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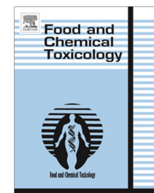

Anti-inflammatory activity of edible brown alga *Eisenia bicyclis* and its constituents fucosterol and phlorotannins in LPS-stimulated RAW264.7 macrophages

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ABSTRACT

Although individual phlorotannins contained in the edible brown algae have been reported to possess strong anti-inflammatory activity, the responsible components of *Eisenia bicyclis* have yet to be fully studied. Thus, we evaluated their anti-inflammatory activity via inhibition against production of lipopolysaccharide (LPS)-induced nitric oxide (NO) and *tert*-butylhydroperoxide (*t*-BHP)-induced reactive oxygen species (ROS), along with suppression against expression of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), in RAW 264.7 cells. The anti-inflammatory activity potential of the methanolic extract and its fractions of *E. bicyclis* was in the order of dichloromethane > methanol > ethyl acetate > *n*-butanol. The strong anti-inflammatory dichloromethane fraction was further purified to yield fucosterol. From the ethyl acetate fraction, six known phlorotannins were isolated: phloroglucinol, eckol, dieckol, 7-phloroeckol, phlorofucofuroeckol A and dioxinodehydroeckol. We found that these compounds, at non-toxic concentrations, dose-dependently inhibited LPS-induced NO production. Fucosterol also inhibited *t*-BHP-induced ROS generation and suppressed the expression of iNOS and COX-2. These results indicate that *E. bicyclis* and its constituents exhibited anti-inflammatory activity which might attribute to inhibition of NO and ROS generation and suppression of the NF-κB pathway and can therefore be considered as a useful therapeutic and preventive approach to various inflammatory and oxidative stress-related diseases.

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1. Introduction

Eisenia bicyclis (Kjellman) Setchell is a perennial brown alga (Phaeophyta) which belongs to the Laminareaceae family and is distributed in the middle Pacific coast around Korea and Japan. It is consumed as a raw material for sodium alginate and phlorotannin-rich raw materials (Okada et al., 2004). Phlorotannins are secondary metabolites of phloroglucinol (1,3,5-trihydroxybenzene) that has been polymerized through ether, phenyl, or 1,4-dibenzodioxin linkages (Okada et al., 2004). In particular, these polyphenolic compounds are responsible for a variety of bioactivities, including anti-diabetic complication (Okada et al., 2004; Jung et al., 2008), antitumor (Noda et al., 1989), hepatoprotective (Kim et al., 2005), anti-plasmin inhibitory (Fukuyama et al., 1989), algicidal (Nagayama et al., 2003), tyrosinase inhibitory (Kang et al., 2004a), anti-inflammatory (Kim et al., 2009, 2011; Ryu et al., 2009), anti-skin aging (Kim et al., 2006; Joe et al., 2006), antioxidant (Kang et al., 2004b, 2003; Li et al., 2009), anti-cholinesterase (Yoon et al., 2008a; Myung et al., 2005), anti-Alzheimer (Jung et al., 2010), anti-hyperlipidemic (Yoon et al., 2008b), anti-diabetic (Iwai, 2008; Lee et al., 2010a,b, 2009), anti-allergic (Sugiura et al., 2006), and angiotensin converting enzyme-I inhibitory activities (Jung et al., 2006). Phlorotannins derived from the brown alga *E. bicyclis* have also been suggested to be associated with the inhibitory activities of hyaluronidase, phospholipase A_2, lipoxygenase, and COX enzymes, which are involved in the inflammatory response (Shibata et al., 2003). Furthermore, phlorofucofuroeckol B

Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride; COX-2, cyclooxygenase-2; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's Modified Eagle's Medium; DMSO-d_6, deuterated dimethylsulfoxide; FBS, fetal bovine serum; HRP, horseradish peroxidase; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; *t*-BHP, *tert*-butylhydroperoxide; TLC, thin layer chromatography; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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isolated from *E. arborea* suppresses histamine release from rat basophile leukemia cells (Sugiura et al., 2006), further supporting the anti-inflammatory activities of phlorotannins. Phlorotannins exert anti-inflammatory activity by inhibition of iNOS and COX-2 expression and suppress the activity of matrix metalloproteinase (MMPs) by activating the mitogen-activated protein kinases (MAPK) pathway (Kim et al., 2011; Ryu et al., 2009). Although individual phlorotannins contained in the edible brown algae *Ecklonia* sp. and *Eisenia* sp. have been reported to possess strong anti-inflammatory activity, the anti-inflammation activity of isolated components of *E. bicyclis* have yet to be scrutinized. In addition, phytochemical research on nonpolar dichloromethane (CH_2Cl_2) fraction is limited.

Therefore, the anti-inflammatory activity of the methanol (MeOH) extract and its organic solvent soluble fractions including those from CH_2Cl_2, ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and water (H_2O) layers from *E. bicyclis* and its active components were determined by *in vitro* inhibitory activities against lipopolysaccharide (LPS)-induced NO production, iNOS, COX-2 protein expression, and *tert*-butylhydroperoxide (*t*-BHP)-induced ROS generation in RAW 264.7 cells. Isolation and identification of the compounds from *E. bicyclis* yielded six known phlorotannins from the weak anti-inflammatory EtOAc fraction (phloroglucinol, eckol, dieckol, dioxyinodehydroeckol, phlorofucofuroeckol A, 7-phloroeckol) and a known sterol from the high anti-inflammatory CH_2Cl_2 fraction (fucosterol). Considering the results of these above experiments, we evaluated the prospect of *E. bicyclis* as a source of anti-inflammatory agents.

2. Materials and methods

2.1. General experimental procedures

Melting points were measured on a Mitamura-Riken apparatus and are uncorrected. All ^1H and ^{13}C NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide (DMSO-d_6). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm) and RP-18 F_{254s} plates (5 × 10 cm, Merck), using 50% H_2SO_4 as a spray reagent. All solvents for column chromatography were of reagent grade and acquired from commercial sources.

