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Anti-inflammatory Activities of an Ethanol Extract of *Ecklonia stolonifera* in Lipopolysaccharide-Stimulated RAW 264.7 Murine Macrophage Cells

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ABSTRACT: *Ecklonia stolonifera* is a brown alga that was shown to have antioxidant, anti-inflammatory, tyrosinase inhibitory, and chemopreventive activities. However, the molecular mechanisms underlying its anti-inflammatory activity remain unclear. In this study, we investigated the molecular mechanism of the anti-inflammatory action of *E. stolonifera* ethanolic extracts (ESE) using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. ESE inhibited LPS-induced nitric oxide (IC_{50} = 72 ± 1.9 μg/mL) and prostaglandin E_2 (IC_{50} = 98 ± 5.3 μg/mL) production in a dose-dependent manner and suppressed the expression of inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 cells. ESE also reduced the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. LPS-induced nuclear factor-κB (NF-κB) transcriptional activity and NF-κB translocation into the nucleus were significantly inhibited by ESE treatment through the prevention of the degradation of inhibitor κB-α. Moreover, ESE inhibited the activation of Akt, ERK, JNK1/2, and p38 MAPK in LPS-stimulated RAW 264.7 cells. The main components with anti-inflammatory activity in ESE were identified as phlorofucofuroeckol A and B based on the inhibition of NO production. Our results indicate that ESE can be considered as a potential source of therapeutic agents for inflammatory diseases.

KEYWORDS: *Ecklonia stolonifera, anti-inflammation, NF-κB, MAP kinases, RAW 264.7 cells*

■ INTRODUCTION

Macrophages are known to play an important role in host defense mechanism. Activations of macrophages are detected in inflammed tissues and are induced after exposure of interferon-γ, tumor necrosis factor (TNF), and a microbial lipopolysaccharide (LPS).[1] Activated macrophages play a pivotal role in inflammatory diseases via excess production of cytokines including TNF-α, interleukin (IL)-1β, IL-6, and inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2).[2,3] Excessive production of these cytokines is involved in the pathogenesis of chronic diseases, such as atherosclerosis, inflammatory arthritis, and cancer.[4,5] Thus, identification of any compound or extract from a natural product that can inhibit the production of these inflammatory cytokines would be attractive in terms of the development of nutraceuticals for inflammatory diseases.

Gene expressions of inflammatory proteins, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, are primarily controlled at transcriptional levels.[6,7] Transcriptional induction of iNOS or COX-2 is largely dependent on the activity of the transcription factor, nuclear factor-κB (NF-κB).[7,8] NF-κB plays pivotal roles in immune responses as well as in cell survival.[9] In the unstimulated condition, NF-κB is located in the cytoplasm as an inactive complex bound to inhibitor κB-α (IκB-α).[10] The activation of NF-κB in response to LPS or pro-inflammatory stimuli leads to degradation of IκB-α, resulting in the translocation of NF-κB into the nucleus, and induces the transcription of inflammatory mediators and pro-inflammatory cytokines.[10] The activation of NF-κB is also regulated by cellular kinases such as mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways. The MAPKs, extracellular signal regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK, have been implicated in the transcriptional regulation of inflammatory gene.[11,12] PI3K is also involved in cytokine expression and NF-κB activation in mouse macrophage.[13,14] Hence, any substances that inhibit the activation of NF-κB pathway are considered as potential anti-inflammatory agents.

Ecklonia stolonifera is a marine brown alga that is commonly used as a culinary item in Korea and Japan. *Ecklonia* spp. is used as an herbal medicine in China to treat goiter, scrofula, urinary disease, dropsy, stomach ailments, and hemorrhoids.[15] Phlorotannins, marine polyphenols, from *E. stolonifera* have been reported to have several biological activities, such as antioxidation,[16,17] antidiabetic complications,[18] antihyperlipidemia,[19] antiskin aging,[20] and hepatoprotection.[21] As part of our ongoing effort to develop anti-inflammatory agents from brown algae, we found strong antioxidant and anti-inflammatory activities in *E. stolonifera* ethanolic extract (ESE). This led us to investigate the inhibitory activity of ESE on LPS-stimulated

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inflammatory responses in RAW 264.7 cells as well as to explore the possible molecular mechanisms underlying its anti-inflammatory action.

■ MATERIALS AND METHODS

Plant Material and Reagents. *E. stolonifera* was collected from Busan aquaculture farm, South Korea, in August 2010, and a voucher specimen was deposited in our laboratory (H.R.K.). LPS, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and the specific kinase inhibitors (LY294002, PD98059, SP600125, and SB203580) were purchased from Sigma Chemical Co. (St. Louis, MO). CellTiter^{96} AQ$_{ueous}$ One Solution Cell Proliferation assay kit, dual luciferase assay kit, murine NF-κB promoter/luciferase DNA, pRL-TK DNA, and superscript reverse transcriptase were obtained from Promega (Madison, WI). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine/Plus, TRIzol, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 4,6′-diamidino-2-phenylindole (DAPI), Alexa Fluor 488-conjugated secondary antibody, penicillin, and streptomycin were purchase from Invitrogen (Carlsbad, CA).

Preparation of ESE. *E. stolonifera* was rinsed in tap water to remove salt and dried in an air dryer at 50 °C for 72 h. A dried sample was ground with a hammer mill, and the powder was stored at −20 °C until used. The dried powder (1 kg) of *E. stolonifera* was extracted three times with 96% ethanol (5 L/each) for 3 h at 70 °C. The combined extracts were concentrated using a rotary vacuum evaporator (Tokyo Eyela, Tokyo, Japan) at 40 °C and lyophilized. Two hundred seventy-five grams of extract were obtained from 1 kg of dried powder (yield = 27.5%).

Cell Culture and Viability Assay. Murine macrophage RAW 264.7 cells (ATCC, Rockville, MD) were cultured at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) under a humidified atmosphere of 5% CO_2. The cell viability was determined by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using CellTiter^{96} AQ$_{ueous}$ One Solution Cell Proliferation assay kit according to the manufacturer's manual. Briefly, cells were inoculated at a density of 1×10^5 cells/well into 96-well plates and cultured at 37 °C for 24 h. The culture media were replaced by 200 μL of DMEM containing serial dilutions (0−200 μg/mL) of ESE, and the cells were incubated for 24 h. The culture media were removed and replaced by 95 μL of fresh culture medium and 5 μL of kit solution. After 1 h, the absorbance was measured using a microplate reader (Glomax Multi Detection System, Promega) at 490 nm.

Measurement of Intracellular Reactive Oxygen Species (ROS). The intracellular ROS scavenging activity of the sample was measured using the oxidant-sensitive fluorescent probe DCFH-DA. RAW 264.7 cells (1×10^6 cells per well in a 12-well plate) were incubated with 0−200 μg/mL of ESE for 1 h and then stimulated without or with 1 μg/mL LPS for 2 h. Cells were harvested by trypsin−EDTA solution [0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS)] and washed with PBS twice. The cells were treated with 20 μM DCFH-DA for 30 min at 37 °C. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corp., Sunnyvale, CA).

