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Isolation and Identification of Phlorotannins from *Ecklonia stolonifera* with Antioxidant and Anti-inflammatory Properties

A-Reum Kim,[†] Tai-Sun Shin,[‡] Min-Sup Lee,[†] Ji-Young Park,[†] Kyoung-Eun Park,[†] Na-Young Yoon,[†] Jong-Soon Kim,[†] Jae-Sue Choi,[†] Byeong-Churl Jang,[§] Dae-Seok Byun,[†] Nam-Kyu Park,[†] and Hyeung-Rak Kim*,[†]

[†]Division of Food Science and Biotechnology, Pukyong National University, Busan 608-737, [‡]Department of Food Science and Nutrition, Chunnam National University, Yosu 550-749, and [§]Chronic Disease Research Center, Keimyung University School of Medicine, 194 Dongsan-dong, Jung-gu, Daegu 700-712, South Korea

Bioactivity-guided fractionation of *Ecklonia stolonifera* was used to determine the chemical identity of bioactive constituents, with potent antioxidant activities. The structures of the phlorotannins were determined on the basis of spectroscopic analysis, including NMR and mass spectrometry analysis. The antioxidant activities of the isolated compounds were evaluated by free radical scavenging activities in both in vitro and cellular systems. The anti-inflammatory effects of the isolated compounds were evaluated by determining their inhibitory effects on the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophage cells. The results indicated that phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol showed potential radical scavenging activities against 2,2-diphenyl-1-picrylhydrazyl. Among them, phlorofucofuroeckol A and dieckol significantly suppressed the intracellular reactive oxygen species level assayed by 2′, 7′-dichlorofluorescein diacetate assay in LPS-induced RAW 264.7 cells. Phlorofucofuroeckol A significantly inhibited the LPS-induced production of NO and PGE_2 through the down-regulation of inducible nitric oxide synthase and cyclooxygenase 2 protein expressions. In conclusion, these results suggest that phlorofucofuroeckol A has a potential for functional foods with antioxidant and anti-inflammatory activities.

KEYWORDS: *Ecklonia stolonifera*; antioxidant; anti-inflammatory; phlorotannin; lipopolysaccharide; RAW 264.7 cells

INTRODUCTION

Oxidative damage is thought to be one of the causes of chronic diseases such as cancer, heart disease, and inflammation. The relationship between inflammation and oxidative damage has generated interest in the benefits of antioxidant supplements in health and disease. Antioxidants are found in vegetables, fruits, and cereals, and they can be taken in the form of dietary supplements. Among dietary antioxidants, polyphenols are the most abundant ones and exert antioxidant properties through various mechanisms of action including the scavenging of free radicals and the inhibition of the generation of reactive species during the course of normal cell metabolism, thereby preventing damage to lipids, proteins, and nucleic acids and eventually cell damage and death (*1*). Recent studies showed that polyphenols are promising compounds that may help to control oxidative stress and consequently inflammatory response (*2*).

Recently, there has been remarkable increments in scientific knowledge dealing with the beneficial role of polyphenols during oxidative stress. Even though many studies have been performed on polyphenolic antioxidants derived from terrestrial plants, only very limited information has been available for such compounds from marine plants. Brown algae are very popular foods, and many people ingest them as a health food in Korea and Japan. *Ecklonia stolonifera* Okamura, the representative brown algae of *Ecklonia* species, is a member of the family of Laminariaceae, belonging to the order Laminariales as a perennial brown algae (*3*). Marine algal polyphenols, phlorotannins, which are only known in brown algae, are restricted to polymers of phloroglucinol (1,3,5-trihydroxybenzene) (*4*). Phlorotannins have been reported to have several biological activities, such as antioxidation (*5*−*7*), antidiabetic complications (*8*), antiamnesia (*9*), and chemoprevention against several vascular diseases (*10*). A few anti-inflammatory activities of phlorotannins have also been reported. Phlorotannins derived from the brown algae *Eisenia bicyclis* have been suggested to be associated with the inhibition of several enzymes such as hyaluronidase, phospholipase A2, lipoxygenase, and cyclooxygenases, which are involved in the inflammatory response (*11*). Furthermore, phlorofucofuroeckol B isolated from *Eisenia arborea* suppressed

*To whom correspondence should be addressed. Tel: +82-51-629-5847. Fax: +82-51-629-5842. E-mail: hrkim@pknu.ac.kr.

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histamine release from rat basophile leukemia cells (*12*), suggesting anti-inflammatory activities of phlorotannins. However, the anti-inflammatory effect of phlorotannins from marine plants on the suppression of inflammatory protein [inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2)] expression is still unknown.