2.2. Chemicals

LPS from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *t*-BHP, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fetal bovine serum (FBS), and antibiotics were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), and Dulbecco's Modified Eagle's Medium (DMEM) was from Hyclone (Logan, Utah, USA). Various primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidenefluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billelica, MA, USA). Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All chemicals and solvents used were purchased from E. Merck, Fluka, and Sigma–Aldrich Co., unless stated otherwise.

2.3. Plant materials

The leafy thalli of *E. bicyclis* were purchased from a local telemarketing company in Gangwon Province, Korea (www.ulleumgdomall.com) in February 2009. The alga was authenticated by Emeritus professor C.H. Son of Pukyong National University. Voucher specimens (no. 20090218) have been deposited in the author's laboratory (J.S. Choi).

2.4. Extraction, fractionation, and isolation of E. bicyclis

The powdered leafy thallus of *E. bicyclis* (500 g) was refluxed with MeOH for 3 h (2 L × 3). The total filtrate was then concentrated to dryness *in vacuo* at 40 °C in order to render the MeOH extract (175.0 g). This extract was suspended in distilled H_2O and then successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH to yield the CH_2Cl_2 (16.8 g), EtOAc (51.5 g), and *n*-BuOH (39.6 g) fractions, respectively, as well as H_2O residue (62.5 g). The weak active EtOAc fraction (51.5 g) obtained from *E. bicyclis* was subjected to chromatography on a silica gel column, with EtOAc–MeOH (50:1–5:1) as the eluent, yielding 10 subfractions (EF01–EF10). Repeated column chromatography of EF01 (6.8 g) was conducted with a solvent mixture of *n*-hexane and EtOAc, yielding 11 subfractions (EF0101–EF0111). Compound **1** (35 mg) was purified from EF0104 (0.6 g) on an RP-18 column, eluted with aqueous

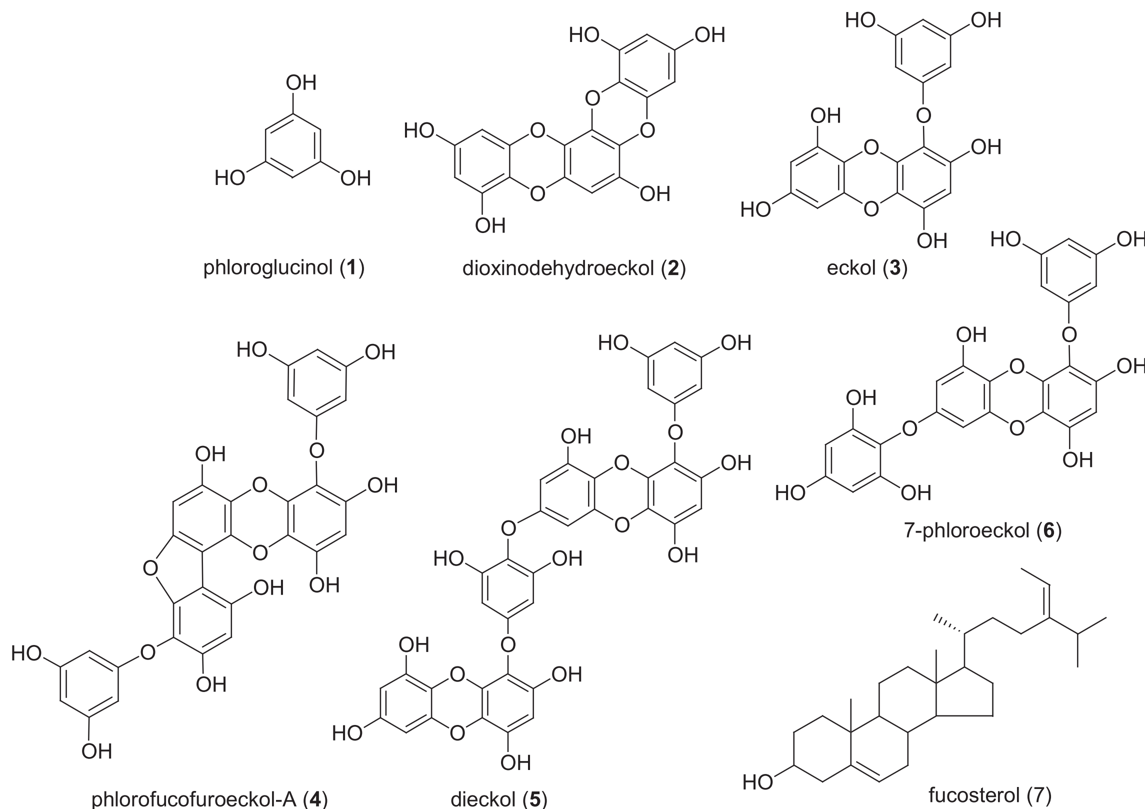

Fig. 1. Chemical structures of the phlorotannins and fucosterol isolated from *E. bicyclis*.

MeOH (20% MeOH to 100% MeOH, gradient elution). RP-18 column chromatography of EF0105 (3.8 g), using identical solvent conditions to EF0104, led to the isolation of **2** (19 mg) and **3** (160 mg). Compounds **4** (200 mg), **5** (300 mg), and **6** (8.5 mg) were purified from EF0106 (2.2 g) on RP-18 (20% MeOH to 100% MeOH, gradient elution) and Sephadex LH-20 columns (100% MeOH). Isolated compounds **1–6** were identified and characterized as phloroglucinol (Lee et al., 1996), dioxinodehydroeckol, eckol, phlorofucofuroeckol-A, dieckol (Kang et al., 2003), and 7-phloroeckol (Okada et al., 2004; Lee et al., 2009), respectively, by spectroscopic methods, as well as by comparisons with published data. The CH_2Cl_2 fraction (16.0 g) was subjected to chromatography over a Si gel column (8 × 80 cm), using a mixed solvent of *n*-hexane and EtOAc (*n*-hexane:EtOAc 20:1 → 0:1, gradient) to afford 9 subfractions (F01–F09). Fraction 7 (F07, 2.3 g) was repeatedly recrystallized from MeOH to yield **7** (1.23 g), which was identified and characterized as fucosterol (Sheu et al., 1997) by spectroscopic methods, including ^1H and ^{13}C NMR as well as by comparisons with published spectral data and TLC. The respective spectral data of isolates **1–7** are available upon request from the corresponding authors. The structures are shown in Fig. 1.

