven steroidal saponins (15). In addition, the polysaccharide composition of *A. cochinchinensis* roots is reported to have several therapeutic properties, including antioxidant and anti-aging properties (16-18), antibacterial-inflammatory effects (16), antitumor effects (19-21), blood sugar reducing activity (22) and improvement of cough (23,24).

Previous studies have reported the anti-inflammatory activity of *A. cochinchinensis* extract. Secretion of the pro-inflammatory cytokine, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), in lipopolysaccharide (LPS)-and substance P-stimulated mouse astrocytes was significantly inhibited by *A. cochinchinensis* extract (25). Aspacochinosides N, O and P extracted from ethanol-treated *A. cochinchinensis* decreased the nitric oxide (NO) concentration in LPS-stimulated BV-2 microglial cells (26). Furthermore, ethanol extract from *A. cochinchinensis* decreased the degree of ectopic edema, ear thickness, cytokine secretion TNF- $\alpha$  and interleukin (IL)-1 $\beta$  and myeloperoxidase activity in a skin inflammation-induced mouse model treated with 12-O-tetradecanoyl-phorbol-13-acetate, all of which are considered indicators of skin inflammation progression (27). A crude aqueous extract of *A. cochinchinensis* effectively inhibited TNF- $\alpha$ -induced cytotoxicity (21), increased the spleen index and superoxide dismutase (SOD) activity and decreased malondialdehyde (MDA) in mice (1). A recent study reported the inhibitory effects of *A. cochinchinensis* in allergic asthma-associated airway remodeling. The standardized herbal formula PM014, which includes the roots of *A. cochinchinensis*, efficiently inhibited the number of total cells, eosinophils, neutrophils, macrophages and lymphocytes in the bronchoalveolar lavage fluid of cockroach allergen-induced mice (28). Meanwhile, LPS used to induce an inflammatory response, is recognized by toll-like receptors (TLRs) distributed on the membrane of macrophages. The engagement of the TLR results in a potent inflammatory response characterized by the release of inflammatory cytokines, NO production and cell cycle regulation through the activation of the MAPK signaling pathway. Therefore, these markers and pathways may be considered as key factors for the analysis of the inflammatory response, to identify novel drugs with anti-inflammatory properties (29). However, the underlying mechanism by which *A. cochinchinensis* exerts its anti-inflammatory effects in macrophages has not yet been clearly identified, even though the induction of inflammatory lung diseases including asthma, cystic fibrosis, emphysema, and chronic obstructive pulmonary disorder have been implicated in the activation of macrophages.

The present study investigated the fundamental mechanisms responsible for anti-inflammatory activities of ethyl acetate extract from *A. cochinchinensis* root (EaEAC) in LPS-induced RAW264.7 microphage cells.

## Materials and methods

**Preparation of EaEAC.** Roots of *A. cochinchinensis* were collected from plantations in the Go-Chang county of North Jeolla (Korea) and were dried in a drying machine (model

FD5510S-FD5520S; IIShinBioBase Co., Dongducheon, Korea) at 60°C. Voucher specimens of *A. cochinchinensis* (WPC-14-003) were deposited in the Functional Materials Bank of the PNU-Wellbeing RIS Center at Pusan National University (Pusan, Korea). Dry roots of *A. cochinchinensis* were reduced to powder using a pulverizer (model MF-3100S; Hanil Electric Group Co., Ltd., Seoul, Korea), followed by extraction of EaEAC at 50°C for 24 h in a fixed liquor ratio (solid powder of *A. cochinchinensis*/ethyl acetate solvent ratio, 1:10) using circulating extraction equipment (SHWB-30/45, Woori Science Instrument Co., Pocheon, Korea). Extract solutions were subsequently passed through a 0.4  $\mu$ m filter, and concentrated by vacuum evaporation and lyophilized using circulating extraction equipment (IKA Labortechnik, Staufen im Breisgau, Germany). Finally, the EaEAC powder was dissolved in dimethyl sulfoxide (DMSO; Duchefa Biochemie B.V., Haarlem, Netherlands) to 1 mg/ml, then further diluted to the required concentration.

**Free radical scavenging activity.** The scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured as previously described (30). Briefly, each 100  $\mu$ l sample in eight different concentrations of EaEAC (250-2,000  $\mu$ g/ml) was mixed with 100  $\mu$ l DPPH (0.1 mM; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in 95% ethanol solution or 100  $\mu$ l 95% ethanol solution, then incubated for 30 min at room temperature. The absorbance of the reaction mixture was measured at 517 nm using a VersaMax plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The DPPH radical scavenging activity of the EaEAC was expressed as the percent decrease in absorbance relative to the control. The half maximal inhibitory concentration (IC<sub>50</sub>) is defined as the concentration of substrate that causes a 50% loss in DPPH activity.

**Cell culture.** The RAW264.7 cell line used in the present study is an Abelson murine leukemia virus-transformed macrophage cell line, provided by the Korean Cell Line Bank (Seoul, Korea). RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; cat. no. S001-01; Welgene, Gyeongsan, Korea), L-glutamine, penicillin, and streptomycin (Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO<sub>2</sub> and 95% air.

**Cell viability assay.** Cell viability was determined using the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; Merck Millipore). To determine cell viability, RAW264.7 cells were seeded at a density of 5x10<sup>4</sup> cells/0.2 ml and cultured for 12 h in a 37°C incubator. When the cells reached 70-80% confluence, they were either untreated (NC group), treated with vehicle (DMSO), or pretreated with 100 or 200  $\mu$ g/ml EaEAC dissolved in DMSO for 2 h. Cells were subsequently incubated for 24 h with 1  $\mu$ g/ml LPS (derived from *Escherichia coli*, serotype 055:B5; Sigma-Aldrich; Merck Millipore), then the supernatants were discarded and 0.2 ml fresh DMEM and 50  $\mu$ l MTT solution [2 mg/ml in phosphate-buffered saline (PBS)] was added to each well. Cells were incubated at 37°C for a further 4 h. Formazan precipitate was dissolved in

DMSO and the absorbance at 570 nm was read directly in the wells using a VersaMax plate reader (Molecular Devices, LLC). The morphological features of RAW264.7 cells in each treated group were also observed using an inverted light microscope [Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland].