In the present study, three phlorotannins showing high antioxidant activity were isolated from *E. stolonifera* through gel, silica, and reverse-phase column chromatographies based on bioactivity-guided fractionation. Among them, only phlorofucofuroeckol A showed high anti-inflammatory activity in the lipopolysaccharide (LPS)-induced RAW 264.7 cells, and the potential mechanism was also suggested by inhibiting the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) through suppression of iNOS and COX-2 protein expression.

MATERIALS AND METHODS

Plant Material. *E. stolonifera* was collected along the coast of Busan, Korea, in August 2007. The samples were rinsed using tap water to remove salt. Samples were air-dried under shade for 2 weeks and ground with a hammer grinder, and the dried powder was stored at room temperature until used.

Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and LPS were purchased from Sigma Chemical Co. (St. Louis, MO). CellTiter96 AQueous One Solution Cell Proliferation Assay Kit was obtained from Promega (Madison, WI). Mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD). Cell culture medium and all of the other materials required for cell culture were purchased from Gibco BRL Life Technologies (United States). All solvents were of high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA). Polyclonal antibodies against iNOS and COX-2 and horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein standard marker was purchased from Amersham Bioscience (Piscataway, NJ), and the enhanced chemiluminescence (ECL) detection kit was purchased from PerkinElmer Life Science (Waltham, MA).

Extraction and Isolation of Phlorotannins. The dried powder (4 kg) of *E. stolonifera* was refluxed with MeOH (3 × 9 L) for 3 h. The extract (1.1 kg) was suspended in water (1 L) and partitioned with dichloromethane, ethyl acetate (EtOAc), *n*-butanol, and solvents in sequence, yielding the dichloromethane (114.8 g), EtOAc (314.7 g), *n*-butanol (141.5 g), and water (528.2 g) fractions. The EtOAc fraction, which exhibited the most potent antioxidant activity on DPPH radical scavenging activity, was dissolved in dichloromethane and applied to a silica gel (70−230 mesh, Merck) column (100 cm × 10 cm) and eluted with a stepwise mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 1.2 L). The eluates were pooled into 17 fractions based on silica gel thin-layer chromatography (TLC) (250 μm, silica gel GF Uniplate, Analtech, Inc., Newark, DE). The TLC plates were developed in a dichloromethane/methanol/water (65:35:10, v/v/v) solvent system. Fractions 7−9 showed high antioxidant activity on DPPH radical scavenging activities, were pooled, and were dried (25 g). The dried sample was dissolved in dichloromethane and applied on the second silica gel column (100 cm × 5 cm) to enhance the antioxidant activity. The column was eluted with a mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 0.5 L) and separated into nine subfractions (Fr.1−Fr.9).

Fraction 4 (5.80 g), which showed the highest antioxidant activity, was subjected to preparative size exclusion column of Asahipak GS-310 (500 mm × 20 mm, Showa Denko, Tokyo, Japan). An exclusion HPLC apparatus consisted of a pump (Shimadzu LC-6AD), a photodiode array detector (Shimadzu SPD-M20A), an online degasser (Shimadzu DUG-20A_3), an autosampler (SIL-20A), a fraction collector (Shimadzu FRC-10A), a system controller (CBM-20A), and a Shimadzu LCsolution (ver. 1.22sp). Fraction 4 was chromatographed on an Asahipak GS-310 column eluting with methanol at a flow rate of 5.0 mL/min and monitored at 245 nm. The fraction was separated into five fractions (GS1−GS5). The GS3 fraction (1.110 g) showing high antioxidant activity was chromatographed over Shim-pack PREP-ODS (5 μm, 100 Å, 250 mm × 20 mm, Shimadzu Co., Tokyo, Japan). A preparative ODS HPLC system was similar to the exclusion HPLC system except for a binary pump (Shimadzu LC-6AD) and a column oven (35 °C, Shimadzu CTO-20A). The separation of GS3 fraction was conducted using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution profile consisted of a linear gradient from 20 to 100% B solvent for 40 min and re-equilibration of the column with 20% B solvent for 10 min. The flow rate was 7.0 mL/min, and detection was performed at 245 nm. The fraction gave eight subfractions (GS3-ODS1−GS3-ODS8). GS3-ODS1 (67 mg), ODS-3 (150 mg), and ODS7 (144 mg) were purified by the same HPLC system with a Luna RP-18 column [Luna C18(2), 5 μm, 250 mm × 10 mm, Phenomenex] and with the same mobile phase systems at a flow rate of 3.0 mL/min. The isolated fractions such as GS-ODS1 (phlorofucofuroeckol A), GS-ODS3 (dieckol), and GS-ODS7 (dioxinodehydroeckol) were used in this study.

Spectrometry. ^{1}H and ^{13}C NMR spectra were determined on a JNM ECP-400 spectrometer (JEOL, Japan), using DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded using pulsed field gradients.