2.5. Cell culture

RAW 264.7 murine macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2-humidified air.

2.6. Assay for cell viability

Cell viability was assessed using the MTT assay as described previously (Mossman, 1983). In brief, RAW 264.7 cells were seeded into a 96-well plate at a density of 1.0×10^4 cells per well and incubated at 37 °C for 24 h. The cells were then treated with various concentrations of the samples. After an additional 24 h incubation at 37 °C, 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells, and the incubation continued for another 2 h. The resulting color was assayed at 540 nm using a microplate spectrophotometer (Molecular Devices, CA, USA).

2.7. Assay for inhibition of cellular NO production

The nitrite concentration in the medium was measured by Griess reagent as an indicator of NO production as previously described (Shin et al., 2008). Briefly, RAW 264.7 cells (1.0×10^5 cells/well in a 24-well plate with 500 μL of culture medium) were pretreated with samples for 2 h and incubated for 18 h with LPS (1.0 μg/mL). After incubation, the nitrite concentration of the supernatants (100 μL/well) was measured by adding 100 μL of Griess reagent. To quantify the nitrite concentration, standard nitrite solutions were prepared, and the absorbance of the mixtures was determined using a microplate spectrophotometer (Molecular Devices, CA, USA) at a wavelength of 540 nm. The iNOS inhibitor AMT was used as a positive control.

2.8. Analysis for inhibition of iNOS and COX-2 protein expression

To measure the protein levels of iNOS and COX-2, a Western blotting technique was used. RAW 264.7 cells were cultured in 100 mm culture dishes in the presence or absence of LPS (1.0 μg/mL) and with/without test samples for 18 h. Afterward, the cells were washed twice with ice-cold PBS and lysed with a buffer on ice for 30 min. Cell extracts were obtained by centrifugation at 14,000*g* at 4 °C for 20 min. Cytosolic proteins were electrophoretically separated on SDS–PAGE and transferred into PVDF membranes. The membranes were immediately blocked with 5% (w/v) non-fat dry milk in TBST buffer [Tris-buffered saline containing 0.1% Tween-20 (pH 7.4)] at room temperature for 1 h. The membranes were washed three times (10 min) in TBST buffer and incubated with primary antibody, diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST buffer at 4 °C overnight. After three washes in TBST buffer (10 min), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted 1:2000 in 5% (w/v) non-fat dry milk in TBST buffer at room temperature for 1 h. After another three washes in TBST buffer (10 min), the antibody labeling was visualized with a Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacture's instructions and exposed to X-ray film (GE Healthcare Ltd., UK). Pre-stained blue protein markers were used for molecular-weight determination.

2.9. Assay for inhibition of intracellular ROS generation

ROS generation was assessed using a ROS-sensitive fluorescence indicator DCFH-DA (Lebel and Bondy, 1990). To determine intracellular ROS scavenging activity, RAW 264.7 cells (2.0×10^4 cells/well) were seeded in black 96-well plates. After 24 h, the cells were treated with samples and *t*-BHP (200 μM) for 2 h to induce ROS generation. After the cells were incubated with DCFH-DA (20 μM) for 30 min, and the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (Bio-Tek Instruments Inc., FLx 800, Winooski, UT, USA).

2.10. Statistics

The data are presented as mean ± SD of more than three independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by Student's t test.

3. Results

3.1. Effect of the MeOH extract and its solvent-soluble fractions obtained from E. bicyclis on cell viability and NO production in LPS-induced RAW 264.7 cells

The cytotoxicity of the MeOH extract and its solvent soluble fractions obtained from *E. bicyclis* were measured by MTT assay. Although the data are not shown, the MeOH extract and its solvent soluble fractions of *E. bicyclis* exerted no cytotoxic effect at concentrations up to 100 μg/mL. Increased NO production is a typical phenomenon that occurs in LPS-stimulated macrophages and is used as an indicator of a typical inflammatory response. The inhibitory effect of the MeOH extract and its solvent-soluble fractions obtained from *E. bicyclis* on the production of NO in LPS-stimulated RAW 264.7 cells was investigated. The amount of nitrite, a stable metabolite of NO, was used as an indicator of NO production in the medium. As demonstrated in Fig. 2, cells stimulated with LPS (1.0 μg/mL) during an 18 h incubation exhibited increased accumulation of nitrite in the culture medium compared with control treatments in RAW 264.7 cells. This increase was significantly reduced by pretreatment with the MeOH extract and the CH_2Cl_2 soluble fraction of the *E. bicyclis* at a concentration of 50 μg/mL by 38.5% and 41.2%, respectively, as compared with that of the positive control, AMT (89.33% inhibition at 10 μM). However, the EtOAc, *n*-BuOH, and H_2O fractions did not inhibit NO production in the LPS-induced cells at the same concentration. As shown in Fig. 3, the EtOAc fraction exhibited NO inhibitory activity by 45.3% at a non-toxic concentration of 400 μg/mL.

