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Antioxidant Effects of Phlorotannins Isolated from *Ishige okamurae* in Free Radical Mediated Oxidative Systems

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Three phlorotannins, including phloroglucinol, diphlorethohydroxycarmalol, and 6,6′-bieckol, were isolated from *Ishige okamurae* by column chromatography. The structures of the phlorotannins were determined on the basis of spectroscopic analysis, including NMR and mass spectrometry (MS) techniques. Antioxidant effects of phlorotannins were measured by direct free radical scavenging activities using the electron spin resonance spectrometry (ESR) technique and cellular systems in vitro. The results indicated that diphlorethohydroxycarmalol and 6,6′-bieckol showed potential radical scavenging activities against the 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, alkyl, and superoxide radicals. Moreover, no cytotoxicities of the phlorotannins on human fetal lung fibroblasts cell line (MRC-5), mouse macrophages cell line (RAW264.7), and human leukemic cell line (HL-60) were observed. In addition, diphlorethohydroxycarmalol and 6,6′-bieckol significantly reduced the intracellular reactive oxygen species level assessed by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay in RAW264.7 cells, and myeloperoxide (MPO) activity in HL-60 cells and radical-mediated oxidation of cell membrane proteins in RAW264.7 cells were dose-dependently inhibited in the presence of diphlorethohydroxycarmalol and 6,6′-bieckol. In conclusion, these results suggested that phlorotannins could be used as novel functional foodstuffs or antioxidants in the cosmetic and drug industries.

KEYWORDS: Antioxidant activities; *Ishige okamurae*; phlorotannins; free radicals; electron spin resonance (ESR)

INTRODUCTION

Reactive oxygen species (ROS), a class of highly reactive molecules, includes free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl (HO•), peroxyl (ROO•), alkoxyl (RO•), and nitric oxide, and non-free-radicals such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (*1*). Although ROS play an important role in the origin of life and the evolution of biology, they are damaging to organisms when overproduced because they can attack lipids, proteins, enzymes, carbohydrates, and DNA, thus inducing oxidative stress, an imbalance between ROS and the defense and repair antioxidant system (*2–4*). ROS can be produced by endogenous sources such as normal aerobic respiration, simulated polymorphonuclear leukocytes and macrophages, peroxisomes, and exogenous sources such as smoking, ionizing radiation, certain pollutants, organic solvents, pesticides, and a high polyunsaturated fatty acid diet (*5, 6*). Beneficial effects of ROS in biological systems are that they serve in energy protection, phagocytosis, regulation of cell growth and intercellular signaling, and synthesis of biologically important compounds at physiologic levels. On the other hand, they are highly deleterious and cytotoxic oxidants at pathologic levels which leads to cell injury and death (*7–9*). More than 100 diseases are related to ROS. The ROS contribute to cellar aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes, muscular dystrophy, and neurodegeneration (*10–13*). Nevertheless, all aerobic organisms have evolved with an antioxidant defense system to counteract oxidative stress from ROS. These systems include some antioxidants produced in the body and others obtained from the diet. When antioxidants produced in the body are inefficient to protect against oxidative stress, it is needed to intake dietary antioxidants to diminish the cumulative effects of oxidative damage over the life span (*4, 14, 15*).

Recently, there is an increasing interest concerned about the dietary intake of antioxidant-rich food and oxidative stress and

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age-dependent diseases (*16*). Polyphenols, common secondary metabolites, are abundant in terrestrial plants such as fruits, vegetables, and medicinal plants, and marine plants such as seaweeds. Those substances have received the greatest attention and have been investigated extensively since they are high free radical scavengers and less toxic and have less side effects than synthetic antioxidants such as BHA and BHT (*17–19*). Polyphenols from marine brown algae, called phlorotannins, are structurally different from those obtained from terrestrial plants, in which they are strictly restricted to polymers of phloroglucinol (*20*). Phlorotannins have functions including antioxidant, antiallergic, antibacterial, and antitumor activities, and studies about phlorotannins are of great interest among researchers (*21–23*).