Measurement of NO, PGE$_2$, TNF-α, IL-1β, and IL-6. RAW 264.7 cells were placed in a 12-well plate at a density of 1×10^6 cells per well and incubated for 24 h. Cultured cells were treated with DMSO or various concentrations of ESE for 1 h and then stimulated without or with 1 μg/mL LPS for 24 h. Cultured media were collected after centrifugation at 2000*g* for 10 min and stored at −70 °C until tested. The nitrite concentration in the cultured media was measured as an indicator of NO production. Culture media (100 μL) were mixed with the same volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). Absorbance of the mixture at 540 nm was measured with a microplate reader. Levels of PGE$_2$, IL-1β, IL-6, and TNF-α in cultured media were quantitated by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). RAW 264.7 cells plated in a 12-well plate were pretreated with ESE for 1 h and then stimulated with LPS for 6 h. Total RNA from each group was isolated with the TRIzol reagent. Five micrograms of total RNA were used for reverse transcription using random hexamer and superscript reverse transcriptase. PCR was carried out using gene-specific primers: COX-2 sense, 5′-CAG CAA ATC CTT GCT GTT CC-3′; COX-2 antisense, 5′-TGG GCA AAG AAT GCA AAC ATC-3′; iNOS sense, 5′-TCT TCG AAA TCC CAC CTG AC-3′; iNOS antisense, 5′-CCA TGA TGG TCA CAT TCT GC-3′; glyceraldehydes 3-phosphate dehydrogenase (GAPDH) sense, 5′-TGG CAC AGT CAA GGC TGA GA-3′; and GAPDH antisense, 5′-CTT CTG AGT GGC AGT GAT GG-3′. GAPDH was used as an internal standard to evaluate the relative expression of COX-2 and iNOS.

Transient Transfection and Luciferase Assay. Murine NF-κB promoter/luciferase DNA (1 μg) along with 20 ng of control pRL-TK DNA was transiently transfected into 1×10^6 RAW 264.7 cells/well in a 24-well plate using Lipofectamine/Plus reagents for 40 h. Cells pretreated with 0−200 μg/mL ESE for 1 h were stimulated with LPS (1 μg/mL) for 6 h. Each well was washed twice with cold PBS, harvested in 100 μL of lysis buffer (0.5 mM HEPES, pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$), and used for the measurement of luciferase activity using a luciferase assay kit. Luminescence was measured on a TopCount microplate scintillation and luminescence counter (PerkinElmer, Santa Clara, CA) in single-photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. The luciferase activity was normalized to the expression of control pRL-TK.

Preparation of Cytosolic and Nuclear Extracts. RAW 264.7 cells (1×10^6 cells/well) pretreated with ESE for 1 h were stimulated with LPS for 0.5 h. Cells were washed twice with cold PBS and harvested with 500 μL of cold PBS. Cell pellets were resuspended in 300 μL of hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9) and incubated on ice for 15 min. After they were vortexed for 10 s, homogenates were divided into supernatants (cytoplasmic compartments) and pellets (nuclear components) by centrifugation at 13000*g* for 10 min. The pellet was gently resuspended in 40 μL of complete lysis buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF, pH 7.9) and centrifuged at 13000*g* for 20 min at 4 °C. The supernatant was used as the nuclear extract.

Western Immunoblot Analysis. RAW 264.7 cells were incubated with various concentrations of ESE for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. Control and ESE-treated RAW 264.7 cells were washed twice with cold PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/mL leupeptin, 50 mM NaF, and protease inhibitor cocktail) on ice for 1 h. After centrifugation at 18000*g* for 10 min, the protein concentrations in supernatants were determined, and aliquots of protein (40 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline with Tween 20 (TBST) for 1 h and then incubated with various primary antibodies: goat polyclonal anti-COX-2 (1:2000), rabbit polyclonal anti-iNOS (1:2000), mouse monoclonal anti-IκB-α (1:2000), mouse monoclonal anti-NF-κB (1:2000), rabbit polyclonal anti-poly-ADP-ribose polymerase (PARP) (1:2000), rabbit polyclonal anti-p-Akt (1:1000), rabbit monoclonal anti-p-p38 (1:2000), mouse monoclonal anti-p-ERK (1:2000), mouse monoclonal anti-p-JNK(1:1000), and mouse monoclonal anti-β-actin (1:5000). The blots were treated with horseradish peroxidase-conjugated secondary antigoat (1:5000), antirabbit (1:5000), or antimouse (1:5000) antibody in TBST buffer containing 5% nonfat dried milk for 1 h, and immune complexes were detected using an ECL detection kit (GE Healthcare Bio-Sciences,

Piscataway, NJ). Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II (ATTO Co., Tokyo, Japan) and CS analyzer version 3.00 software (ATTO Co.).

Immunofluorescence Analysis. To analyze nuclear localization of NF-κB in RAW 264.7 cells, cells were maintained on glass coverslips (SPL Lifesciences Co., Gyeonggi-do, Korea) in 24-well plates for 24 h. Cells pretreated with ESE for 1 h were incubated with LPS (1 μg/mL) for 1 h. Cells were fixed in 4.0% (w/v) paraformaldehyde in PBS for 15 min at room temperature and then permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min. Permeabilized cells were washed with PBS and blocked with 3% (w/v) BSA in PBS for 30 min. Thereafter, cells were incubated with rabbit polyclonal anti-NF-κB antibody diluted in 3% BSA/PBS (1:75) for 2 h and incubated in Alexa Fluor 488-conjugated secondary antirabbit antibody diluted in 3% BSA/PBS (1:200) for 1 h. Cells were stained with 2 μg/mL DAPI, and images were captured using an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Chromatographic Conditions. Chromatographic analysis was performed with a Shimadzu high-performance liquid chromatography (HPLC) system (Shimadzu Co., Kyoto, Japan), consisting of a pump (Shimadzu LC-20AD), a photodiode array detector (Shimadzu SPD-M20A), an autosampler (SIL-20A), a system controller (CBM-20A), and a Shimadzu LCsolution (ver.1.22sp). ESE powder was dissolved in methanol at a concentration of 100 ppm. Two microliters of ESE solution were separated by HPLC system with Luna RP-18 column [Luna C18(2), 3 μm, 150 mm × 3.0 mm, Phenomenex, Torrence, CA]. The separation of ESE was conducted using as the mobile phase of 0.1% formic acid in methanol (solvent A) and 0.1% formic acid in water (solvent B). The elution profile consisted of a linear gradient from 8 to 27.4% solvent B for 90 min and was held for 10 min and then re-equilibration of the column with 8% solvent B for 18 min. The flow rate was 0.34 mL/min at 35 °C oven temperature, and detection was performed at 270 nm. Compounds were identified by comparison of their retention times to those of authentic standards as described previously.[16,21]

Statistical Analysis. Data were expressed as the means ± standard deviations (SDs). Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair of Student's t tests for multiple comparisons. Differences were considered significant of $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL).

RESULTS

Effect of ESE on the Production of NO and PGE_2. To evaluate the effect of ESE on NO production in LPS-stimulated RAW 264.7 cells, we measured the nitrite concentration in the cultured media using Griess reagent. As shown in Figure 1A, NO production, measured as nitrite, was increased by LPS alone; however, ESE significantly inhibited the levels of NO production from LPS-stimulated cells in a dose-dependent manner (IC_{50} = 72 ± 1.9 μg/mL, $P < 0.05$) (Figure 1B). In addition to NO, ESE also significantly inhibited PGE_2 production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner (IC_{50} = 98 ± 5.3 μg/mL, $P < 0.05$). The MTS assay showed that ESE did not have any cytotoxicity at the concentrations (0−200 μg/mL) (Figure 1C). Thus, the inhibitory effects of ESE on NO and PGE_2 productions are not attributed to cytotoxic effects.