3.2. Effect of the MeOH extract and its solvent-soluble fractions obtained from E. bicyclis on t-BHP-induced ROS generation in RAW 264.7 cells

t-BHP stimulates the redox status of cells resulting in generation of ROS such as alkoxy and hydroxyl radicals (Elliott et al.,

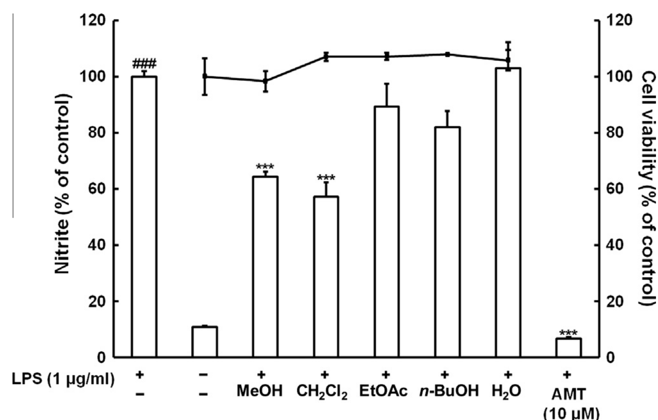

Fig. 2. Effect of the MeOH extract and its solvent soluble fractions of *E. bicyclis* on LPS-induced NO production and cell viability in RAW 264.7 cells. RAW 264.7 cells were pretreated with 50 μg/ml of the MeOH extract (A) and its solvent soluble fractions (CH_2Cl_2 (B), EtOAc (C), *n*-BuOH (D), and H_2O (E)) of *E. bicyclis* for 2 h followed by LPS (1.0 μg/ml) treatment. After 18 h of incubation, the amount of NO in the culture supernatants was measured by Griess reaction assay. Cell viability was measured by a MTT assay. The data represent the mean ± SD of triplicate experiments. $^{###}P < 0.001$ indicates significant differences from the unstimulated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences from the LPS-treated group.

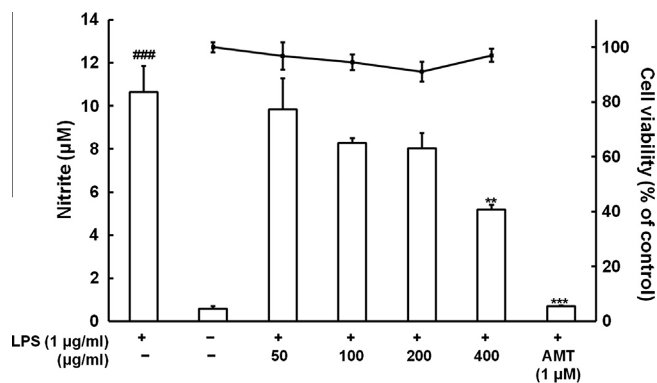

Fig. 3. Concentration-dependent inhibitory effect of the EtOAc-soluble fraction of *E. bicyclis* on LPS-induced NO production and cell viability in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of the EtOAc-soluble fraction of *E.bicyclis* for 2 h followed by LPS (1.0 μg/ml) treatment. After 18 h of incubation, the amount of NO in the culture supernatants was measured by Griess reaction assay. Cell viability was determined using the MTT method. The data represent the mean ± SD of triplicate experiments. $^{###}P < 0.001$ indicates significant differences from the unstimulated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences from the LPS-treated group.

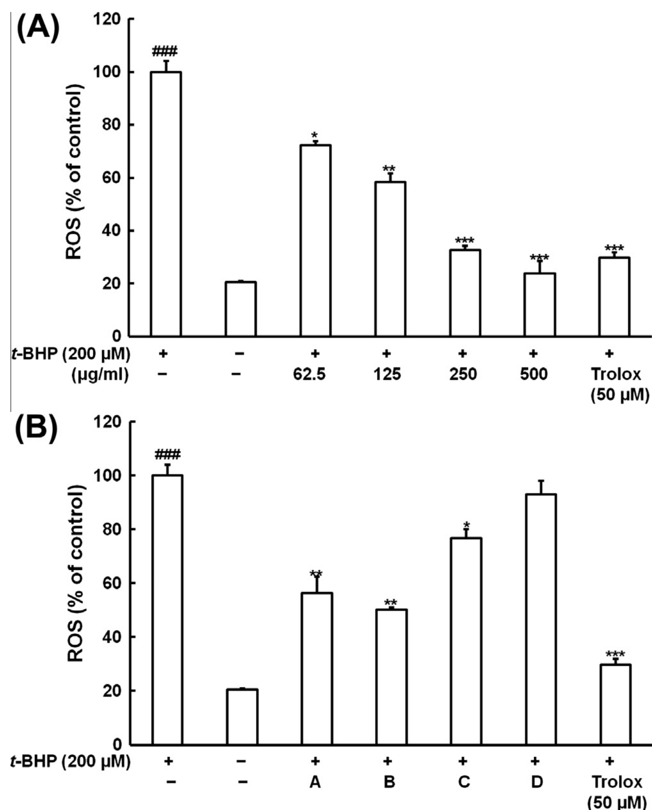

Fig. 4. Effects of the MeOH extract (A) and solvent-soluble fractions (B) of *E. bicyclis* on *t*-BHP-induced ROS generation in RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with the indicated concentrations of MeOH extract of *E. bicyclis* and *t*-BHP (200 μM) and incubated for 1 h. (B) RAW 264.7 cells were pretreated with the 100 μg/ml concentration of solvent-soluble fractions (CH_2Cl_2 (A), EtOAc (B), *n*-BuOH (C), and H_2O (D)) of *E. bicyclis* and *t*-BHP (200 μM) and incubated for 1 h. Control values were obtained in the absence of *t*-BHP and the MeOH extract of *E. bicyclis* and after addition of *t*-BHP (200 μM). The data represent the mean ± SD of triplicate experiments. $^{###}P < 0.001$ indicates significant differences from the unstimulated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences from the LPS-treated group.