Ishige okamurae, a kind of brown macroalga with narrow fronds, a thick cortical layer, and acute apexes, belongs to the family of Ishigeaceae (Chordariales, Phaeophyta) and grows on rocks in the upper and middle intertidal zone on rough open coasts (*24*). To our knowledge, few reports were conducted on the antioxidant activity of constituents from this alga apart from some studies regarding its biological characteristics such as antifouling activity and cytotoxic activity (*24, 25*). In the present study, three phlorotannins were isolated by kinds of column chromatographies. Free radical scavenging activities on 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, hydroxyl, and alkyl radicals were assessed by electron spin resonance (ESR) spectroscopy. Furthermore, we investigated their antioxidant activities in cellular systems, including 2′,7′-dichlorofluorescein diacetate (DCFH-DA) fluorescence probe by ROS, and inhibitory effects on myeloperoxide (MPO) activity and membrane protein oxidation.

MATERIALS AND METHODS

Plant Material. *I. okamurae* was collected along the coast of Busan, Korea in October 2007. The samples were washed three times using tap water to remove salt, epiphytes, and sand attached to the surfaces of the samples. Finally, the samples were air-dried and ground with a coffee grinder, and the alga powders were stored in a freezer at −20 °C until use.

Materials and Chemicals. Silica gel 60 (0.036−0.2 mm, Merck), Sephadex LH-20 (GE Healthcare Bioscience AB, Uppsala, Sweden), and thin-layer chromatography (TLC) plates (Kieselgel 60 F254 5715, Merck) were used for column chromatography and analytical TLC, respectively. DPPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), $FeSO_4$, H_2O_2, 2,2-azobis-(2-amidinopropane)-hydrochloride (AAPH), α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), DCFH-DA, α-tocopherol, and epigallocatechin gallate (EGCG) were purchased from Sigma Chemical Co. (St. Louis, MO). Human fetal lung fibroblasts cell line MRC-5, mouse macrophages cell line RAW264.7, and human leukemic cell line HL-60 were obtained from American Type Culture Collection (Manassas, VA). Cell culture medium and all the other materials required for culturing were obtained from Gibco BRL, Life Technologies (U.S.A.). All other reagents were of the highest grade available commercially.

Extraction and Isolation of Phlorotannins. The powders of *I. okamurae* (500 g) were extracted with 2 L of methanol for three times at room temperature. After removal of the solvents by evaporation, the extract (96 g) was suspended in H_2O and then partitioned successively with hexane, dichloromethane, EtOAc, and *n*-BuOH. The EtOAC-soluble part (22 g) was subjected to column chromatography over silica gel, eluting successively with hexane (4 L), mixtures of hexane and ethyl acetate (20:1, 10:1, 5:1, 1:1, v/v, each 5 L), dichloromethane (3 L), and mixtures of dichloromethane and methanol (10:1, 5:1, 1:1, v/v, each 5 L) to give 29 fractions (Fr.1−Fr.29) based on TLC analysis. Fr.17 was further chromatographed over silica gel and Sephadex LH-20 to afford compound **1** (142 mg). Fr.20 was separated over silica gel eluting with chloroform−methanol−water (6:1:0.1 v/v/v) to give five subfractions (Fr.20.A−Fr.20.E). Fr.20.C was further purified over Sephadex LH-20 to afford compound **2** (24 mg). Compound **3** (5 mg) was obtained by further purification from fraction Fr.25 over silica gel and Sephadex LH-20.

Structural Elucidation of Isolated Phlorotannins. *Phloroglucinol* (**1**). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.02 (s, 3, H-1, 3, 5), 5.68 (s, 3, H-2, 4, 6). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.8 (C-1, 3, 5), 95.4 (C-2, 4, 6). EI-MS *m/z*: 126 $[M]^+$ (100), 111 (9), 97 (20), 85 (30), 69 (41), 52 (26).

Diphlorethohydroxycarmalol (**2**). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.29−9.11 (m, 9, OH-1, 2, 6, 8, 2′, 4′, 6′, 3″, 5″), 6.09 (s, 1, H-4), 5.90 (br s, 2, H-3′, 5′), 5.81 (t, 1, *J* = 1.8 Hz, H-4″), 5.71 (s, 1, H-9), 5.70 (d, 2, *J* = 1.8 Hz, H-2″, 6″). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.2 (C-1″), 159.0 (C-3″, 5′), 155.0 (C-4′), 151.4 (C-2′, 6′), 146.1 (C-3), 143.1 (C-7), 139.7 (C-1), 138.9 (C-4a), 135.2 (C-6), 133.9 (C-9a), 130.8 (C-5a), 126.4 (C-2), 125.5 (C-8), 124.2 (C-10a), 122.9 (C-1′), 96.2 (C-4″), 95.1 (C-3′, 5′), 94.4 (C-4), 93.8 (C-2″, 6″), 92.4 (C-9). FAB-MS *m/z*: 512 $[M]^+$.