Structural Elucidation of Isolated Phlorotannins. *Phlorofucofuroeckol A*. $C_{30}H_{18}O_{14}$ (MW = 602). ^{1}H NMR (400 MHz, CD_3OD) δ: 6.63 (1H, s, H-7), 6.40 (1H, s, H-11), 6.26 (1H, s, H-2), 5.97 (2H, d, J = 2.1 Hz, H-2″, 6″), 5.94 (1H, t, J = 1.9 Hz, H-4′), 5.92 (1H, t, J = 2.0 Hz, H-4″), 5.88 (2H, d, J = 2.1 Hz, H-2′, 6′). ^{13}C NMR (100 MHz, CD_3OD) δ: 162.7 (C-1″), 162.6 (C-1′ 161.0 (C-3′, 5′), 161.0 (C-3″, 5″), 154.0 (C-7a), 152.5 (C-10), 152.0 (C-8a), 149.1 (C-3), 149.0 (C-12), 146.7 (C-6), 144.7 (C-1), 139.2 (C-4a), 136.2 (C-12c), 128.9 (C-5a), 125.9 (C-13a), 125.6 (C-4), 123.2 (C-9), 106.2 (C-12a), 106.1 (C-12b), 100.8 (C-11), 100.2 (C-2), 98.6 (C-4′), 98.5 (C-4″), 97.0 (C-7), 96.2 (C-2″, 6″), 96.2 (C-2′, 6′).

Dieckol. $C_{36}H_{22}O_{18}$ (MW = 742). ^{1}H NMR (400 MHz, CD_3OD) δ: 6.15 (1H, s, H-3″), 6.13 (1H, s, H-3), 6.09 (2H, s, H-2″, 6″), 6.06 (1H, d, J = 2.9 Hz, H-8), 6.05 (1H, d, J = 2.9 Hz, H-6″), 5.98 (1H, d, J = 2.8 Hz, H-6), 5.95 (1H, d, J = 2.8 Hz, H-6), 5.92 (3H, s, H-2′, 4′, 6′). ^{13}C NMR (100 MHz, CD_3OD) δ: 162.7 (C-1′), 161.0 (C-3′, 5′), 158.6 (C-1‴), 156.8 (C-7), 155.3 (C-7″), 153.2 (C-3‴, 5‴), 148.1 (C-2″), 148.01 (C-2), 147.9 (C-9″), 147.7 (C-9), 145.1 (C-5a″), 145.0 (C-5a), 144.2 (C-4″), 144.1 (C-4‴), 139.4 (C-10a), 139.3 (C-10a″), 127.3 (C-4‴), 127.0 (C-9a), 126.5 (C-1), 126.4 (C-1″), 125.7 (C-9a″), 125.5 (C-4a″), 125.4 (C-4a), 100.7 (C-8″), 100.6 (C-8), 100.3 (C-3), 100.2 (C-3″), 98.5 (C-4′), 97.0 (C-2‴, 6‴), 96.7 (C-6″), 96.6 (C-6′), 96.2 (C-2′, 6′).

Dioxinodehydroeckol. $C_{18}H_{10}O_9$ (MW = 370). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.77 (1-OH), 9.64 (9-OH), 9.60 (6-OH), 9.27 (3-OH), 9.26 (11-OH), 6.10 (1H, s, H-7), 6.04 (1H, d, J = 2.7 Hz, H-2), 6.01 (1H, d, J = 2.7 Hz, H-10), 5.84 (1H, d, J = 2.7 Hz, H-4), 5.82 (1H, d, J = 2.7 Hz, H-12). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 153.3 (C-3), 153.0 (C-11), 146.1 (C-1), 146.0 (C-9), 142.1 (C-4a), 141.7 (C-12a), 140.1 (C-6), 137.2 (C-7a), 131.6 (C-13b), 125.9 (C-5a), 122.7 (C-8a), 122.5 (C-13a), 122.3 (C-14a), 98.8 (C-2, 10), 97.6 (C-7), 93.9 (C-4, 12).

DPPH Radical Scavenging Assay. DPPH radical scavenging activities of fractions from *E. stolonifera* and isolated compounds were tested according to the modified method of Nanjo et al. (*13*). Twenty microliters of sample solution (or DMSO as control) was mixed with 40 μL of DPPH (100 μM) in a 96 well microtiter plate and incubated at room temperature for 30 min. The absorbance at 520 nm was measured with a microplate reader (Ultraspec 2100 Pro, Amersham Biosciences). EC_{50} values were determined as the mean effective concentration to exert half of the antioxidant and were calculated by three different concentrations of sample.