1989). Therefore, in our study, cells were treated with *t*-BHP to induce oxidative stress. ROS generation was assessed using the ROS-sensitive fluorescence indicator DCFH-DA (Lebel and Bondy, 1990). When DCFH-DA is applied to viable cells, it penetrates into the cell membrane and is deacetylated by intracellular esterases to form non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF), which is readily detected by a fluorescent spectrophotometer. As shown in Fig. 4A, pretreatment of cells with different concentrations of the MeOH extract of *E. bicyclis* exerted good inhibitory activities (IC_{50} = 178.5 μg/mL) in a dose-dependent manner on *t*-BHP-induced ROS generation in RAW 264.7 cells. Among the several solvent-soluble fractions obtained from *E. bicyclis*, the EtOAc fraction exhibited a 48.6% higher *t*-BHP-induced ROS inhibitory activity compared to the other fractions by at 100 μg/mL concentration (Fig. 4B). The ROS generation inhibitory activity potential of the individual fractions at a concentration of 100 μg/mL was in the order of EtOAc > CH_2Cl_2 > *n*-BuOH > H_2O fractions with the inhibition % of 48.6 ± 2.58, 42.2 ± 1.66, 27.5 ± 1.78, and 5.2 ± 0.74, respectively.

3.3. Isolation of fucosterol from the CH_2Cl_2 fraction of E. bicyclis and its effect on cell viability and NO production in LPS-induced RAW 264.7 cells

The MeOH extract of *E. bicyclis* revealed strong anti-inflammatory activity and was thus subjected to solvent partitioning using CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O phases. Among the several solvent-soluble fractions, the CH_2Cl_2 fraction exhibiting strong anti-inflammatory activity was further purified by repeated column chromatography to yield the known compound fucosterol as an active constituent in a substantial quantity of 1.23 g. This compound was characterized and identified by spectroscopic methods, including ^{1}H and ^{13}C NMR, as well as through comparison with published spectral data and TLC analysis. As shown in Fig. 5A, fucosterol produced significantly ($^{**}P < 0.01$) greater cytotoxicity at concentrations higher than 50 μM. However, fucosterol did not significantly affect cell viability at concentrations up to 20 μM. Therefore, fucosterol was treated at a range of respective non-cytotoxic concentrations in subsequent experiments. As shown in Fig. 5B, fucosterol exhibited significant dose-dependent inhibitory activities on LPS-induced NO production in RAW 264.7 cells at concentrations of 5–20 μM.

3.4. Effect of fucosterol isolated from the CH_2Cl_2 fraction of E. bicyclis on t-BHP-induced ROS generation in RAW 264.7 cells

The effects of fucosterol on *t*-BHP-induced ROS generation in RAW 264.7 cells are shown in Fig. 5C. During a 30 min incubation with *t*-BHP (200 μM), ROS generation in RAW 264.7 cells increased dramatically. Fig. 5C demonstrates the results of pretreatment with fucosterol (25, 50, 100, 200 and 400 μM) for 1 h. Both approaches exhibited dose-dependent inhibitory effects with IC_{50} values of 125 μM for *t*-BHP-induced ROS levels in RAW 264.7 cells.

3.5. Effect of fucosterol isolated from E. bicyclis on iNOS and COX-2 expression in RAW 264.7 cells

iNOS and COX-2 are well-characterized markers of NF-κB-responsive inflammation (Sharif et al., 2007) and are involved in the pathogenesis of many human diseases (Lee et al., 2003b, 2006). The effect of fucosterol on the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells was examined by Western blot analysis. As shown in Fig. 6, LPS treatment (1 μg/mL) induced a significant increase in iNOS and COX-2 protein expression.