6,6′-Biecko (**3**). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.28 (s, 2, HO-9, 9′), 9.16 (br s, 6, HO-2, 2′, 3″, 3‴, 5″, 5‴), 9.09 (s, 2, HO-4, 4′), 8.65 (s, 2, HO-7, 7′), 6.08 (s, 2, H-3, 3′), 6.04 (s, 2, H-8, 8′), 5.79 (t, 2, *J* = 1.8 Hz, H-4″, 4‴), 5.74 (d, 4, *J* = 1.8 Hz, H-2″, 2‴, 6″, 6‴). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.4 (C-1″, 1‴), 158.7 (C-3″, 3‴, 5″, 5‴), 151.3 (C-7, 7′), 145.4 (C-2, 2′), 144.4 (C-9, 9′), 141.7 (C-4, 4′), 141.3 (C-5a, 5′a), 137.1 (C-10a, 10′a), 123.5 (C-1, 1′), 122.6 (C-9a, 9′a), 121.9 (C-4a, 4′a), 99.6 (C-6″, 6‴), 97.8 (C-8, 8′), 97.7 (C-3, 3′), 96.1 (C-4″, 4‴), 93.6 (C-2″, 2″, 6″, 6‴). FAB-MS *m/z*: 742 $[M]^+$.

DPPH Radical Assay. DPPH radical scavenging activity was measured using the method described by Nanjo et al. (*26*). A 30 μL phlorotannins solution (or ethanol itself as control) was added to 30 μL of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 μL quartz capillary tube, and the scavenging activity of phlorotannins on DPPH radical was measured using a JESFA ESR spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 30 s. DPPH radical scavenging ability was calculated according to the following equation: DPPH scavenging activity (%) = $(1 - A/A_0) \times 100$, in which A and A_0 were the relative peak heights of radical signals with and without sample, respectively. The percentage of scavenging activity was plotted against the sample concentration to obtain the EC_{50}.

Hydroxyl Radical Assay. Hydroxyl radicals were generated by iron-catalyzed Fenton Haber−Weiss reaction, and the generated hydroxyl radicals rapidly reacted with nitrone spin trap DMPO. The resultant DMPO−OH adducts were detected with an ESR spectrometer (*27*). The phlorotannins solution (20 μL) was mixed with DMPO (0.3 M, 20 μL), $FeSO_4$ (10 mM, 20 μL), and H_2O_2 (10 mM, 20 μL) in a phosphate buffer solution (pH 7.4) and then transferred into a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 200; sweep time, 4 min. The scavenging activity was calculated as follows: scavenging activity (%) = $(1 - A/A_0) \times 100$, in which A and A_0 were the relative peak heights of radical signals with and without sample, respectively.

Alkyl Radical Assay. Alkyl radicals were generated by AAPH according to the method of Hiramoto et al. (*28*). Briefly, 20 μL of 40 mM AAPH was mixed with 20 μL of phosphate-buffered saline (PBS), 20 μL of 40 mM 4-POBN, and 20 μL of the indicated concentrations of tested samples. The mixture was vortexed and incubated at 37 °C for 30 min. Subsequently, the reaction mixture was transferred to a sealed capillary tube and the spin adduct was recorded with controlled spectrometric conditions: modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9441 MHz; magnetic field, 336.5 ± 5 mT; sweep time, 30 s. Alkyl radical scavenging ability was calculated as follows: scavenging activity (%) = $(1- A/A_0) \times 100$, in which A and A_0 were the relative peak heights of radical signals with and without sample, respectively.

Superoxide Radical Scavenging Activity. Superoxide radicals were

generated by a UV-irradiated riboflavin/EDTA system (*29*). The reaction mixture containing 0.3 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO, and the indicated concentrations of tested samples was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was transferred to a 100 μL quartz capillary tube of the ESR spectrometer for measurement. Experimental conditions: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 1 min. Superoxide radical scavenging ability was calculated as follows: scavenging activity (%) = $(1 - A/A_0) \times 100$, in which A and A_0 were the relative peak heights of radical signals with and without sample, respectively.