Cell Culture and Viability Assay. Murine macrophage RAW 264.7 (ATCC) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum,

penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. The cell viability was determined by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter96 AQueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's manual. Cells were inoculated at a density of 10^5 cells/well into 96 well plates and cultured at 37 °C for 24 h. The culture medium was replaced by 200 μL of serial dilutions (0−20 μM) of isolated compounds, and the cells were incubated for 24 h. The final concentration of DMSO was less than 0.1% in the cell culture medium. The culture medium was removed and replaced by 95 μL of fresh culture medium and 5 μL of MTS solution. After 3 h, the absorbance was measured using a microplate reader (Ultraspec 2100 pro, Amersham Biosciences) 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. DCFH converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. RAW 264.7 cells were incubated with 0−20 μM phlorotannins in the absence or presence of LPS (1 μg/mL) 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 Corporation, Sunnyvale, CA).

Measurement of NO and PGE_2. RAW 264.7 cells (5×10^5) were plated and incubated with 0−20 μM phlorotannins in the absence or presence of LPS (1 μg/mL) for 24 h. After treatment of LPS and phlorotannins, RAW 264.7 cell culture medium was saved for the measurement of nitrite and PGE_2. The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction (*14*). One hundred microliters of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Ultraspec 2100 pro) at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

PGE_2 was measured using the PGE_2 enzyme-linked immunosorbant assay kit (Amersham Biosciences). The concentration of PGE_2 was photometrically determined using a microplate reader at 405 nm.

Western Immunoblot. RAW 264.7 cells were washed twice with ice-cold PBS and lysed with a buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet-40, 1% Tween-20, 0.1% SDS, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] on ice for 1 h. After centrifugation at 18000*g* for 10 min, the protein content of supernatant was measured, and aliquots (20 μg) of protein 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 Tween-20 (TBST) buffer for 1 h and incubated for 2 h with primary antibody in TBST buffer containing 5% nonfat dried milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer containing 5% nonfat dried milk for 1 h, and an immune complex was detected using the ECL detection kit.

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 if $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Isolation and Structural Determination of Phlorotannins. The EtOAc-soluble fraction of the *E. stolonifera* led to the isolation of three phlorotannins (**Figure 1**). The structures of isolated three compounds were identified by comparison with published spectral data as phlorofucofuroeckol A (*15*), dieckol (*16*), and dioxinodehydroeckol (*3*). Recently, several phlorotannins were isolated from brown macroalgae (*5*). 6′,6′-Bieckol and diphlorethohydroxycarmalol isolated from *Ishige okamurae* showed high antioxidant activities (*5*), and fucodiphloroethol and phlorofucofuroeckol A isolated from *Ecklonia cava* had an antiallergic activity by reducing histamine release from human basophilic leukemia and rat basophilic leukemia cells (*17*) through the suppression of binding between IgE and Fcε RI receptors.

Cytotoxicity of Phlorotannins. The cytotoxicities of isolated phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol were measured using MTS assays on RAW 264.7 cells prior to testing of phlorotannins for their antioxidant and anti-inflammatory activities. As shown in **Figure 2**, cytotoxicities of the isolated phlorotannins were not shown in RAW 264.7 cells up to 25 μM. However, phlorofucofuroeckol A and dieckol showed cytotoxicities of approximately 15 and 20%, respectively, at a 50 μM concentration.

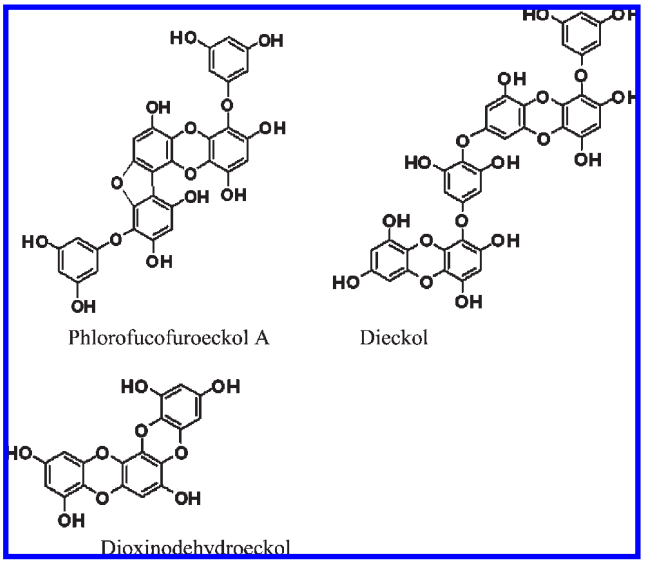

Figure 1. Structures of phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol isolated from *E. stolonifera*.