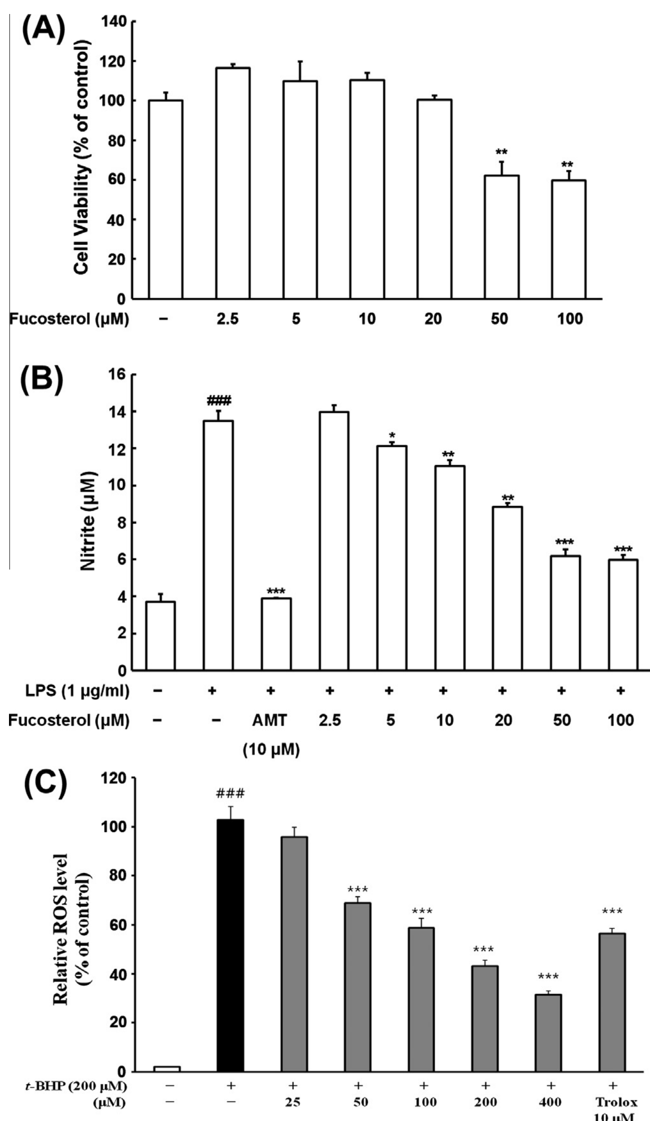

Fig. 5. Effects of fucosterol on cell viability (A), LPS-induced NO production (B), and *t*-BHP-induced ROS generation (C) in RAW 264.7 cells. (A) Cells were incubated for 24 h with fucosterol at indicated concentrations. The cell viability was measured by MTT assay. (B) Cells were pretreated with the indicated concentrations of fucosterol for 2 h followed by treatment with LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by Griess reaction assay. (C) The cells were pre-treated with different concentrations (25, 50, 100, 200 and 400 μM) of fucosterol and *t*-BHP (200 μM) and incubated for 1 h. The control values were obtained in the absence of *t*-BHP (200 μM) and fucosterol and after the addition of *t*-BHP (200 μM). Data are expressed as the mean ± SD of three independent experiments. $^{###}P < 0.001$ indicates significant differences from the unstimulated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences from the LPS-treated group and the *t*-BHP-treated group.

Treatment with fucosterol at 5–20 μM suppressed iNOS and COX-2 proteins in a dose-dependent manners.

3.6. Effect of phlorotannins isolated from the EtOAc fraction of E. bicyclis on cell viability and NO production in LPS-induced RAW 264.7 cells

The effect of individual phlorotannins contained within the EtOAc fraction of *E. bicyclis* on cell viability was measured by MTT assay (Fig. 7A–F). Among them, phlorofucofuroeckol A (Fig. 7D) and dioxyinodehydroeckol (Fig. 7E) exhibited cytotoxicity at concentrations greater than 10 μg/mL, whereas eckol (Fig. 7B), dieckol (Fig. 7C), and 7-phloroeckol (Fig. 7F) did not significantly affect cell viability at concentrations up to 50 μg/mL. Meanwhile, phloroglucinol (Fig. 7A) did not significantly affect cell viability at concentrations up to 100 μg/mL. Therefore, phlorotannins isolated from *E. bicyclis* were treated at a range of respective non-cytotoxic concentrations in subsequent experiments. Among the phlorotannins isolated from the weak anti-inflammatory EtOAc fraction, it was demonstrated that phloroglucinol and phlorofucofuoeckol A showed anti-inflammatory activity (Kim et al., 2009, 2011). As expected, phloroglucinol and phlorofucofuroeckol A revealed good inhibitory activities of cellular NO production in LPS-stimulated RAW 264.7 cells by 24.5% and 66.2% at 10 μg/mL, respectively (Fig. 7A and D); these results matched well with those of a previous study. On the other hand, eckol, dieckol and 7-phloroeckol exhibited dose-dependent inhibitory activities on LPS-induced NO production in RAW 264.7 cells, with IC_{50} values of 52.86, 51.42 and 26.87 μg/mL, respectively (Fig. 7B, C, and F). Nevertheless, dioxyinodehydroeckol showed weak inhibition in the cellular NO production at 10 μg/mL non-cytotoxic concentration (Fig. 7E). As a positive control, AMT at a concentration of 10 μM exhibited significant inhibitory effects, inhibiting NO production by 98.36%.

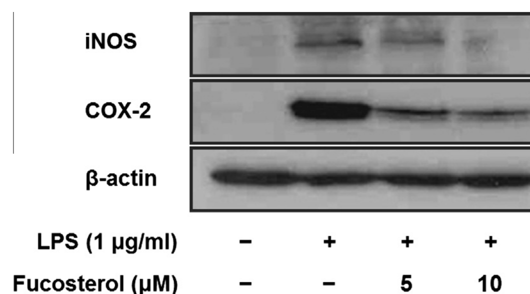

Fig. 6. Effects of fucosterol on iNOS and COX-2 expression. Cells were pretreated with the indicated concentrations of the compounds for 2 h and LPS (1.0 μg/mL) for 18 h. Cytosolic lysates were separated by SDS–PAGE. iNOS, COX-2, and β-actin were detected by Western blot analysis.

4. Discussion

Brown algae are very popular foods as sea vegetables, and many people also ingest them as a health food in China, Japan and South Korea (Nisizawa et al., 1987). Recent studies have focused on the biological and pharmacological activities of marine brown algae and have shown them to be potentially prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents (Ali, 2010). Several varieties of bioactive novel compounds such as phlorotannins, diterpenes, polysaccharides, phytosterols, and phytopigments from brown algae have been isolated, and many of these compounds have been demonstrated to possess numerous biological activities including antioxidant, cytotoxic (Guardia et al., 1999), hepatoprotective (Kim et al., 2005), antiviral (Barbosa et al., 2004), antifungal (Perry et al., 1991), and antidiabetic (Lee et al., 2004). However, the possibility of developing functional foods as neutraceuticals and pharmaceuticals is difficult since the activity of algal extract/fractions themselves sometimes do not correlate to the activity of ingredients. Indeed, the magnitude of the activity of the extract/fractions might be significantly different from the activity of the individual compounds contained therein. Although individual phlorotannins isolated from the EtOAc fraction of the MeOH extract of the edible brown alga *E. bicyclis* have been previously reported to possess strong anti-inflammatory activity, the EtOAc fraction shows poor anti-inflammatory activity compared to the CH_2Cl_2 fraction derived from the MeOH extract. Thus, it is likely that the phlorotannins contained in *E. bicyclis* are not the