**Measurement of NO concentration.** NO accumulation was measured in the cell culture medium using Griess reagent [1% sulfanilamide, 5% phosphoric acid, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; Sigma-Aldrich; Merck Millipore] as described previously (31). Briefly, RAW264.7 cells were treated with EaEAC (100 and 200  $\mu\text{g}/\text{ml}$ ) for 2 h followed by LPS (1  $\mu\text{g}/\text{ml}$ ) for 24 hr. Following the collection of the supernatant, each sample (100  $\mu\text{l}$ ) was mixed with the same volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was read at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Analysis of intracellular ROS level.** ROS levels in cells were measured by staining with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Merck Millipore), a cell permeable and nonfluorescent agent that can be deacetylated by intracellular esterases to nonfluorescent DCFH, and is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of intracellular ROS. When the RAW264.7 cells reached 70-80% confluence, they were either untreated, treated with vehicle, or pretreated with 100 or 200  $\mu\text{g}/\text{ml}$  EaEAC dissolved in DMSO for 2 h. Cells were subsequently incubated for 24 h with 1  $\mu\text{g}/\text{ml}$  LPS. Cells were then incubated with 25  $\mu\text{M}$  DCFH-DA for 30 min at 37°C, before they were washed twice with PBS. Green fluorescence was visualized at x200 and x400 magnification using an Eclipse TX100 fluorescent microscope (Nikon Corporation, Tokyo, Japan).

**Western blotting.** When the RAW264.7 cells reached 70-80% confluence, they were either untreated, treated with vehicle, or pretreated with 100 or 200  $\mu\text{g}/\text{ml}$  EaEAC dissolved in DMSO for 2 h. Cells were subsequently incubated for 24 h with 1  $\mu\text{g}/\text{ml}$  LPS. Total protein for western blotting was extracted from RAW264.7 cells using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea), centrifuged at 11,000  $\times g$  for 5 min, and quantified using a SMART bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Proteins were separated by 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 h (30  $\mu\text{g}$  per gel), then resolved proteins were transferred to nitrocellulose membranes for 2 h at 40 V. After the blocking with 3-5% skim milk in TNT solution (137 mM NaCl, 2.7 mM KCl, 10 mM  $\text{Na}_2\text{HPO}_4$  and 0.05% Tween-20) and incubating at room temperature for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: Anti-SAPK/JNK (cat. no. 9252; dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p-) SAPK/JNK (Thr183/Tyr185; cat. no. 9251; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-ERK1 (K-23; cat. no. sc-94; dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p-ERK1/2 (Thr202/Tyr204; cat. no. 9101; dilution, 1:1,000; Cell Signaling Technology,

Inc.), anti-p38 mitogen-activated protein kinase (MAPK; cat. no. 9212; dilution, 1:1,000; Cell Signaling Technology, Inc.), anti-p-p38 MAPK (Thr180/Tyr182; cat. no. 9211; dilution, 1:1,000; Cell Signaling Technology, Inc.) and anti- $\beta$ -actin (cat. no. A5316; dilution, 1:3,000; Sigma-Aldrich; Merck Millipore). Membranes were then washed with TNT solution and incubated with 1:1,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. no. 81-6120; Zymed; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Blots were developed using Amersham ECL Select Western Blotting detection reagent (GE Healthcare Life Sciences, Chalfont, UK). Protein bands were visualized using the FluorChem<sup>®</sup> FC2 Imaging system (Alpha Innotech Corporation, San Leandro, CA, USA) and the band density was semi-quantified using the AlphaView Program (version, 3.2.2; Cell Biosciences, Inc., Palo Alto, CA, USA). The target protein expression levels of three samples from each group were analyzed in three separate western blot analyses.

**Enzyme-linked immunosorbent assay (ELISA) for IL-6.** The concentration of IL-6 secreted from RAW264.7 cells was determined using an IL-6 ELISA kit (cat. no. 431304; Biolegend, Inc., San Diego, CA, USA) according to the manufacturer's protocols. Briefly, RAW264.7 cells were treated with two different concentrations of EaEAC (100 and 200  $\mu\text{g}/\text{ml}$ ) for 2 h, followed by 1  $\mu\text{g}/\text{ml}$  of LPS for 24 h. Cell supernatant was collected and 100  $\mu\text{l}$  serial dilutions of the standard or the supernatant were added to a 96-well plate coated with anti-IL-6 antibody, then incubated for 2 h at room temperature. Following five washes with wash solution (PBS, 0.05% Tween-20, pH 7.4), 100  $\mu\text{l}$  avidin-HRP solution was added to each well, and the plates incubated at 37°C for 2 h. Following five washes, 100  $\mu\text{l}$  substrate solution was added and the plate was incubated at 37°C for a further 30 min. The reaction was terminated by the addition of 100  $\mu\text{l}$  stop solution (2N  $\text{H}_2\text{SO}_4$ ) and the absorbance at 450 nm was determined with a VersaMax plate reader (Molecular Devices, LLC).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis for cytokine gene expression.** The relative expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 mRNAs were measured by RT-qPCR as previously described (32). Total RNA was purified by removing media from each cultured sample and homogenizing the cells in RNasee (cat. no. CS104; Tel-Test Inc., Friendswood, TX, USA). The isolated RNA concentration was then determined using a NanoDrop spectrophotometer (BioSpec-Nano, Shimadzu Scientific Instruments, Columbia, MD, USA). Total RNA (5  $\mu\text{g}$ ) was used to synthesize cDNA in a reaction mixture consisting of oligo (dT) primers (500 ng; Invitrogen; Thermo Fisher Scientific, Inc.), 10 mM deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine triphosphates, 0.1 M dithiothreitol, 5X reaction buffer and 1  $\mu\text{l}$  Superscript II reverse transcriptase (200 U/ $\mu\text{l}$ ; cat. no. 18064-014; Invitrogen; Thermo Fisher Scientific, Inc.). Thermal cycling conditions for reverse transcription consisted of 70°C for 10 min followed by 42°C for 50 min. RNA was removed from cDNA samples using 3.2 U/ $\mu\text{l}$  RNase H (cat. no. 18021071; Invitrogen; Thermo Fisher Scientific Inc.) at 37°C for 20 min. In order to amplify