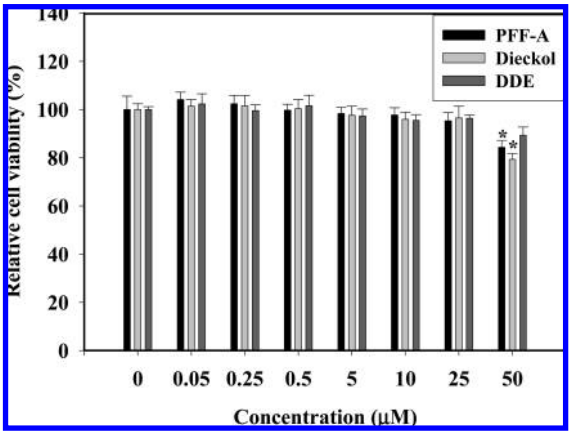

Figure 2. Cytotoxic effect of phlorotannins in RAW 264.7 cells. Cells were incubated for 24 h with phlorofucofuroeckol A (PFF-A), dieckol, and dioxinodehydroeckol (DDE) at indicated concentrations. The cell viability was measured by MTS assay. Values are the means ± SDs of three independent experiments. *$P < 0.05$ indicates significant differences from the control group.

However, 6′,6′-bieckol and diphlorethohydroxycarmalol isolated from *I. okamurae* (*5*) did not shown any cytotoxicities up to 100 μM on MRC-5, RAW 264.7, and HL-60 cells, which is different from our results. Dieckol and 6′,6′-bieckol have the same chemical composition and have different chemical structures. The difference in the cytocoxicities of those compounds is presumed to be a different structure.

Antioxidant Activity of Phlorotannins. The isolated phlorotannins from *E. stolonifera* were tested for their antioxidant activity by measuring their ability to scavenge free radicals with DPPH. The antioxidant capacity of compounds was compared with those shown by a known antioxidant, L-ascorbic acid, treated in the same assay. As shown in **Table 1**, the EC_{50} values for the phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol were estimated to be 4.7 ± 0.3, 6.2 ± 0.4, and 8.8 ± 0.4 μM, respectively, which is lower than that of L-ascorbic acid as a positive control. Although DPPH free radical scavenging activity is being used in the evaluation of the antioxidant activity of dietary polyphenols, in vitro data in cellular system are necessary to apply the results to the physiological environment.

The antioxidant activities of phlorotannins were determined in cellular systems to investigate whether phlorotannins can affect the free radical-mediated oxidation in cellular systems or not. For this purpose, LPS was used as a model compound for the generation of intracellular ROS. **Figure 3** shows the effect of phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol on ROS production in the LPS-induced RAW 264.7 cells. Treatment of cells with phlorofucofuroeckol A and dieckol inhibited the production ROS in a dose-dependent manner; however, dioxinodehydroeckol did not inhibit the production of ROS, although the compound had a higher DPPH radical scavenging activity, comparable to phlorofucofuroeckol A and dieckol. This result suggests that the intracellular antioxidant activities of phlorofucofuroeckol A and dieckol are due to direct scavenging of cellular ROS in LPS-induced RAW 264.7 cells.

ROS are produced in cells or tissues under physiological and pathological conditions. Excessive ROS play a key role in enhancing the inflammation through the activation of inflammatory proteins in various inflammatory diseases (*18*). Such undesired effects of oxidative stress induce oxidative damage in biomolecules causing atherosclerosis, hypertension, diabetes, and cancer (*19*). Compounds derived from botanical sources, such as phenolic compounds, have suppressed inflammatory protein and cytokine expression through the removal of ROS by their antioxidant activities (*20*). Recently, phlorotannins such as diphlorethohydroxycarmalol and 6′,6′-bieckol isolated from *I. okamurae* suppressed ROS production in RAW 264.7 cells in a time-dependent manner and protected cell membrane proteins from oxidation (*5*).

When DCFH-DA was treated in viable cells, it can be penetrated into cell and deacetylated by intracellular esterases to form 2′,7′-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell, and converted to 2′,7′-dichlorofluorescein (DCF), which is detected by a fluorescent spectrophotometer. We can presume that the nonsuppressive effect of dioxinodehydroeckol on ROS production is because it is hard to penetrate itself into RAW cells or hard to stimulate antioxidant system in RAW 264.7 cells. Although dioxinodehydroeckol had a high DPPH free radical scavenging activity, at this point, we cannot conclude why dioxinodehydroeckol fails to suppress ROS production in LPS-induced RAW 264.7 cells.

Table 1. DPPH Radical Scavenging Activity of Phlorotannins Isolated from *E. stolonifera*[a]

compounds	EC_{50} (μM)
phlorofucofuroeckol A	4.7 ± 0.3
dieckol	6.2 ± 0.4
dioxinodehydroeckol	8.8 ± 0.4
L-ascorbic acid	10.3 ± 0.5

[a] Values are the means ± SDs of three independent experiments.