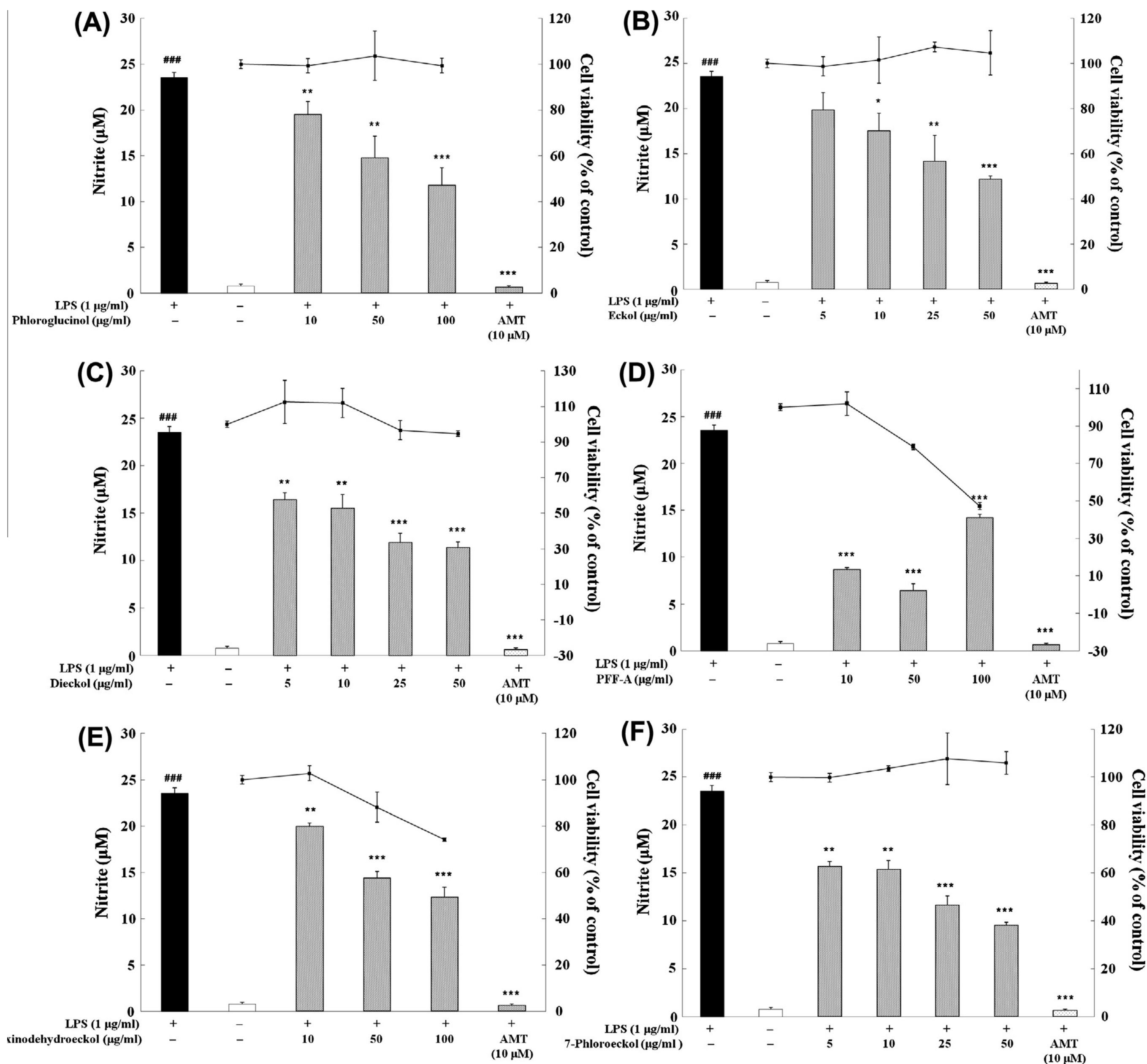

Fig. 7. Effects of compounds isolated from *E. bicyclis* on LPS-induced NO production and cell viability in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of (A) phloroglucinol, (B) eckol, (C) dieckol, (D) phlorofucofuroeckol-A, (E) dioxinodehydroeckol, (F) 7-phloroeckol for 2 h followed by treatment with LPS (1.0 μg/mL). After 18 h of incubation, the amount of NO in the culture supernatants was measured by Griess reaction assay, as described in the materials and methods. Cell viability was determined using the MTT method. The data represent the mean ± SD of triplicate experiments. $^{###}P < 0.001$ indicates significant differences from the unstimulated control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences from the LPS-treated group.

main anti-inflammatory principles; hence, a systematic activity-guided procedure is required to isolate the major anti-inflammatory components from the extract. Therefore, the present study was undertaken to isolate components from *E. bicyclis* by fractionation and determine their anti-inflammatory activity.

Reactive oxygen species (ROS) play an important role in the pathogenesis of inflammatory disease (Conner and Grisham, 1996) and are involved in inflammatory gene expression by mediating redox-based activation of the nuclear factor-kappa B (NF-κB) and cyclooxygenase-2 (COX-2) signaling pathways (Kabe et al., 2005). Nitric oxide (NO), which is synthesized from L-arginine by nitric oxide synthases (NOSs), exerts its inflammatory effects by stimulating enzyme mediators such as COX-2 and inducible nitric oxide synthase (iNOS) (MacMicking et al., 1997). Therefore, pharmacological inhibitors that regulate reactive oxygen species production and inflammatory gene expression can be used as therapeutic drugs for inflammatory diseases.

Since the MeOH extract of *E. bicyclis* exerted good inhibition of cellular NO production in LPS-stimulated RAW 264.7 cells, bioactivity-guided fractionation of the MeOH extract was performed to obtain the CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O fractions. Among several solvent-soluble fractions obtained from *E. bicyclis*, the CH_2Cl_2 fraction showed higher cellular NO production inhibitory activity than the EtOAc fraction, which contains phlorotannins (Fig. 2). The EtOAc fraction exhibited a 45.3% NO production inhibitory activity at 400 μg/mL, which was a non-toxic concentration and higher than that of the CH_2Cl_2 fraction. However, *n*-BuOH, and H_2O fractions did not inhibit NO production in the LPS-induced cells at the tested concentrations. The comparative inhibitory effects of several fractions derived from the MeOH extract of

E. bicyclis were explained by their compositional and content differences. Non-polar compounds such as fatty acids, pigments, and sterols, as well as polyphenols such as phlorotannins, were expected to be present in the CH_2Cl_2 and EtOAc fractions in large quantities, respectively.