target genes, 2.5  $\mu$ l cDNA, 10 pmol specific sense and anti-sense primers (Macrogen Co., Seoul, South Korea), 5 U/ $\mu$ l Taq polymerase (0.2  $\mu$ l; PCR Core kit; Roche Diagnostics, Basel, Switzerland), 2 mM deoxynucleotide triphosphate (2.5  $\mu$ l), and 10X reaction buffer containing 15 mM MgCl<sub>2</sub> (2.5  $\mu$ l) were combined. A thermal cycler (Perkin-Elmer, Inc., Waltham, MA, USA) was used to amplify target genes using the following parameters: A pre-denaturation step of 7 min at 94°C, followed by 25-32 cycles of 30 sec at 94°C, 30 sec at 62°C, and 45 sec at 72°C and a final post-elongation step of 7 min at 72°C. The primer sequences for target gene expression identification were as follows: iNOS, sense 5'-CACTTG GAGTTCACCCAGT-3' and anti-sense, 5'-ACCACTCGTACT TGGGATGC-3'; COX-2, sense 5'-CAGGTCATTGGTGGG GAGGTGTATC-3' and anti-sense, 5'-CCAGGAGGATGG AGTTGTTGTAGAG-3'; TNF- $\alpha$ , sense 5'-CCTGTAGCCCAC GTCGTAGC-3' and anti-sense, 5'-TTGACCTCAGCGCTG ACTTG-3'; IL-1 $\beta$ , sense 5'-GCACATCAACAAGAGCTT CAGGCAG-3' and anti-sense, 5'-GCTGCTTGTGAGGTG CTGATGTAC-3'; IL-10, sense 5'-CCAAGCCTTATCGGA AATGA-3' and anti-sense, 5'-TTTTTCACAGGGGAGAAAT CG-3'; IL-6, sense 5'-TTGGGACTGATGTTGTTGACA-3' and anti-sense, 5'-TCATCGCTGTTGATACAATCAGA-3'. The final PCR products were separated on 1% agarose gel and then visualized by ethidium bromide staining. The band signal for each sample was visualized using the AE-9000 E-Graph gel documentation system (ATTO Corporation, Tokyo, Japan) and the band density was quantified using Image Saver 5 software (version 5.0; ATTO Corporation). The experiment was repeated three times and all samples were PCR-analyzed in triplicate.

**Cell cycle assay.** The cell cycle was measured using a Muse™ Cell Cycle kit (cat. no. MCH100106; EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, RAW264.7 cells were divided into 100 mm<sup>2</sup> dishes (2.5x10<sup>6</sup> cells/dish), then pretreated with 100 or 200  $\mu$ g/ml of EaEAC for 2 h. Following treatment with 1  $\mu$ g/ml of LPS for 24 h, cells from subset groups were harvested by centrifugation at 3,000 x g for 5 min, then fixed with 70% ethanol at -20°C for 3 h. The fixed cells were washed with 1X PBS, then added to 200  $\mu$ l Cell Cycle Reagent. Following incubation at 37°C in a CO<sub>2</sub> incubator for 30 min, cell cycles were analyzed using a Muse® Cell Analyzer with the Muse 1.3.1 analysis program (EMD Millipore).

**Statistical analysis.** Statistical tests were performed using SPSS version 10.10 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to identify significant differences between NC- and LPS-treated groups. Differences in the responses of the Vehicle + LPS treated group and EaEAC + LPS treated groups were evaluated using a post hoc Tukey's test. All values are reported as the mean  $\pm$  standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Anti-oxidant activity of EaEAC.** The anti-oxidant activity of EaEAC was measured by DPPH scavenging analysis.

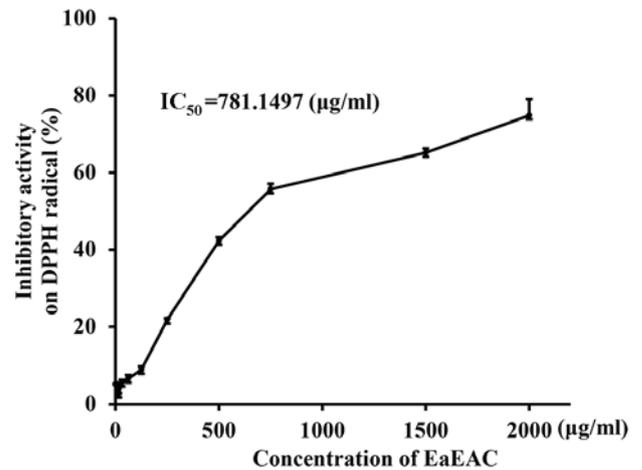


Figure 1. Free radical scavenging activity of EaEAC. DPPH radical scavenging activity was assayed in a mixture containing 0.1 mM DPPH and a range of concentrations of EaEAC (250-2,000  $\mu$ g/ml). DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; IC<sub>50</sub>, half maximal inhibitory concentration; EaEAC, ethyl acetate extract from *Asparagus cochinchinensis* root. Values are presented as the mean  $\pm$  standard deviation of three replicates.

Scavenging activity of EaEAC against DPPH radical was increased in a dose-dependent manner, with an IC<sub>50</sub> of 781.1497  $\mu$ g/ml (Fig. 1). This indicated that EaEAC exhibited DPPH radical scavenging activity.

**Toxicity of EaEAC.** To determine the toxicity of EaEAC, cell viability was measured by MTT assay. No marked differences in morphological features (Fig. 2A) or significant changes to cell viability (Fig. 2B) were observed in RAW264.7 cells treated with 100 and 200  $\mu$ g/ml EaEAC for 24 h compared with NC or vehicle group cells, indicating EaEAC is not toxic at concentrations up to 200  $\mu$ g/ml.

**Effects of EaEAC on NO production, iNOS and COX-2 expression.** To examine the anti-inflammatory properties of EaEAC, alterations in NO concentration, and iNOS and COX-2 transcription were measured in LPS-stimulated RAW264.7 cells following EaEAC pretreatment. The concentration of NO was significantly increased in the vehicle + LPS-treated group compared with the NC group (P=0.01; Fig. 3A). However, the concentration of NO decreased by 18 and 33% in cells pretreated with 100 and 200  $\mu$ g/ml EaEAC, respectively, with the concentration in the cells that were pretreated with 200  $\mu$ g/ml EaEAC significantly reduced compared with the vehicle + LPS group (P=0.002; Fig. 3A).