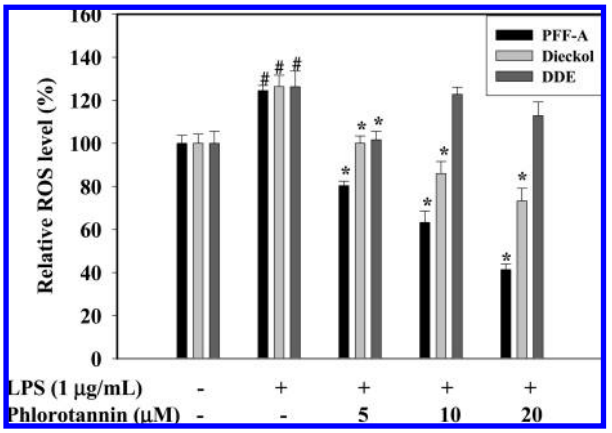

Figure 3. Effect of phlorotannins on the LPS-induced ROS production in RAW 264.7 cells. Cells were cotreated with different concentrations (5, 10, and 20 μM) of phlorofucofuroeckol A, dieckol, or dioxinodehydroeckol (DDE) and LPS (1 μg/mL) and incubated for 2 h. Control values were obtained in the absence of LPS and phlorotannins. $^{\#}P < 0.05$ indicates significant differences from the control group. $^{*}P < 0.05$ indicates significant differences from the LPS-treated group.

Effect of Phlorotannins on NO and PGE_2 Production. NO is synthesized from L-arginine by nitric oxide synthases (NOSs). Under pathological conditions, a significant increase of NO synthesized by the inducible NOS (iNOS) participates in provoking inflammatory process and acts synergistically with other inflammatory mediators (*21*). Also, the iNOS is strongly stimulated upon exposure to bacterial endotoxin and pro-inflammatory cytokines (*22*). Compounds able to reduce NO production by iNOS may be attractive as anti-inflammatory agents, and for this reason, the effects of polyphenols on iNOS activity have been intensively studied to develop anti-inflammatory drugs (*20*). **Figure 4** shows the effect of phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol on nitrite production in the LPS-induced RAW 264.7 cells. NO production, measured as nitrite, was increased up to 47.6 ± 2.6 μM by treatment of 1 μg/mL LPS in RAW 264.7 cells, as compared to a 11.2 ± 0.8 μM concentration of the control without LPS. Phlorofucofuroeckol A was found to significantly reduce LPS-induced NO production in a dose-dependent manner at the concentration of 5−20 μM. However, dieckol and dioxinodehydroeckol did not suppress NO production in the LPS-induced RAW 264.7 cells, even though both compounds had high antioxidant activities. This result implied that phlorofucofuroeckol A had an inhibitory activity on iNOS or suppressive effect on the expression of iNOS protein in LPS-induced RAW 264.7 cells.

Cyclooxygenases regulate the conversion of arachidonic acid to prostaglandin E_2 (PGE_2) and are rate-limiting enzymes in the biosynthesis of prostaglandins (*23*). COX-1 is constitutively expressed in many tissues, while COX-2 is

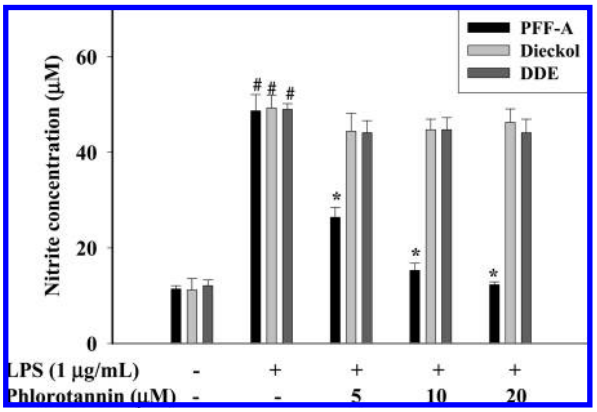

Figure 4. Effect of phlorotannins on the LPS-induced nitrite production in RAW 264.7 cells. Cells were cotreated with different concentrations (5, 10, and 20 μM) of phlorofucofuroeckol A, dieckol, or dioxinodehydroeckol (DDE) and LPS (1 μg/mL) and incubated for 24 h. Control values were obtained in the absence of LPS and phlorotannins and after addition of LPS (1 μg/mL). $^{\#}P < 0.05$ indicates significant differences from the control group. $^{*}P < 0.05$ indicates significant differences from the LPS-treated group.