Cell Cultures and Cytotoxic Effect of Phlorotannins on MRC-5, RAW264.7, and HL-60 Cell Lines. MRC-5 and RAW264.7 cells, two anchorage-dependent cell lines, were cultured in Dulbecco's modified Eagle's medium (DMEM), and HL-60 cells, a suspension-dependent cell line, were cultured in Roswell Park Memorial Institute medium (RPMI), respectively, supplemented with 10% heated-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every other day. Cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (*30*). The cells were cultured in microtiter 96-well plates (1.5×10^5 cells/well) with serum-free media and treated with different concentrations of phlorotannins for 24 h. Phlorotannins were dissolved in 10% dimethyl sulfoxide (DMSO) and sterilized by filtering through a 0.22 μm filter membrane. The final concentration of DMSO in culture media never exceeded 0.1%. Sequentially, 20 μL of MTT dye solution was added to each well. After 4 h of incubation, 200 μL of DMSO solution was added for dissolving the formazan crystals and the absorbance was read using Genios multifunction microplate reader (TECAN Austria Gmbh, Austria) at 540 nm.

Determination of Intracellular ROS by DCFH-DA. The DCFH-DA method was used to measure intracellular ROS production as described previously (*31*). RAW264.7 cells growing in fluorescence microtiter 96-well plates were labeled with 20 mM DCFH-DA in Hank's balanced salt solution (HBSS) for 20 min in the dark. Cells were then treated with different concentrations of phlorotannins and incubated for another 1 h. After washing the cells with PBS for three times, 500 mM H_2O_2 was added. The intensity of the fluorescence signal was detected time-dependently with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Genios fluorescence microplate reader. Effects of treatments were plotted and compared with the fluorescence intensity of control and blank groups.

Membrane Protein Oxidation Assay. The amount of membrane protein carbonyl group was assessed by a previously described method with modifications (*32*). RAW264.7 cells collected by centrifugation were washed with PBS and lysed with 20 mL of lysis buffer without reducing agents (25 mM Tris−HCl pH 7.8, 2 mM EDTA, 180 mM NaCl, 1% Triton X-100). The lysate was aliquoted into microtubes (0.5 mL) and incubated with the indicated concentrations of samples for 30 min at 37 °C. Then 100 μL of 0.1 M $FeSO_4$ and 100 μL of 2 mM H_2O_2 were added, and the mixture was incubated at 37 °C for 1 h. After addition of 400 μL of 20% trichloroacetic acid, solubilized protein (1 mg) was precipitated by centrifugation. The supernatant was discarded, and the pellet was resuspended in 150 μL of 0.2% 2,4-dinitrophenyl hydrazine in 2 mM HCl and allowed to stand at room temperature for 40 min. The reaction mixture was vortexed every 10 min to facilitate the reaction with proteins. The protein was precipitated again with 20% trichloroacetic acid, and the pellet was washed three times with ethanol/ethyl acetate (1:1 v/v) solution. The pellet was then dissolved in 500 μL of 6 N guanidine hydrochloride and incubated for 15 min at 37 °C. After centrifugation at 6000*g* for 5 min, the absorbance of the supernatant was read against a complementary blank at 370 nm. A blank was prepared with a parallel procedure using 2 mM HCl alone instead of 2,4-dinitrophenyl hydrazine reagent. The carbonyl group content was expressed in nmol/mg of protein, using a molar absorption coefficient of 22 000 M^{-1} cm^{-1}.

MPO Activity Assay. Activity of myeloperoxidase, a marker enzyme of leukocytes, is thought to represent leukocyte migration to