Similarly, increased levels of iNOS and COX-2 mRNA expression were detected in the vehicle + LPS-treated group compared with the NC group (P=0.011 and P=0.001, respectively; Fig. 3B). However, these levels were reduced by 21% (COX-2) and 33% (iNOS) in LPS-stimulated RAW264.7 cells that were pretreated with 200  $\mu$ g/ml of EaEAC compared with the vehicle + LPS group (P=0.012 and P=0.003, respectively; Fig. 3B). EaEAC pretreatment was, therefore, demonstrated to inhibit the increase of NO concentration, and COX-2 and iNOS mRNA expression in LPS-activated RAW264.7 cells.

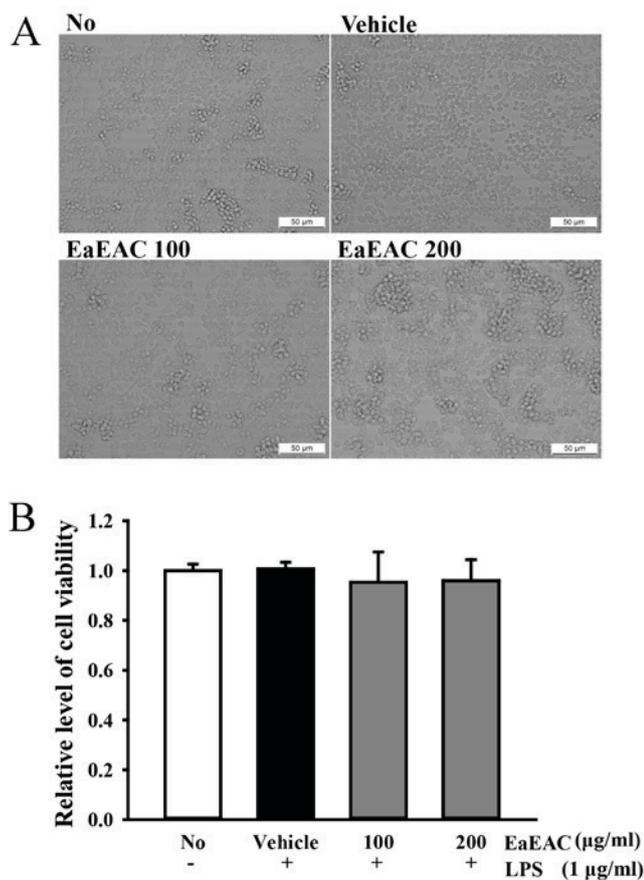


Figure 2. Toxicity of EaEAC. (A) Cell morphologies were observed under an inverted light microscope at x400 magnification following incubation with LPS and 0 (vehicle), 100 or 200  $\mu\text{g/ml}$  EaEAC in dimethyl sulfoxide for 24 h. (B) Cell viability of RAW264.7 cells treated with EaEAC + LPS was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Values are presented as the mean  $\pm$  standard deviation of three replicates. NC, untreated control; EaEAC, ethyl acetate extract from *Asparagus cochinchinensis* root; LPS, lipopolysaccharide.

#### Effects of EaEAC on the expression of inflammatory cytokines.

EaEAC-induced alterations to the mRNA expression levels of pro-inflammatory (TNF- $\alpha$  and IL-1 $\beta$ ) and anti-inflammatory (IL-10 and IL-6) cytokines in LPS-activated RAW264.7 cells were then investigated by semi-quantitative RT-PCR. The mRNA levels of the cytokines were significantly increased in the vehicle + LPS-treated group compared with the NC group ( $P=0.002$ ; Fig. 4A). Pretreatment with EaEAC had no significant effect on the mRNA expression levels of TNF- $\alpha$  at either 100 or 200  $\mu\text{g/ml}$  (Fig. 4A). However, pretreatment with 100 and 200  $\mu\text{g/ml}$  EaEAC significantly reduced mRNA expression of IL-1 $\beta$  ( $P=0.003$  and  $P=0.003$ , respectively; Fig. 4A), while 200  $\mu\text{g/ml}$  EaEAC significantly reduced mRNA levels of IL-10 ( $P=0.008$ ) and IL-6 ( $P=0.002$ ) compared with vehicle + LPS (Fig. 4A).

The concentration of IL-6 in the culture supernatant of LPS-activated RAW264.7 cells was also measured by ELISA. Similarly to the mRNA expression levels, the concentration of IL-6 was significantly increased in the Vehicle + LPS treated group compared with the NC group ( $P=0.005$ ; Fig. 4B). However, no significant differences were observed between the Vehicle + LPS group and the EaEAC + LPS groups, despite a

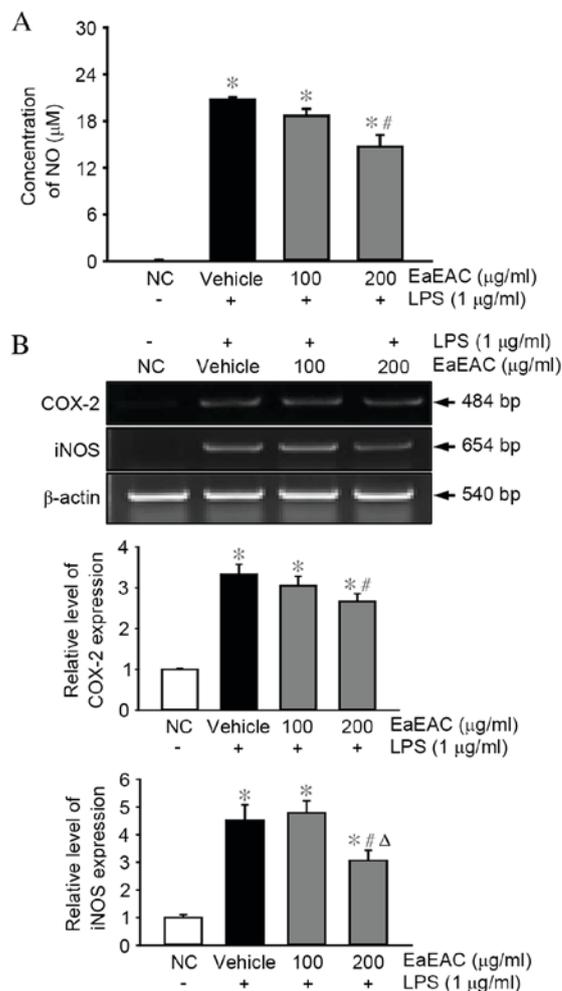


Figure 3. Determination of NO concentration, COX-2 and iNOS expression. (A) NO concentration was determined using supernatant collected from LPS-activated RAW264.7 cells treated with 0 (vehicle), 100 or 200  $\mu\text{g/ml}$  EaEAC. (B) COX-2 and iNOS mRNA transcript levels in the NC, vehicle + LPS and EaEAC + LPS treated groups were examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using transcript-specific primers. Values are presented as the mean  $\pm$  standard deviation of three replicates. \* $P<0.05$  vs. NC; \*\* $P<0.05$  vs. vehicle + LPS-treated group; # $P<0.05$  vs. 100  $\mu\text{g/ml}$  EaEAC + LPS-treated group. NO, nitric oxide; NC, untreated control; EaEAC, ethyl acetate extract from *Asparagus cochinchinensis* root; LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.

trend towards lower concentrations following EaEAC treatment (Fig. 4B). EaEAC pretreatment is, therefore, suggested to suppress enhanced anti- and pro-inflammatory cytokine expression in RAW264.7 cells stimulated by LPS treatment.