Effect of ESE on the Production of iNOS and COX-2. Because iNOS and COX-2 are the key enzymes for the production of NO and PGE_2, respectively, we analyzed the expression level of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 cells by Western blotting. As shown in Figure 2A, ESE strongly suppressed the expressions of iNOS (IC_{50} = 64.2 ± 2.7 μg/mL) and COX-2 (IC_{50} = 133.5 ± 12.5 μg/mL) proteins in a dose-dependent manner ($P < 0.05$). In addition to protein expression, ESE inhibited iNOS (IC_{50} = 77.9 ± 4.0 μg/mL) and COX-2 (IC_{50} = 101.6 ± 4.0 μg/mL) mRNA expressions in a dose-dependent manner in LPS-stimulated RAW 264.7 cells ($P < 0.05$) (Figure 2B). These results suggest that ESE-mediated inhibition of NO and PGE_2 production is associated with transcriptional down-regulation of iNOS and COX-2 genes.

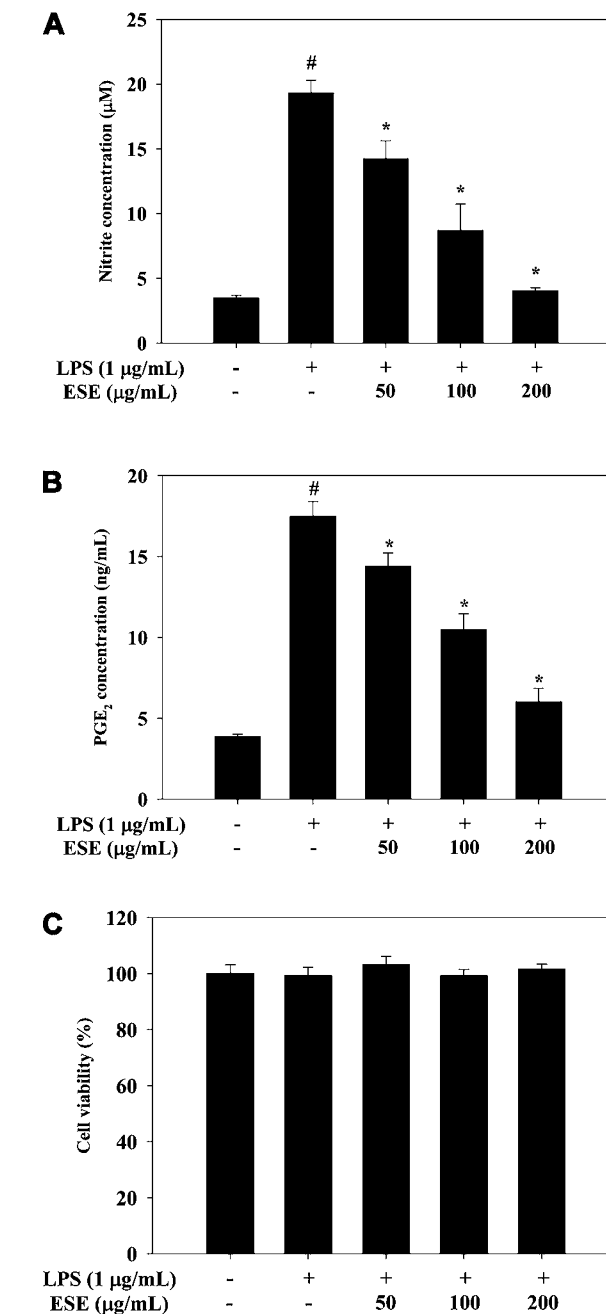

Figure 1. Effect of ESE on the LPS-induced NO and PGE_2 production in RAW 264.7 cells. Cells pretreated with indicated concentrations of ESE for 1 h were stimulated without or with LPS (1 μg/mL) for 24 h. The treated culture media were used to measure the amount of nitrite to evaluate NO production (A) and PGE_2 production (B). The cytotoxic effect of ESE was measured by MTS assay (C). Values are the means ± SDs of three independent experiments. $^{\#}P < 0.05$ indicates significant differences as compared to the nontreated control group. $^{*}P < 0.05$ indicates significant differences as compared to the LPS-only group.

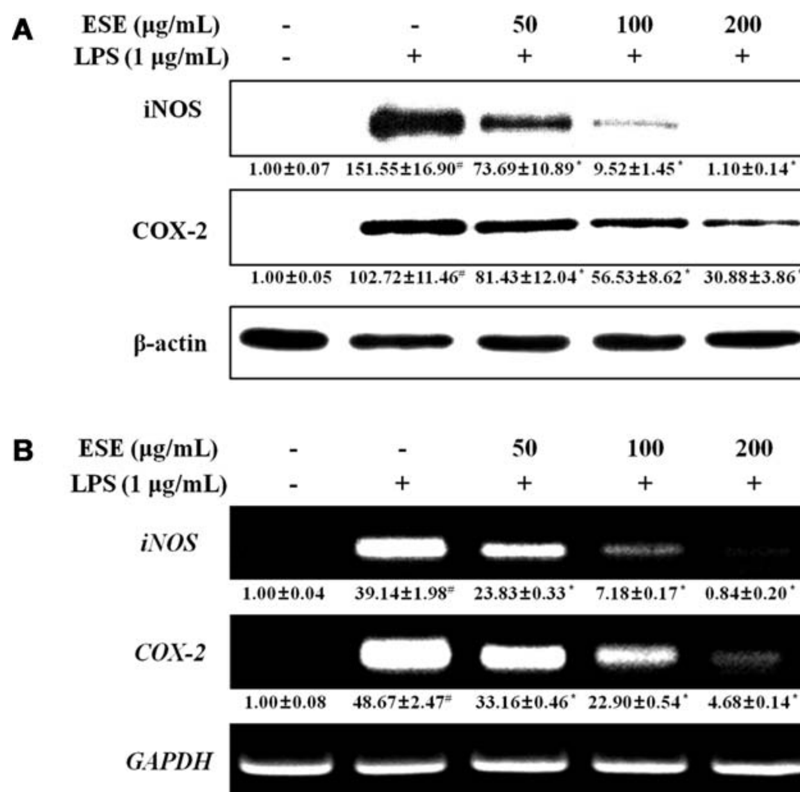

Figure 2. Effect of ESE on LPS-stimulated iNOS and COX-2 expression in RAW 264.7 cells. (A) Cells pretreated with indicated concentrations of ESE for 1 h were stimulated without or with LPS (1 μg/mL) for 16 h. An equal amount of total proteins was subjected to 10% SDS-PAGE. The expressions of iNOS, COX-2, and β-actin proteins were detected by Western blot using corresponding antibodies. (B) Cells were pretreated with ESE for 1 h and stimulated with LPS for 6 h, and then, total RNA was prepared for RT-PCR. The results are representative of those obtained from three independent experiments. $^{\#}P < 0.05$ indicates significant differences as compared to the nontreated control group. $^{*}P < 0.05$ indicates significant differences as compared to the LPS-only group.