In the present study, six known phlorotannins (phloroglucinol, eckol, dieckol, dioxinodehydroeckol, phlorofucofuroeckol A, and 7-phloroeckol) were isolated from the lower anti-inflammatory EtOAc fraction, and a known sterol (fucosterol) was isolated from the higher anti-inflammatory CH_2Cl_2 fraction and characterized on the basis of spectral analysis. Among them, phloroglucinol, eckol, dieckol, phlorofucofuroeckol A are known to possess anti-inflammatory activity (Kim et al., 2009, 2011). In the present study, phloroglucinol, phlorofucofuroeckol A, eckol, dieckol, and 7-phloroeckol exhibited dose-dependent inhibitory activities on LPS-induced NO production at a range of non-cytotoxic concentrations in RAW 264.7 cells (Fig. 7D). Phlorofucofuroeckol A contained within the EtOAc fraction showed potent inhibitory activities of cellular NO production (Fig. 7D), similar to those reported by Kim et al. (2009, 2011). It has been reported that anti-inflammatory effects of phlorofucofuroeckol A are associated with reduced iNOS and COX-2 protein expression in LPS-stimulated macrophages via inhibition of NF-κB, Akt, and p38 MAPK (Kim et al., 2011). We report here, for the first time, that 7-phloroeckol has an inhibitory activity toward NO production in LPS-stimulated RAW 264.7 cells (Fig. 7F).

Sterols are an important family of lipids present in the majority of eukaryotic cells. Because of different routes of synthesis, sterols from plants, fungi, and animals exhibit marked differences. Brown seaweed lipids are rich in highly unsaturated fatty acids such as α-linolenic acid (18:3, n-3), stearidonic acid (18:4, n-3), arachidonic acid (20:4, n-6), and eicosapentaenoic acid (20:5, n-3) as well as sterols, tocopherols, and pigments (carotenoids) (Terasaki et al., 2009). It has been reported that fucosterol is the primary sterol found in brown algae (Ganchevakamenarska et al., 2003), while fucoxanthin is the dominant carotenoid (Dembitsky and Maoka, 2007). Indeed, fucosterol contents are reported to range between 82.9 ± 1.8% and 97.3 ± 0.6% in brown seaweed (Sánchez-Machado et al., 2004). Although fucosterol is the most abundant sterol found in seaweeds, investigations into the involvement of anti-inflammatory activity are limited, but only recently has the maritime sterol, fucosterol been emerged as anti-inflammatory agent (Yoo et al., 2012). Apart from phlorotannins, we demonstrated that fucosterol exhibited significant dose-dependent inhibitory activities on LPS-induced NO production in RAW 264.7 cells at concentrations of 5–20 μM. Among known inflammatory mediators, iNOS and COX-2 proteins are responsible for increased levels of NO. Thus, we demonstrated the suppression of both iNOS and COX-2 by fucosterol in the present study. Similar to previous works (Yoo et al., 2012), fucosterol inhibited both NO production and ROS generation through suppression of both iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Although there has been much research on the cholinesterase inhibitory (Ahmed et al., 2006; Yoon et al., 2008a), antioxidant (Lee et al., 2003a), anti-diabetic (Lee et al., 2004), anti-osteoporotic (Bang et al., 2011), and antifungal (Kumar et al., 2010) effects of fucosterol, it was not until recently that there has been a growing interest in anti-inflammatory activity and the action mechanism of fucosterol. In particular, Yoo et al. (2012) revealed that significant anti-inflammatory activity of fucosterol might attribute to the suppression of the NF-κB and p38 MAPK pathways.

Recent anti-inflammatory studies on spinasterol, a kind of fucosterol, have shown that it suppresses LPS-induced expression of pro-inflammatory enzymes and inflammatory mediators in BV2 microglia through the extracellular signal-regulated kinase (ERK) pathway-dependent expression of heme oxygenase-1 (Jeong et al., 2010). Plant sterols, including β-sitosterol, campesterol, and stigmasterol have been reported to possess anti-inflammatory activity by inhibition of NF-κB transcription (Valerio et al., 2011; Chao et al., 2010). Similar to plant sterols, fucosterol also showed anti-inflammatory activity, indicating that this activity might be due in part to similarity in sterol nucleus and aliphatic side chain of these compounds. It has also recently been reported that fucoxanthin, which was present in the CH_2Cl_2 soluble fraction from *E. bicyclis*, exhibits strong anti-inflammatory activity (Shiratori et al., 2005; Heo et al., 2010; Kim et al., 2010; Kim et al., 2011). In the present work, the inhibitory effects of *E. bicyclis* – derived fucosterol against NO production and protein abundance of iNOS and COX-2 in RAW 264.7 cells have been elucidated in LPS-stimulated RAW 264.7 cells. Therefore, fucosterol, together with fucoxanthin, appears to be the primary anti-inflammatory component of the CH_2Cl_2 soluble fraction of *E. bicyclis* and may be useful as potential anti-inflammatory agents.

5. Conclusions

In summary, the MeOH extract and its CH_2Cl_2 and EtOAc fractions, as well as its constituents fucosterol and phlorotannins, contained in *E. bicyclis* possess anti-inflammatory activity sufficient to inhibit LPS-induced NO production, protein levels of iNOS and COX-2, and *t*-BHP-induced ROS generation in RAW 264.7 murine macrophage cells. Thus, these components, either separately or in combination, may function as potent preventive and therapeutic candidates for various inflammatory diseases and oxidative stress-related disease. Further *in vivo* and cellular-based studies will be necessary to help clarify the detailed mechanism and bioavailability of fucosterol and phlorotannins in target organs.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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