**Effects of EaEAC on MAPK signaling.** The MAPK pathway is critical in the regulation of cell growth and differentiation, and in the control of cellular responses to various cytokines and stresses (33). To investigate whether EaEAC pretreatment influences activation of the MAPK pathway, the phosphorylation levels of ERK, JNK and p38 were assessed in LPS-stimulated RAW264.7 cells by western blotting (Fig. 5A and B). Levels of phosphorylated ERK and p38 were significantly higher in cells stimulated with LPS (vehicle + LPS group) compared with untreated control cells ( $P=0.001$  and  $P=0.002$ , respectively; Fig. 5B). EaEAC pretreatment significantly suppressed

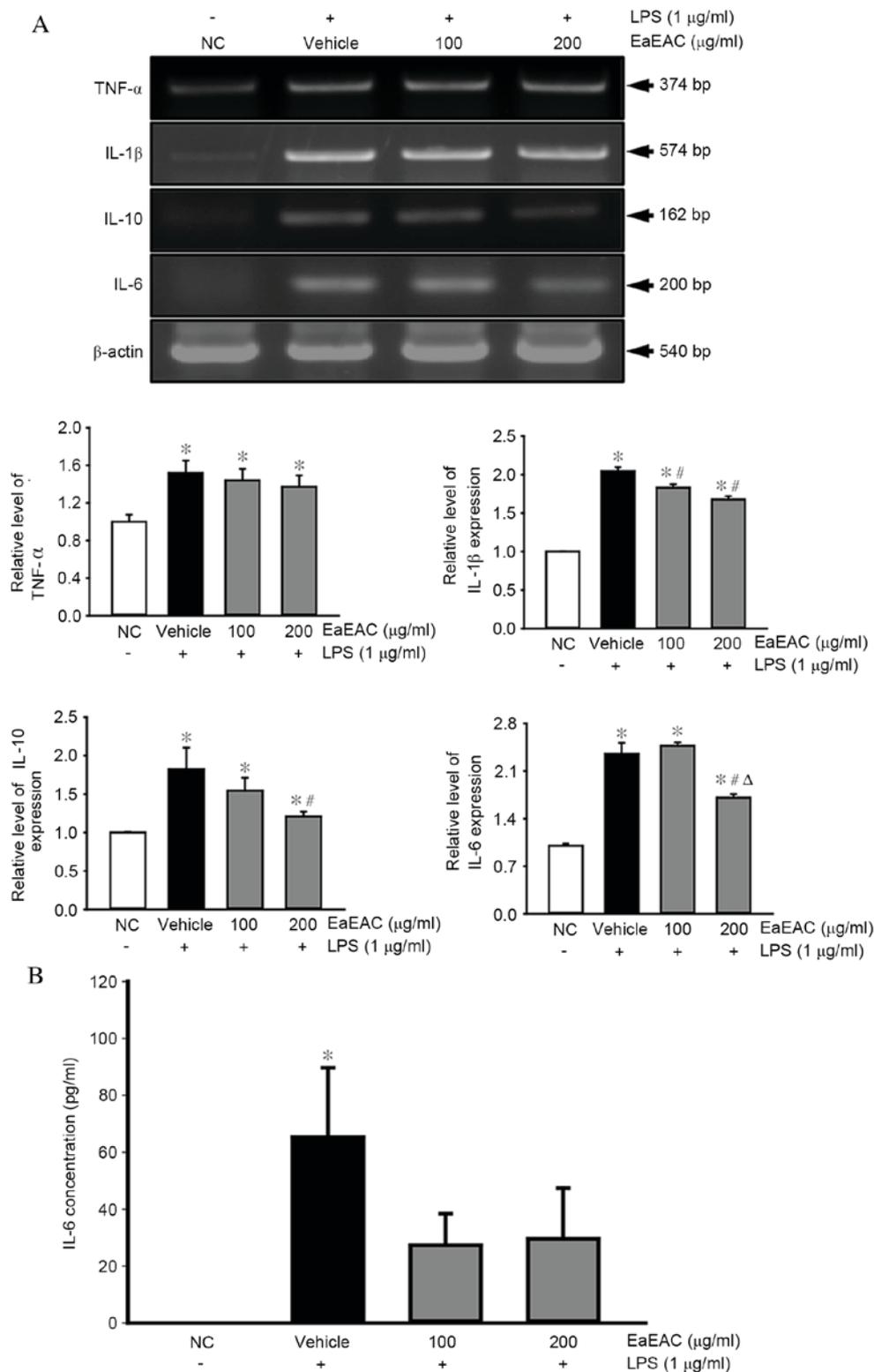


Figure 4. Analysis of pro- and anti-inflammatory cytokine expression. Following treatment of RAW264.7 cells with LPS and 0 (vehicle), 100 or 200  $\mu\text{g/ml}$  of EaEAC, (A) IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-10 mRNA levels were assessed by semi-quantitative reverse transcription polymerase chain reaction using transcript-specific primers, and (B) IL-6 concentration was detected using an enzyme-linked immunosorbent assay kit with a minimum detection threshold of 9.3 pg/ml. Values are presented as the mean  $\pm$  standard deviation of three replicates. \* $P < 0.05$  vs. NC; # $P < 0.05$  vs. vehicle + LPS-treated group;  $\Delta P < 0.05$  vs. 100  $\mu\text{g/ml}$  EaEAC + LPS-treated group. LPS, lipopolysaccharide; NC, untreated control; EaEAC, ethyl acetate extract from *A. cochinchinensis* root; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin.