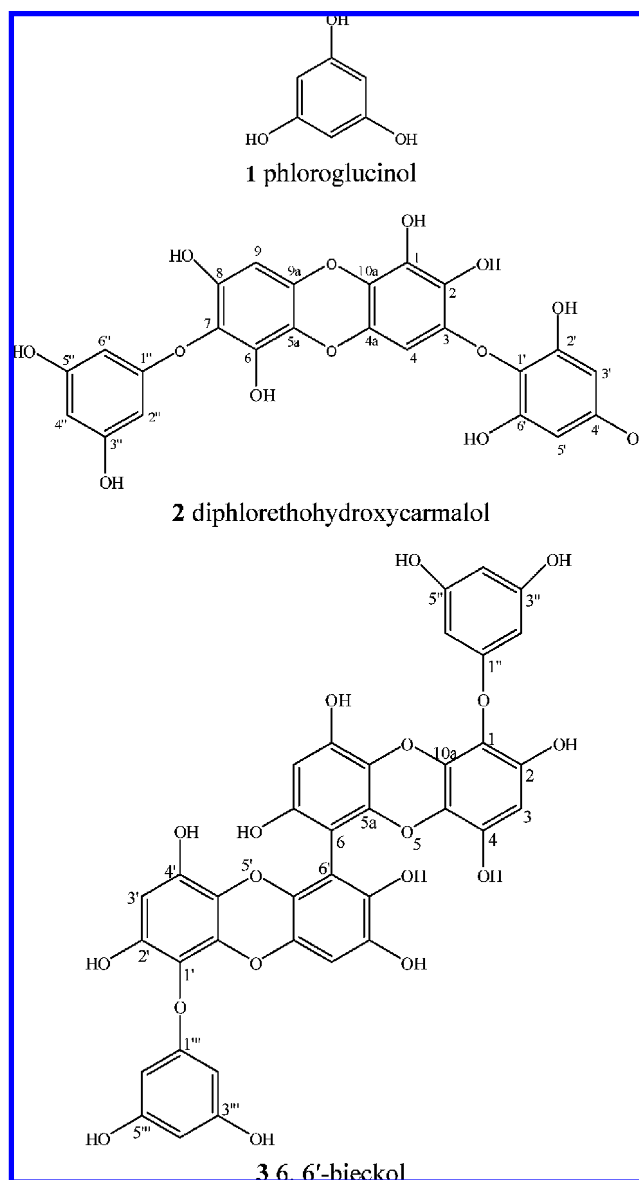

Figure 1. Structures of phlorotannins isolated from *I. okamurae*.

injured tissues. The amount of MPO released in stimulated neutrophils was determined by the *o*-dianisidine method with modifications (*33*). HL-60 cells were suspended in RPMI without phenol red and FBS and seeded into 96-well plates. Cells were preincubated with various concentrations of samples for 30 min followed by stimulation with TNF-α (0.05 μg/mL) at 37 °C for 30 min. Cells were then added with the assay mixture containing 0.05 mL of 2 mM H_2O_2 in 0.1 M phosphate buffer (pH 6.0) and 0.05 mL of 0.02 M *o*-dianisidine (freshly prepared) in deionized water. The amount of MPO released was measured spectrophotometrically at 460 nm, and the inhibition percent of phlorotannins on MPO activity was calculated as 100 × (1 − (absorbance of sample − absorbance of blank)/(absorbance of control − absorbance of blank). Cells in the control and blank were grown in the absence of phlorotannins, and a blank was made without the stimulation with TNF-α.

Statistical Analysis. The data were expressed as the mean of three replicate determinations and standard deviation (SD); statistical comparisons were made with Student's *t* test. P values of < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Isolation and Structural Determination of Phlorotannins. In the present study, three phlorotannins were isolated from *I. okamurae* by column chromatography (**Figure 1**). Each com-