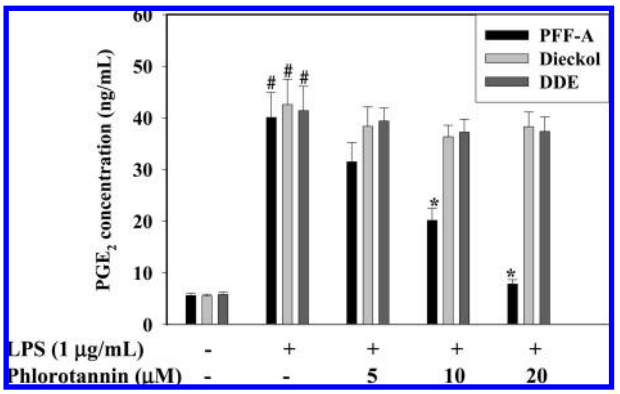

Figure 5. Effect of phlorotannins on the LPS-induced PGE_2 production in RAW 264.7 cells. Cells were cotreated with different concentrations (5, 10, and 20 μM) of phlorofucofuroeckol A, dieckol, or dioxinodehydroeckol (DDE) and LPS (1 μg/mL) and incubated for 24 h. Control values were obtained in the absence of LPS and phlorotannins. $^{\#}P < 0.05$ indicates significant differences from the control group. $^{*}P < 0.05$ indicates significant differences from the LPS-treated group.

known as an inducible enzyme that produces, in most cases, large amounts of prostaglandins. COX-2 is highly expressed in the inflammation-related cell types including macrophages and mast cells after stimulation by pro-inflammatory cytokines and/or lipopolysaccharide (*20*, *22*). The effect of phlorotannins on PGE_2 production in LPS-induced RAW 264.7 cells is shown in **Figure 5**. Phlorofucofuroeckol A efficiently inhibited the production of PGE_2 in the LPS-induced RAW 264.7 cells. However, dieckol and dioxinodehydroeckol did not reduce the PGE_2 production in the LPS-induced RAW 264.7 cells, which is a similar pattern to NO production (**Figure 4**).

An examination of the cell viability of isolated compounds in RAW 264.7 cells by MTS assay indicated that even a 25 μM concentration of the isolated compounds did not affect the viability of the RAW 264.7 cells (**Figure 2**). Thus, the inhibitory effect of phlorofucofuroeckol A on NO and PGE_2 production was not attributed to cytotoxic effects. In this study, we found that phlorofucofuroeckol A showed the great inhibition of NO and PGE_2 production in the LPS-induced RAW 264.7 cells.

Polyphenols from terrestrial plants are well-known to have anti-inflammatory activity by the suppression of NO and/or PGE_2 production (*20*). However, those from marine plants are not studied for their anti-inflammatory effect related to the suppression of NO and PGE_2 production to our best knowledge. Phlorofucofuroeckol A, dieckol, and 8′,8′-bieckol from brown algae *E. bicyclis* were reported to have inhibitory activities on hyaluronidase, which is known to be involved in allergic and inflammatory responses (*24*). Furthermore, phlorofucofuroeckol B isolated from brown algae *E. arborea* showed antiallergic activity by inhibition of histamine release from rat basophile leukemia cells (*12*). As an anti-inflammatory effect of phlorotannins, such as bieckol, dieckol, phlorofucofuroeckol A, and eckol, the phlorotannins showed higher inhibitory activities on soybean and human lipoxygenase than the well-known lipoxygenase inhibitors, resveratrol and epigallocatechin gallate (*11*). However, the literature regarding the anti-inflammatory activities of phlorotannins from brown algae were not published with respect to the production of NO and PGE_2 in the LPS-induced RAW 264.7 cells.