Effect of ESE on the Production of Pro-inflammatory Cytokines. Because TNF-α, IL-1β, and IL-6 are early secreted pro-inflammatory cytokines and their elevated levels can be detected in a variety of acute and chronic inflammatory diseases, we determined the effects of ESE on secreted levels of TNF-α, IL-1β, and IL-6 in LPS-treated cells. To reach detectable ranges of secreted cytokines in cultured media, LPS stimulations were extended up to 24 h. The stimulation of RAW 264.7 cells with LPS noteworthy increased levels of TNF-α (Figure 3A), IL-1β (Figure 3B), and IL-6 (Figure 3C). Induced levels of pro-inflammatory cytokine were significantly decreased by ESE in a dose-dependent manner ($P < 0.05$). The IC_{50} values of TNF-α, IL-1β, and IL-6 were estimated to be 131.1 ± 11.8, 85.7 ± 2.5, and 82.8 ± 3.5 μg/mL, respectively. This result indicates that ESE efficiently suppressed LPS-induced TNF-α, IL-1β, and IL-6 production, supporting that ESE inhibits the initial phase of the LPS-stimulated inflammatory response.

Effect of ESE on the Activation and Translocation of NF-κB. To assess transcriptional control of ESE on regulation of inflammatory response, we measured the effects of ESE on the translocation of NF-κB/p65 subunit in LPS-stimulated cells. Observation with confocal microscopy revealed that NF-κB/p65 protein was mostly distributed in the cytoplasm in unstimulated cells. After stimulation with LPS, p65 was translocated to the nucleus (Figure 4A); however, the protein in the nucleus was markedly reduced by pretreatment with ESE in immunofluorescence assay. To assess the molecular mechanisms underlying translocation of NF-κB/p65 from the cytosol to the nucleus in LPS-stimulated RAW 264.7 cells, we also investigated the inhibitory effect of ESE on LPS-stimulated degradation of IκB-α, which is responsible for the activation of NF-κB, by Western blotting. LPS treatment resulted in increased IκB-α degradation as compared to controls, and ESE pretreatment recovered the level of cytosolic IκB-α in a dose-dependent manner (Figure 4B). As a result of IκB-α degradation, the increased nuclear NF-κB level after LPS stimulation was reduced by ESE pretreatment in a dose-dependent manner (IC_{50} = 152.9 ± 14.1 μg/mL, Figure 4B). Considering the inhibitory effects of ESE on LPS-stimulated NF-κB translocation, we next determined the effect of ESE on the promoter activity of NF-κB in LPS-stimulated macrophages. Data suggested that ESE treatment significantly inhibited LPS-induced NF-κB promoter-driven luciferase expression in macrophages (IC_{50} = 104.0 ± 5.0 μg/mL, $P < 0.05$; Figure 4C). These results indicate that the ESE-mediated inhibition of iNOS, COX-2, and pro-inflammatory cytokine expression levels was regulated by the NF-κB pathway in LPS-stimulated macrophages.

Effect of ESE on the Phosphorylation of MAPKs and Akt. To further investigate whether ESE regulates signaling proteins responsible to NF-κB activation, we measured the phosphorylation levels of MAPKs including JNK, p38 MAPK, and ERK in LPS-stimulated macrophages. As shown in Figure 5A, ESE treatment strongly inhibited the phosphorylation of p38 MAPK, JNK, and ERK in LPS-stimulated cells. Since, a signaling molecule such as Akt is linked to COX-2 or iNOS expression via NF-κB activation, we examined the effects of

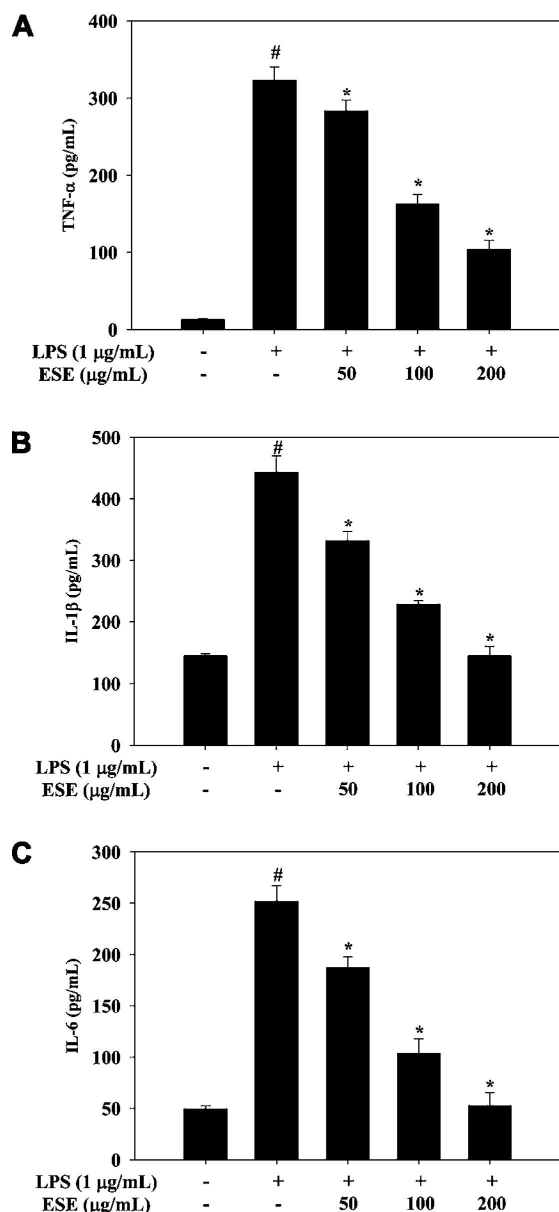

Figure 3. Effects of ESE on pro-inflammatory cytokine productions in RAW 264.7 cells. Cells pretreated with indicated concentrations of ESE for 1 h were stimulated without or with LPS (1 μg/mL) for 24 h. TNF-α (A), IL-1β (B), and IL-6 (C) in the culture media were measured by ELISA. Data are the means ± SDs of three independent experiments. $^{\#}P < 0.05$ indicates significant differences as compared to the nontreated control group. $^{*}P < 0.05$ indicates significant differences as compared to the LPS-only group.

ESE on LPS-induced activation of Akt. ESE markedly inhibited phosphorylation of Akt in LPS-stimulated cells (Figure 5A). These results collectively suggest that phosphorylations of p38 MAPK, JNK, ERK, and Akt are cooperatively involved in the inhibitory effects of ESE on LPS-induced NF-κB activation in LPS-stimulated RAW 264.7 cells.

To further confirm the association of these signaling molecules with the ESE's anti-inflammatory effect, we compared NO secretion in the presence of various kinase inhibitors: LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor). As shown in Figure 5B, secretion of NO was significantly decreased by specific kinase inhibitors as well as by ESE in LPS-treated RAW 264.7 cells ($P < 0.05$). These data suggest that ESE's regulatory effect on NF-κB pathway is mediated by the inhibition of the phosphorylation of MAPKs and Akt proteins in response to LPS.