the LPS-induced phosphorylation of ERK at concentrations of 100 ( $P = 0.002$ ) and 200  $\mu\text{g/ml}$  ( $P = 0.003$ ) compared with the vehicle + LPS group (Fig. 5B). JNK phosphorylation was

also suppressed by 100 ( $P = 0.015$ ) and 200  $\mu\text{g/ml}$  EaEAC ( $P = 0.033$ ) compared with the vehicle + LPS group (Fig. 5B), however, the JNK phosphorylation level was not induced

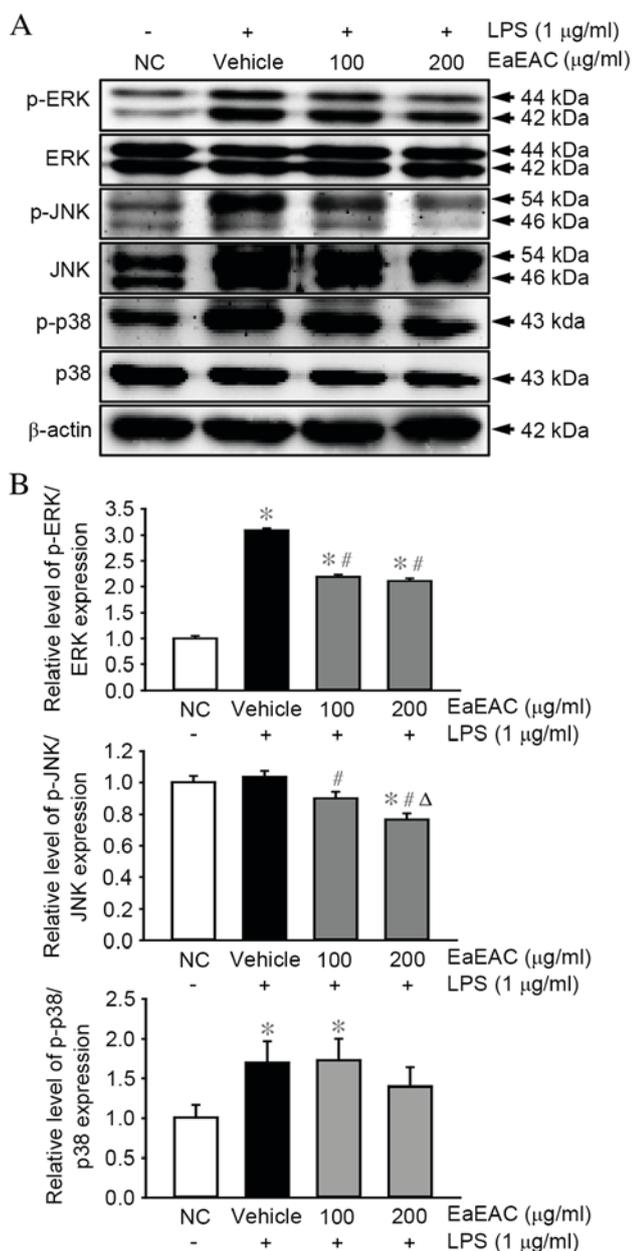


Figure 5. Activation of the mitogen-activated protein kinase signaling pathway proteins ERK, JNK, and p38. (A) p-ERK, ERK, p-JNK, JNK, p38, p-p38 and  $\beta$ -actin protein expression was assessed by western blot following stimulation of RAW264.7 cells with LPS and 0 (vehicle), 100 or 200  $\mu\text{g/ml}$  of EaeAC. (B) Band intensity was determined using an imaging densitometer and expression levels calculated relative to the intensity of  $\beta$ -actin. Values are presented as the mean  $\pm$  standard deviation of three replicates. \* $P < 0.05$  vs. NC; # $P < 0.05$  vs. vehicle + LPS-treated group;  $\Delta P < 0.05$  vs. 100  $\mu\text{g/ml}$  EaeAC + LPS-treated group. LPS, lipopolysaccharide; NC, untreated control; EaeAC, ethyl acetate extract from *Asparagus cochinchinensis* root; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

by LPS stimulation, as demonstrated by the non-significant difference between the NC and vehicle + LPS group (Fig. 5B). EaeAC pretreatment resulted in no significant difference in LPS-induced phosphorylation of p38 at concentrations of 100 or 200  $\mu\text{g/ml}$  compared with the vehicle + LPS group (Fig. 5B). Therefore, EaeAC attenuated the enhanced phosphorylation of some MAPK proteins in LPS-stimulated RAW264.7 macrophage cells.

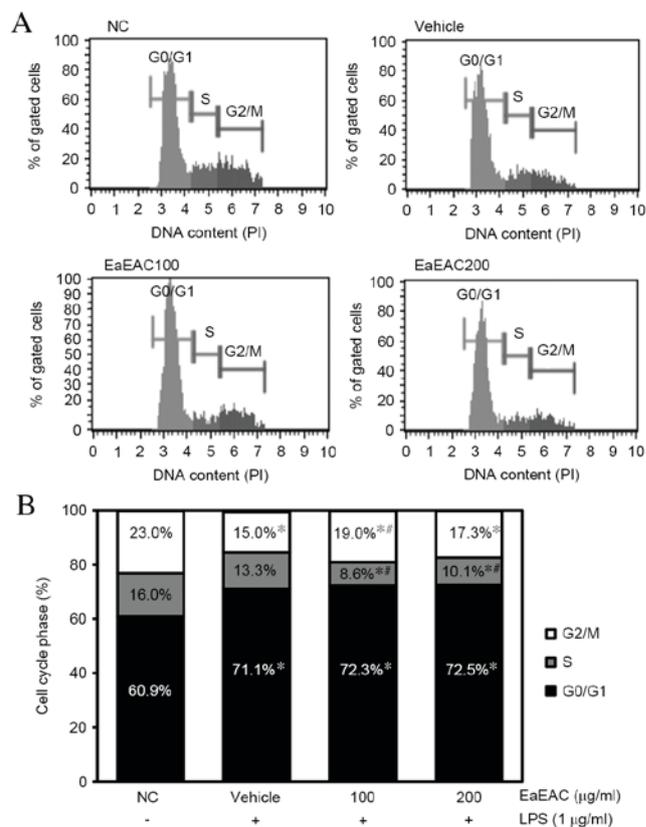


Figure 6. Cell cycle analysis. The cell cycle distribution was determined by flow cytometric analysis of the DNA content of nuclei of RAW264.7 cells following staining with PI. After treatment with EaeAC + LPS, the number of cells in the G0/G1, S and G2/M stage was determined. (A) Fluorescence-activated cell sorting analysis and (B) the relative percentage of cells in each cell cycle phase (G0/G1, S and G2/M). \* $P < 0.05$  vs. NC; # $P < 0.05$  vs. Vehicle + LPS treated group. PI, propidium iodide; EaeAC, ethyl acetate extract from *Asparagus cochinchinensis* root; LPS, lipopolysaccharide; NC, untreated control.