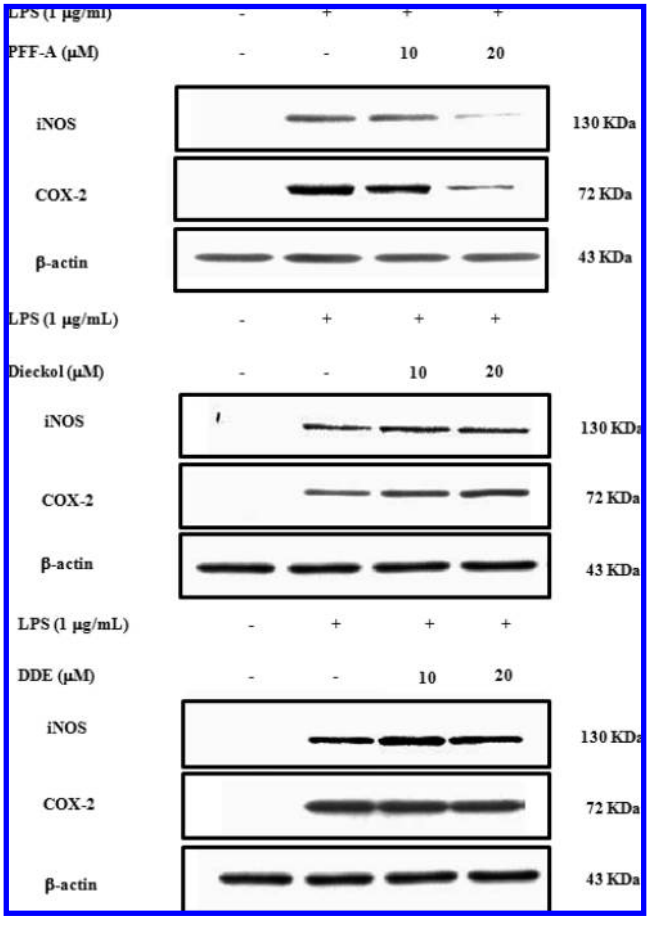

Figure 6. Effect of phlorotannins on LPS-induced iNOS and COX-2 proteins expression in RAW 264.7 cells. A representative immunoblot of three separate experiments is shown.

Effect of Phlorotannins on Expression of iNOS and COX-2 Proteins. The effect of phlorotannins on iNOS and COX-2 proteins expression in the LPS-induced RAW 264.7 cells was examined by Western blot analysis. **Figure 6** shows the effects of phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol on iNOS and COX-2 protein expression in the LPS-induced RAW 264.7 cells. LPS treatment (1 μg/mL) induced

a significant increase in iNOS and COX-2 protein expression, as compared to the negative control. Treatment with 10 and 20 μM phlorofucofuroeckol A suppressed the expression of iNOS and COX-2 proteins, and the result showed that the inhibitory effect of phlorofucofuroeckol A on iNOS and COX-2 protein expression was dose-dependent. However, dieckol and dioxinodehydroeckol did not influence the suppression of iNOS and COX-2 protein expression in the LPS-induced RAW 264.7 cells.

Enhanced expression of iNOS and COX-2 proteins is associated with an inflammatory response. iNOS and COX-2 play a pivotal role in immunity against infectious agents by producing an excess amount of NO and PGE_2, respectively; these enzymes have attracted attention for their detrimental roles in inflammation-related disease (*25*). Recent studies have shown that in vivo or in vitro treatments of polyphenol compounds, such as curcumin, resveratrol, artocarpesin, and sinapic acid, are effective in reducing inflammation and cancer (*26*−*29*), probably by their ROS scavenging ability. Of interest, it has been previously reported that phlorotannin-rich extracts of *E. cava*, which is similar to *E. stolonifera*, have antioxidative and anti-inflammatory effects by scavenging free radicals and reducing PGE_2 in the LPS-treated RAW 24.7 cells (*30*). To further extend it, in this study, we have demonstrated that phlorofucofuroeckol A itself, isolated from *E. stolonifera*, has a strong ability to inhibit production of NO and PGE_2 in the LPS-induced RAW 264.7 cells (**Figures 4** and **5**). Little is known about the relationship between phlorofucofuroeckol A and expression of iNOS and COX-2 proteins, the enzymes responsible for the production of NO and PGE_2, respectively. Importantly, the present findings that treatment with phlorofucofuroeckol A results in strong down-regulation of iNOS and COX-2 protein expressions in the LPS-stimulated RAW 264.7 cells suggest that the phlorofucofuroeckol A-mediated reduction of NO and PGE_2 in the LPS-stimulated RAW 264.7 cells is associated with the ability of phlorofucofuroeckol A to inhibit both iNOS and COX-2 protein expression.

In conclusion, three phlorotannins, such as phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol, were isolated from the brown macroalgae *E. stolonifera* by a series of column chromatographies. The structures of the three isolated compounds were identified by comparison with published spectral data. The three compounds exhibited strong DPPH radical scavenging activity and phlorofucofuroeckol A and dieckol showed inhibition of intracellular ROS production. Only phlorofucofuroeckol A exhibited strong anti-inflammatory activity in the LPS-induced RAW 264.7 cells. An anti-inflammatory effect of phlorofucofuroeckol A resulted from the inhibition of NO and PGE_2 production through down-expression of iNOS and COX-2 proteins in LPS-induced RAW 264.7 cells through its antioxidant activity. The brown marine macroalgae *E. stolonifera* may provide a beneficial effect for inflammatory-mediated diseases and have potential for the development of functional foods for anti-inflammatory effects.

ABBREVIATIONS USED

COX-2, cyclooxygenase 2; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EtOAc, ethyl acetate; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MS, mass spectrometry; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NO, nitric oxide; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBST, Tris-buffered saline Tween-20; TLC, thin layer chromatography; TMS, tetramethylsilane.

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