Identification of Anti-inflammatory Compounds in ESE. The representative chromatogram of ESE is shown in Figure 6. Identification of the phlorotannins in ESE was based on the retention time of the standard phlorotannins isolated from *E. stolonifera*.[16,21] Four components, including eckol, phlorofucofuroeckol A, dieckol, and phlorofucofuroeckol B, were identified (Figure 6). The yields of each compound from 1 kg of *E. stolonifera* are as follows: 66.7 mg of dieckol (0.067%), 200 mg of eckol (0.020%), 83.3 mg of phlorofucofuroeckol A (0.008%), and 33.3 mg of phlorofucofuroeckol B (0.003%). As shown in Table 1, all phlorotannins showed strong ROS scavenging activities against LPS-stimulated RAW 264.7 cells, and dieckol and phlorofucofuroeckol A showed higher ROS scavenging activity than eckol and phlorofucofuroeckol B. In addition, EC_{50} (50% effective concentration) values of phlorofucofuroeckol A and B for the inhibition of NO production in LPS-stimulated cells were determined to be 6.95 ± 0.35 and 12.03 ± 0.79 μM, respectively, indicating that both compounds could be major anti-inflammatory components in ESE.

DISCUSSION

Various studies have focused on natural compounds or extracts since they are beneficial to human health because of their easy availability and low side effects or toxicity. Numerous phytochemicals from terrestrial plants have shown an anti-inflammatory effect, but only a few studies have been reported on the molecular mechanisms of anti-inflammatory action of extracts from marine brown algae, such as *Ishige okamura* and *Ecklonia cava*.[22,23] *I. okamura* ethanolic extract exerted an anti-inflammatory effect on LPS-treated RAW 264.7 cells via inactivation of NF-κB transcription factor; however, the regulation of protein kinases responsible for the activation of NF-κB was not demonstrated.[22] Additionally, *E. cava* ethanolic extract showed anti-inflammatory activity by suppressing the induction of inflammatory cytokines via blocking NF-κB and MAPK activation in LPS-treated microglial cells.[23] In this study, we found that ESE effectively inhibits the production of NO, PGE_2, TNF-α, IL-1β, and IL-6 through inhibition of the NF-κB pathway via an inactivation of MAPKs and Akt in RAW 264.7 cells. Moreover, we found that phlorofucofuroeckol A and B are active compounds, which are responsible, at least in part, for ESE's anti-inflammatory activity. The inhibitory effects of ESE on the expression of inflammatory mediators suggest a mechanism responsible for its anti-inflammatory action and its potential to be a therapeutic agent or nutraceutical for inflammatory diseases.

Excessive inflammatory mediators (NO and PGE_2) and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) produced by activated macrophages induce inflammatory process and act synergistically with other inflammatory mediators.[7,8,24] Natural compounds able to reduce inflammatory mediators may be attractive as anti-inflammatory agents, and for this reason, the inhibitory effects of natural compounds on NO or PGE_2 production have been intensively studied to develop therapeutic agents for treating inflammatory diseases.[25,26] Also, excessive production of pro-inflammatory cytokines plays a critical role in acute inflammatory response as well as chronic inflammatory diseases, including atherosclerosis, liver

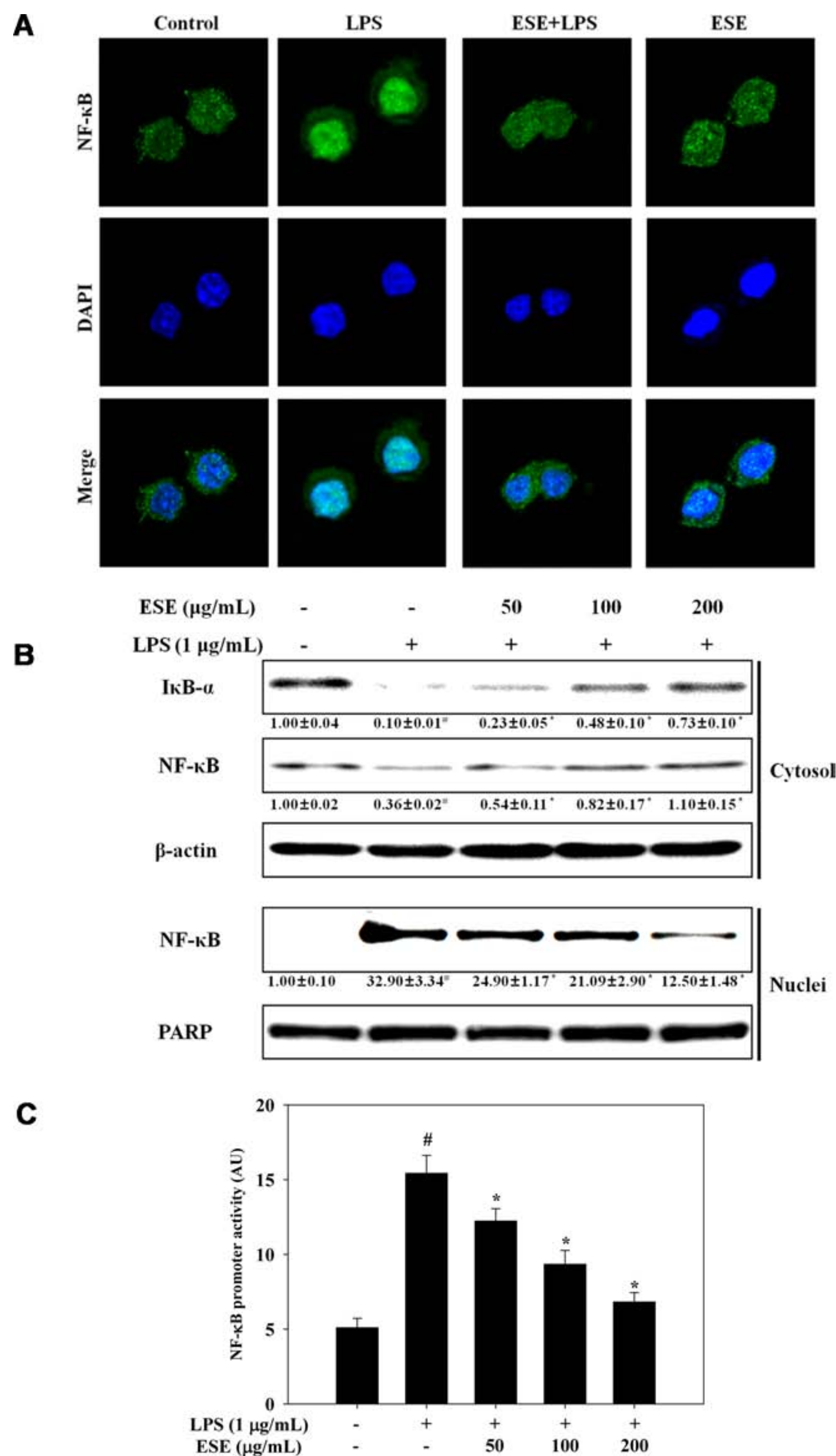

Figure 4. Effect of ESE on activation of NF-κB in RAW 264.7 cells. (A) Cells pretreated with ESE (100 μg/mL) for 1 h were stimulated with 1 μg/mL of LPS for 1 h. Cells were stained by anti-NF-κB/p65 antibody and DAPI, and then prepared for confocal microscopy analysis. The results shown (×100) are representative of those obtained from three independent experiments. (B) Cells pretreated with indicated concentrations of ESE for 1 h were stimulated without or with 1 μg/mL of LPS for 30 min. The phosphorylation of IκB-α and nuclear translocation of NF-κB were determined by a Western blot analysis. The results are representative of those obtained from three independent experiments. (C) Cells were cotransfected with 1 μg of NF-κB promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Transfected cells were pretreated with indicated concentrations of ESE for 1 h and then stimulated without or with LPS (1 μg/mL) for 6 h. Data are the means ± SDs of three independent experiments. $^{\#}P < 0.05$ indicates significant differences as compared to the nontreated control group. $^{*}P < 0.05$ indicates significant differences as compared to the LPS-only group.