**Effect of EaeAC on regulation of the cell cycle.** To examine the effect of EaeAC treatment on the cell cycle, the number of cells in each stage of the cell cycle was counted (Fig. 6A and B). The number of cells in the G0/G1 stage was increased in the vehicle + LPS group compared with untreated control cells ( $P = 0.003$ ; Fig. 6B), whereas those in the S and G2/M stage were decreased ( $P = 0.001$ ; Fig. 6B). However, a significant increase in the number of cells in the G2/M stage was induced by treatment with 100  $\mu\text{g/ml}$  EaeAC compared with the vehicle + LPS group ( $P = 0.023$ ; Fig. 6B), and a similar but non-significant trend towards increased G2/M stage cells was induced by treatment with 200  $\mu\text{g/ml}$  EaeAC (Fig. 6B). A significant decrease in the number of S stage cells was also observed following treatment with 100 ( $P = 0.05$ ) and 200  $\mu\text{g/ml}$  EaeAC ( $P = 0.008$ ) compared with the vehicle + LPS (Fig. 6B). EaeAC treatment was, therefore, demonstrated to induce arrest of the cell cycle in the G2/M stage and stimulate progression from the S stage to the G2/M stage.

**Inhibitory effects of EaeAC treatment on ROS production.** Finally, the inhibitory effects of EaeAC against LPS-induced ROS production in RAW264.7 macrophage cells were determined using a fluorescent oxidation-sensitive dye, DCFH-DA, to measure ROS levels. ROS production increased rapidly

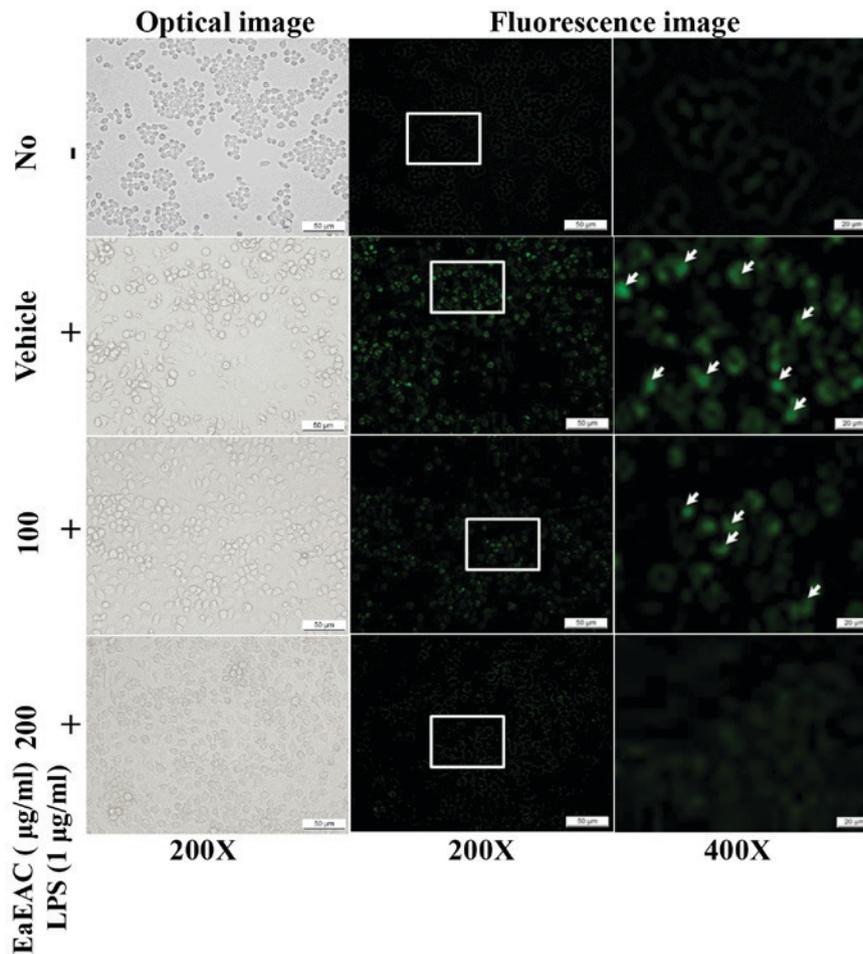


Figure 7. Determination of intracellular reactive oxygen species production. Cells were pretreated with LPS and 0 (vehicle), 100 or 200  $\mu\text{g/ml}$  of EaEAC in dimethyl sulfoxide, then treated with DCFH-DA. Optical images (left column) were observed by light microscopy, and green fluorescence in cells was observed by fluorescence microscopy. Boxed cells in each x200 magnification image (center column) were further examined under x400 magnification (right column). Arrows indicate cells stained with DCFH-DA. DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; NC, untreated control; EaEAC, ethyl acetate extract from *Asparagus cochinchinensis* root; LPS, lipopolysaccharide.

in the vehicle + LPS-treated group compared with the NC group. However, the level of ROS production decreased in an apparently concentration-dependent manner, despite cell morphologies remaining consistent. Therefore, increased ROS levels induced by LPS stimulation were effectively suppressed by EaEAC pretreatment.

## Discussion

Extracts of *A. cochinchinensis* roots have been used in Korea to treat neuro-inflammatory and skin-inflammatory diseases for many years (1,25,27). Several studies have, therefore, focused on *A. cochinchinensis* to develop therapies against lung-inflammatory disease (28,34). In an effort to identify candidate natural products for the treatment of chronic lung disease and examine the mechanism of action, the present study investigated the therapeutic effects of EaEAC in LPS-activated RAW264.7 cells. EaEAC was demonstrated to significantly suppress inflammatory responses through decreased NO, COX-2, iNOS, IL-6, IL-10 and IL-1 $\beta$  expression levels, and attenuation of ROS production in LPS-activated RAW264.7 macrophages.