disease, and inflammatory arthritis.[5,25,27] Recent studies have shown that natural compounds from marine organisms are effective in reducing inflammation by the suppression of iNOS and COX-2 at the transcriptional level.[28−30] Our results in the

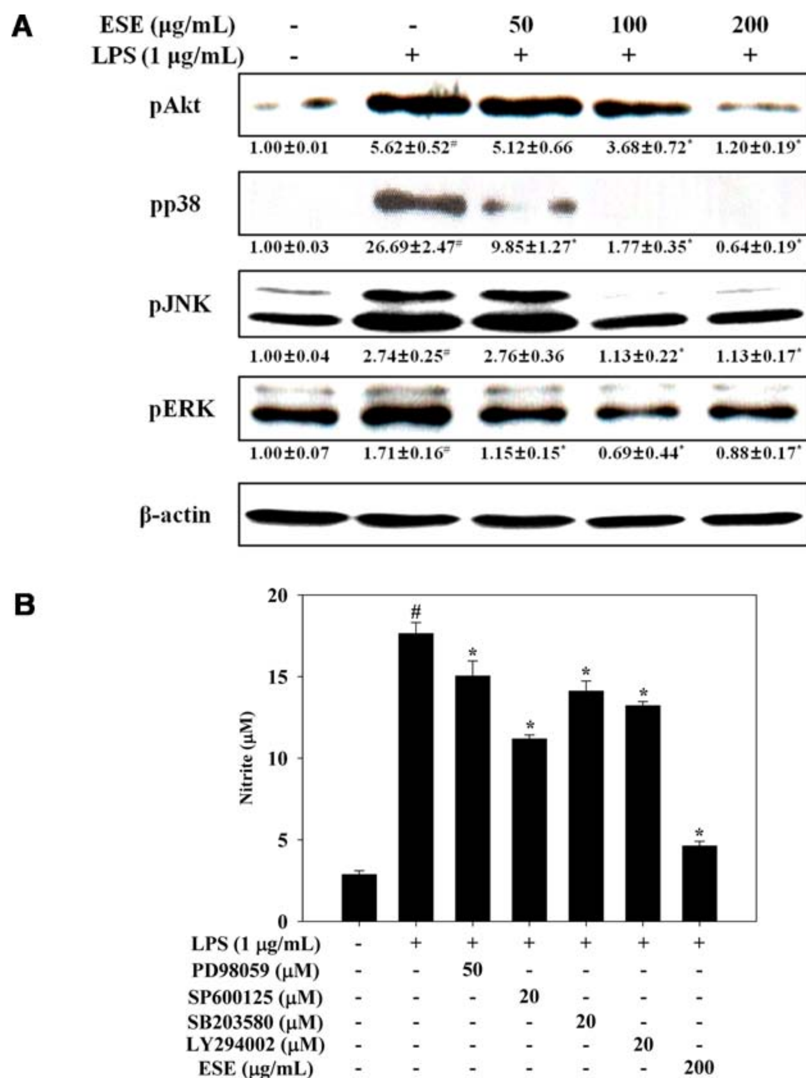

Figure 5. Effects of ESE on phosphorylation of Akt and MAPKs in RAW 264.7 cells. (A) Cells were incubated with various concentrations of ESE for 1 h and then stimulated without or with LPS (1 μg/mL) for 30 min. Whole cell lysates were prepared and analyzed by Western blotting for measuring the phosphorylation of Akt and MAPKs using corresponding antibodies. The results presented are representative of three independent experiments. (B) Cells were pretreated with PI3K/Akt pathway inhibitor (LY294002), ERK inhibitor (PD98059), JNK inhibitor (SP600125), or p38 inhibitor (SB203580) for 1 h and then treated with LPS (1 μg/mL) for another 24 h. The culture media were used to measure the amount of nitrite to evaluate NO production. Data are the means ± SDs of three independent experiments. $^{\#}P < 0.05$ indicates significant differences as compared to the nontreated control group. $^{*}P < 0.05$ indicates significant differences as compared to the LPS-only group.

present study suggest that ESE-mediated inhibition of NO and PGE_2 production in LPS-stimulated cells was associated with down-regulation of iNOS and COX-2 genes at the transcriptional level, which seems to be the first addressing transcriptional inhibition of iNOS and COX-2 by ESE.

NF-κB is a transcription factor involved in the transcriptional regulation of iNOS and COX-2 genes.[7,8] NF-κB is bound with an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. The phosphorylation IκB-α by LPS causes proteolytic degradation and leading translocation of NF-κB into the nucleus.[10] Although biochemical actions of ESE on NF-κB activation remain unknown, we observed that down-regulation of IκB-α in cytosol by LPS was recovered by ESE treatment, suggesting that ESE protected the proteolytic degradation of IκB-α. Degradation of IκB-α involves its dissociation from the inactive complex, leading to activation of NF-κB in response to LPS, which is demonstrated by NF-κB promoter activity (Figure 4C). Moreover, the immunofluorescence experiment revealed that nuclear translocation of NF-κB was remarkably inhibited by ESE, supporting the inhibition of NF-κB activation by ESE. From these data, it is likely that the ESE-mediated down-regulation of the LPS-induced iNOS, COX-2, TNF-α, IL-1β, and IL-6 expression in RAW 264.7 cells is mainly associated with the ability of ESE to inhibit NF-κB pathway.

NF-κB activation is also mediated by various cellular kinases including MAPKs and Akt, which are groups of signaling molecules that also appear to play key roles in inflammatory reactions.[7,11,31] MAPKs have been involved in pro-inflammatory signaling cascades, and evidence demonstrated that activation of JNK, p38 MAPK, and ERK is involved in up-regulation of iNOS and COX-2 through the activation of NF-κB in LPS-stimulated immune cells.[3,24,32] Therefore, anti-inflammatory mechanisms are closely related with inhibition of the phosphorylation of MAPKs in activated RAW 264.7 cells. We found that phosphorylation of ERK, p38 MAPK, and JNK by LPS was inhibited by ESE treatment (Figure 5). An

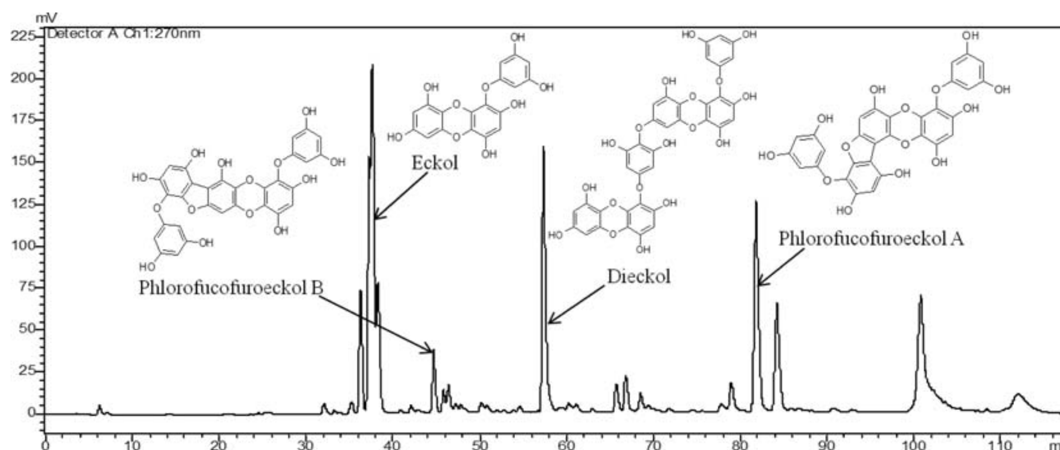

Figure 6. Representative HPLC chromatogram of ESE and chemical structures of phlorotannins. Aliquots of ESE were separated by HPLC system, and each peak was identified by comparison of retention time to the corresponding authentic standard. The chemical structures of four isolated phlorotannins are shown in the inlet.

Table 1. NO and ROS Suppressive Activities of Phlorotannins of *E. stolonifera*

phlorotannins	EC_{50}[b] (μM) ROS[a]	EC_{50}[b] (μM) NO[c]
eckol	26.1 ± 0.97	>200
phlorofucofuroeckol B	17.6 ± 1.06	12.0 ± 0.79
dieckol	4.80 ± 0.38	>200
phlorofucofuroeckol A	3.32 ± 0.10	6.95 ± 0.35

[a]The intracellular ROS level generated by LPS treatment (1 μg/mL) in RAW 264.7 cells was measured by using DCFH-DA. [b]EC_{50}, the half maximal effective concentration. [c]The NO level induced by LPS treatment (1 μg/mL) in RAW 264.7 macrophages was analyzed by Greiss assay.

interesting finding of this study is that ESE inhibited Akt phosphorylation, a downstream regulator of PI3K, in response to LPS signal in RAW 264.7 cells. A role of the PI3K/Akt pathway in NF-κB activation is largely involved in redox-sensitive mechanisms, which indicates a link between the ROS and the PI3K/Akt pathway in regulations of inflammatory genes.[30,33] Regarding an antioxidant component of ESE (Table 1), it is speculated that the antioxidant compounds in ESE may be related to inhibition of Akt activation. Thus, it is likely that inhibition of MAPKs and Akt phosphorylation by ESE may contribute to the ESE-mediated inhibition of NF-κB pathway in LPS-stimulated macrophages.

Various biological activities of phlorotannins have been reported; particularly, their anti-inflammatory actions are partly associated with their antioxidant activities.[16,34] The antioxidant activity of phlorotannins is associated with phenol rings, which act as electron traps to scavenge free radicals.[35] Specific mechanisms of phlorotannins on the anti-inflammatory actions are not clearly defined. Considering cellular signaling of polyphenols, phlorotannins may not merely exert their effects as free radical scavengers but may also modulate inflammatory signaling proteins, including NF-κB and AP-1 transcription factors.[36] Recent studies demonstrated that phlorofucofuroeckol A and 6,6′-bieckol isolated from brown algae suppress inflammatory response to LPS by inhibiting NF-κB pathway with its intrinsic antioxidant activity.[30,34] In this study, we identified four components of phlorotannins, and this is the first report addressing the identification of phlorofucofuroeckol B from *E. stolonifera*. Although phlorofucofuroeckol A and B showed remarkable inhibitory effects on NO production, other unidentified compounds in addition to these two phlorotannins may also contribute to ESE's anti-inflammatory activity.

In conclusion, we have demonstrated that ESE inhibits the production of inflammatory mediators and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Moreover, the inhibitory effects of ESE are found to be associated with an inactivation of NF-κB pathway via a blockade of MAPK and Akt phosphorylation. Verification and confirmation of ESE's anti-inflammation activity and relative mechanism in cellular and molecular levels will be beneficial to the further application of ESE in therapeutic agent for inflammatory diseases.

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Notes

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■ ABBREVIATIONS

Akt, protein kinase B; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; DAPI, 4,6′-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ESE, ethanol extracts of *Ecklonia stolonifera*; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated kinase 1/2; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; iNOS, inducible nitric oxide synthase; IκB-α, inhibitor κB-α; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NF-κB, nuclear factor-κB; NO, nitric oxide; PARP, poly-ADP-ribose polymerase; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinases; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive

oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween 20; TNF, tumor necrosis factor

REFERENCES

(1) Xie, Q. W.; Whisnant, R.; Nathan, C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med.* **1993**, *177*, 1779−1784.

(2) Vane, J. R.; Mitchell, J. A.; Appleton, I.; Tomlinson, A.; Bishop-Bailey, D.; Croxtall, J.; Willoughby, D. A. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2046−2050.

(3) Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **1992**, *6*, 3051−3064.

(4) Maskrey, B. H.; Megson, I. L.; Whitfield, P. D.; Rossi, A. G. Mechanisms of resolution of inflammation: A focus on cardiovascular disease. *Arterioscler., Thromb., Vasc. Biol.* **2011**, *31*, 1001−1006.

(5) Packard, R. R.; Libby, P. Inflammation in atherosclerosis: From vascular biology to biomarker discovery and risk prediction. *Clin. Chem.* **2008**, *54*, 24−38.

(6) Kolyada, A. Y.; Savikovsky, N.; Madias, N. E. Transcriptional regulation of the human iNOS gene in vascular-smooth-muscle cells and macrophages: evidence for tissue specificity. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 600−605.

(7) D'Acquisto, F.; Iuvone, T.; Rombola, L.; Sautebin, L.; Di Rosa, M.; Carnuccio, R. Involvement of NF-κB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett.* **1997**, *418*, 175−178.

(8) Marks-Konczalik, J.; Chu, S. C.; Moss, J. Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor κB-binding sites. *J. Biol. Chem.* **1998**, *273*, 22201−22208.

(9) Makarov, S. S. NF-κB in rheumatoid arthritis: A pivotal regulator of inflammation, hyperplasia, and tissue destruction. *Arthritis Res.* **2001**, *3*, 200−2006.

(10) Chen, Z.; Hagler, J.; Palombella, V. J.; Melandri, F.; Scherer, D.; Ballard, D.; Maniatis, T. Signal-induced site-specific phosphorylation targets IκB-α to the ubiquitin-proteasome pathway. *Genes Dev.* **1995**, *9*, 1586−1597.

(11) Bhat, N. R.; Zhang, P.; Lee, J. C.; Hogan, E. L. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures. *J. Neurosci.* **1998**, *18*, 1633−1641.

(12) Adhikary, G.; Sun, Y.; Pearlman, E. C-Jun NH_2 terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation. *J. Leukocyte Biol.* **2008**, *83*, 991−997.