LPS is the major component of the external membrane of gram-negative bacteria, and an important molecule in the pathogenesis of sepsis and septic shock. LPS has also been widely used as a prototypical endotoxin to activate numerous cell types, including monocytes, dendritic cells, macrophages and B cells (35,36). Among these cells, macrophages are crucial for regulating NO, TNF- $\alpha$ , and IL-6 production, which mediate the development of various inflammatory diseases, including sepsis-related multiple organ dysfunction/multiple organ failure, microbial infection, acute brain/lung/hepatic/renal injuries, neurodegenerative disorders, tumorigenesis, osteoporosis/osteonecrosis, and cardiovascular, metabolic and autoimmune diseases (37-39). Furthermore, various concentrations of LPS (50 ng/ml to 10  $\mu\text{g/ml}$ ) have been applied to activate RAW264.7 cells (40-42). Preliminary data from the present study demonstrated that an inflammatory response was induced in RAW264.7 cells stimulated with 0.1, 0.5, 1.0 and 1.5  $\mu\text{g/ml}$  LPS (data not shown). A concentration of 1  $\mu\text{g/ml}$  LPS was, therefore, selected as the optimal concentration for activation of macrophages in subsequent experiments.

NO, which include nitroglycerine and amyl nitrite precursor, are considered to be important cellular signaling

molecules in various mammalian physiological and pathological processes (33). NO is produced by NOS in the majority of organisms, including bacteria, fungi and mammals. In humans, NO is primarily produced by iNOS in phagocytic cells during an immune response (43). LPS-induced NO production in BV-2 cells has previously been demonstrated to be significantly suppressed by three compounds originating from *A. cochinchinensis*: Aspacochinoside O, Aspacochinoside P and 3-*O*- $\beta$ -D-xylopyranosyl(1 $\rightarrow$ 4)-[ $\beta$ -D-glucopyranosyl(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranosyl-26-*O*- $\beta$ -D-glucopyranosyl-(25S)-5 $\beta$ -furostane-3 $\beta$ , 22 $\alpha$ , 26-triol (44). The present study demonstrated similar inhibitory effects on NO production, although the cell line and solvent used for extraction in the studies differed. EaEAC pretreatment induced the restoration of iNOS and COX-2 transcription levels in LPS-activated macrophage cells. The results of the current study suggest that the inhibitory effects of EaEAC on LPS-stimulated NO production are a result of iNOS and COX-2 transcriptional suppression. However, additional research is required to determine the key compounds within EaEAC that are responsible for the regulation of NO production.

Secretion of cytokines, including interleukins, interferon, colony stimulating factors and numerous growth factors regulate the differentiation, proliferation and development of cells (45). The pro-inflammatory cytokines secreted by stimulated macrophages are important in inflammatory diseases, including sepsis and arthritis (46,47). Furthermore, extracts from *A. cochinchinensis* have previously been demonstrated to effectively regulate the expression of various cytokines; the increase of TNF- $\alpha$  secretion in astrocytes induced by substance P + LPS and ethanol treatment was significantly inhibited by ethanol extracts (70%) of *A. cochinchinensis* (ACE) in a dose dependent manner (21,25). Production of pro-inflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$  has also been previously demonstrated to be suppressed by ACE in mice with phorbol ester-induced dermatitis (27). In the present study, the transcript and protein levels of pro- and anti-inflammatory cytokines in RAW264.7 cells were measured following EaEAC + LPS treatment, revealing similar suppressive activities compared with previous studies, despite certain differences in the reduction rate and the composition of the extracts.

To the best of our knowledge, the present study presents the first evidence of the therapeutic effect of EaEAC in LPS-activated RAW264.7 cells accompanied by alterations in MAPK signaling and the cell cycle. MAPKs are important in the regulation of cell growth and differentiation, and in the control of cellular responses to cytokines and stresses (48). In several previous studies, the expression levels of three major members of the MAPK signaling pathway, JNK, ERK and p38, were significantly increased by LPS treatment in macrophage cells (49,50). LPS also increased the expression of several inflammatory genes, including TNF- $\alpha$ , IL-6, iNOS and COX-2, through regulation of MAPKs (51,52). Furthermore, pathological conditions, including inflammation and oxidative stress, have been demonstrated to induce alteration of the cell cycle in numerous diseases (53,54). Certain natural products have also been previously demonstrated to induce alteration of MAPK expression and the cell cycle in RAW264.7 cells. For example, triterpene extract from *G. lucidum* suppressed

the LPS-induced phosphorylation of ERK1/2 and JNK, and arrested these cells at the G0/G1-M stage (55). Wild grape seed procyanidins inhibited the LPS-induced phosphorylation of p38 (56), and *Rhus verniciflua* Stokes arrested RAW264.7 cells at the G1 stage of the cell cycle (57). In the present study, EaEAC inhibited the phosphorylation of three members of the MAPK signaling pathway and arrested the cell cycle at the G2/M stage. The results of the present study were similar to those of previous studies, although certain differences in the suppression of MAPK signaling were observed. These differences may be due to factors including the innate composition of the extracts and key molecules within the herbal medicine.

Oxidative stress, cell dysfunction and, ultimately, apoptosis and necrosis are induced by excessive production of ROS (58). The activity of SOD and the content of MDA have been demonstrated to be differentially regulated by the roots and stems of *A. cochinchinensis*, and polysaccharide and aqueous extracts of *A. cochinchinensis* roots increased SOD activity, but decreased MDA content (59). By contrast, *A. cochinchinensis* stem extracts decreased SOD activity and enhanced MDA accumulation in the brains and livers of mice (1). Similar effects on anti-oxidant state were detected in the present study: ROS production was increased in the vehicle + LPS-treated group, but was recovered in a dose dependent manner in the EaEAC + LPS-treated group, therefore providing additional evidence that the roots of *A. cochinchinensis* have anti-oxidant activity and may suppress ROS production. In summary, the present study suggests that EaEAC may suppress inflammatory responses in macrophages through stimulation of anti-inflammatory effects, decreased pro-inflammatory cytokine expression and inhibition of ROS production. The inhibitory effects of EaEAC were observed to be associated with attenuation of the enhanced phosphorylation of MAPK proteins following LPS treatment. The MAPK signaling pathway regulates the transcription of a number of genes associated with inflammation, thus, its inhibition by EaEAC offers a potential approach for the treatment of severe inflammatory disease.

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