(13) Cremer, T. J.; Shah, P.; Cormet-Boyaka, E.; Valvano, M. A.; Butchar, J. P.; Tridandapani, S. Akt-Mediated proinflammatory response of mononuclear phagocytes infected with *Burkholderia cenocepacia* occurs by a novel GSK3β-dependent, IκB kinase-independent mechanism. *J. Immunol.* **2011**, *187*, 635−643.

(14) Sheu, M. L.; Chao, K. F.; Sung, Y. J.; Lin, W. W.; Lin-Shiau, S. Y.; Liu, S. H. Activation of phosphoinositide 3-kinase in response to inflammation and nitric oxide leads to the up-regulation of cyclooxygenase-2 expression and subsequent cell proliferation in mesangial cells. *Cell. Signal.* **2005**, *17*, 975−984.

(15) Tseng, C. K. Chinese seaweeds in herbal medicine. *Hydrobiologia* **1984**, *116/117*, 152−154.

(16) Kim, A. R.; Shin, T. S.; Lee, M. S.; Park, J. Y.; Park, K. E.; Yoon, N. Y.; Kim, J. S.; Choi, J. S.; Jang, B. C.; Byun, D. S.; Park, N. K.; Kim, H. R. Isolation and identification of phlorotannins from *Ecklonia stolonifera* with antioxidant and anti-inflammatory properties. *J. Agric. Food Chem.* **2009**, *57*, 3483−3489.

(17) Kang, H. S.; Chung, H. Y.; Kim, J. Y.; Son, B. W.; Jung, H. A.; Choi, J. S. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. *Arch. Pharm. Res.* **2004**, *27*, 194−198.

(18) Moon, H. E.; Islam, N.; Ahn, B. R.; Chowdhury, S. S.; Sohn, H. S.; Jung, H. A.; Choi, J. S. Protein tyrosine phosphatase 1B and α-glucosidase inhibitory phlorotannins from edible brown algae, *Ecklonia stolonifera* and *Eisenia bicyclis*. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 1472−1480.

(19) Yoon, N. Y.; Kim, H. R.; Chung, H. Y.; Choi, J. S. Anti-hyperlipidemic effect of an edible brown algae, *Ecklonia stolonifera*, and its constituents on poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. *Arch. Pharm. Res.* **2008**, *31*, 1564−1571.

(20) Joe, M. J.; Kim, S. N.; Choi, H. Y.; Shin, W. S.; Park, G. M.; Kang, D. W.; Kim, Y. K. The inhibitory effects of eckol and dieckol from *Ecklonia stolonifera* on the expression of matrix metalloproteinase-1 in human dermal fibroblasts. *Biol. Pharm. Bull.* **2006**, *29*, 1735−1739.

(21) Lee, M. S.; Shin, T. S.; Utsuki, T.; Choi, J. S.; Byun, D. S.; Kim, H. R. Isolation and Identification of phlorotannins from *Ecklonia stolonifera* with antioxidant and hepatoprotective properties in tacrine-treated HepG2 cells. *J. Agric. Food Chem.* **2012**, *60*, 5340−5349.

(22) Kim, M. M.; Rajapakse, N.; Kim, S. K. Anti-inflammatory effect of *Ishige okamurae* ethanolic extract via inhibition of NF-κB transcription factor in RAW 264.7 cells. *Phytother. Res.* **2009**, *23*, 628−634.

(23) Jung, W. K.; Ahn, Y. W.; Lee, S. H.; Choi, Y. H.; Kim, S. K.; Yea, S. S.; Choi, I.; Park, S. G.; Seo, S. K.; Lee, S. W.; Choi, I. W. *Ecklonia cava* ethanolic extracts inhibit lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglia via the MAP kinase and NF-κB pathways. *Food Chem. Toxicol.* **2009**, *47*, 410−417.

(24) Guha, M.; Mackman, N. LPS induction of gene expression in human monocytes. *Cell. Signal.* **2001**, *13*, 85−94.

(25) Chao, W. W.; Kuo, Y. H.; Lin, B. F. Anti-inflammatory activity of new compounds from *Andrographis paniculata* by NF-κB transactivation inhibition. *J. Agric. Food Chem.* **2010**, *58*, 2505−2512.

(26) Hwang, P. A.; Chien, S. Y.; Chan, Y. L.; Lu, M. K.; Wu, C. H.; Kong, Z. L.; Wu, C. J. Inhibition of lipopolysaccharide (LPS)-induced inflammatory responses by *Sargassum hemiphyllum* sulfated polysaccharide extract in RAW 264.7 macrophage cells. *J. Agric. Food Chem.* **2011**, *59*, 2062−2068.

(27) Cheng, C. F.; Pan, T. M. Protective effect of *Monascus*-fermented red mold rice against alcoholic liver disease by attenuating oxidative stress and inflammatory response. *J. Agric. Food Chem.* **2011**, *59*, 9950−9957.

(28) Abad, M. J.; Bedoya, L. M.; Bermejo, P. Natural marine anti-inflammatory products. *Mini Rev. Med. Chem.* **2008**, *8*, 740−754.

(29) Jung, W. K.; Heo, S. J.; Jeon, Y. J.; Lee, C. M.; Park, Y. M.; Byun, H. G.; Choi, Y. H.; Park, S. G.; Choi, I. W. Inhibitory effects and molecular mechanism of dieckol isolated from marine brown alga on COX-2 and iNOS in microglial cells. *J. Agric. Food Chem.* **2009**, *57*, 4439−4446.

(30) Kim, A. R.; Lee, M. S.; Shin, T. S.; Hua, H.; Jang, B. C.; Choi, J. S.; Byun, D. S.; Utsuki, T.; Ingram, D.; Kim, H. R. Phlorofucofuroeckol A inhibits the LPS-stimulated iNOS and COX-2 expressions in macrophages via inhibition of NF-κB, Akt, and p38 MAP. *Toxicol In Vitro* **2011**, *25*, 1789−1795.

(31) Madrid, L. V.; Mayo, M. W.; Reuther, J. Y.; Baldwin, A. S., Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-κB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38. *J. Biol. Chem.* **2001**, *276*, 18934−18940.

(32) Li, Q.; Verma, I. M. NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2002**, *2*, 725−734.

(33) Kao, S. J.; Lei, H. C.; Kuo, C. T.; Chang, M. S.; Chen, B. C.; Chang, Y. C.; Chiu, W. T.; Lin, C. H. Lipoteichoic acid induces nuclear factor-κB activation and nitric oxide synthase expression via phosphatidylinositol 3-kinase, Akt, and p38 MAPK in RAW 264.7 macrophages. *Immunology* **2005**, *115*, 366−374.

(34) Yang, Y. I.; Shin, H. C.; Kim, S. H.; Park, W. Y.; Lee, K. T.; Choi, J. H. 6,6′-Bieckol, isolated from marine alga *Ecklonia cava*, suppressed LPS-induced nitric oxide and PGE_2 production and inflammatory cytokine expression in macrophages: The inhibition of NF-κB. *Int. Immunopharmacol.* **2012**, *12*, 510−517.
(35) Wang, T.; Jonsdottir, R.; Olafsdottir, G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem.* **2009**, *116*, 240−248.
(36) Rahman, I.; Biswas, S. K.; Kirkham, P. A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem. Pharmacol.* **2006**, *72*, 1439−1452.