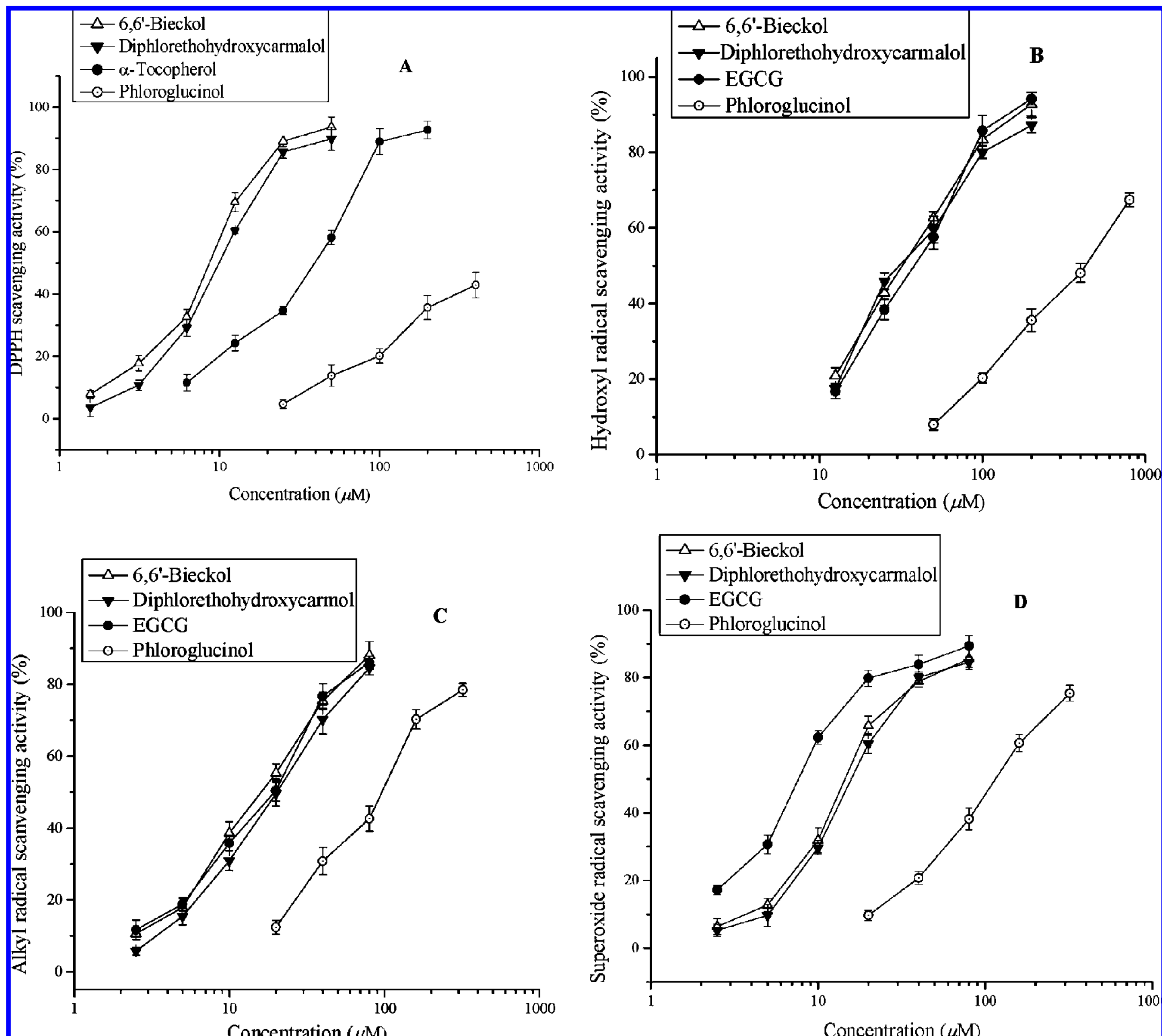

Figure 2. Free radical scavenging activities of phlorotannins isolated from *I. okamurae* determined by the ESR spectrometer. (**A**) DPPH scavenging activity. (**B**) Hydroxyl radical scavenging activity. (**C**) Alkyl radical scavenging activity. (**D**) Superoxide radical scavenging activity. Values are expressed as the mean ± SD of three replicate experiments.

pound was characterized by ^{1}H NMR, ^{13}C NMR, and mass spectrometry (MS). The structures of the phlorotannins were elucidated as phloroglucinol (*34*), diphlorethohydroxycarmalol (*35*), and 6,6′-bieckol (*36*), and the spectra data were in close agreement with those previous reports. 6,6′-Bieckol was isolated from this brown alga for the first time.

Free Radical Scavenging Activities of Phlorotannins by ESR. Recently, the ESR has become the most useful spectrometer to measure accurately radical levels that remain in reactions. Because of its convenience, high sensitivity, and short time consumption, it has been widely used as a powerful method to determine kinds of radicals (*37*). Therefore, the antioxidant activities of the phlorotannins were first determined by scavenging free radicals including DPPH, superoxide, hydroxyl, and alkyl radicals by ESR spectrometer. The radical scavenging activities of phlorotannins are presented in **Figure 2**, and the EC_{50}'s of phlorotannins, defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%, are summarized in **Table 1**.

DPPH radical is a stable organic free radical; when accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant, it can be reduced to a nonradical form DPPH−H by the reaction. Because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening antiradical activities (*38*). The DPPH scavenging activity of three phlorotannins is presented in **Figure 2A**. All phlorotannins showed a dose-dependent manner in scavenging DPPH radical. However, as anticipated, phloroglucinol almost showed no scavenging effect on DPPH radical at lower concentration; even at the concentration of 400 μM, only 42.9% of the DPPH radical was scavenged, and this scavenging activity was not statistically significant compared with the control ($p > 0.05$). The EC_{50}'s of the phlorotannins 6,6′-bieckol and diphlorethohydroxycarmalol were 9.1 and 10.5 μM, respectively. In contrast, The EC_{50} value of α-tocopherol, a positive control, was 45.3 μM. Although 6,6′-bieckol showed the strongest DPPH scavenging

Table 1. EC_{50} Values of Phlorotannins from *I. okamurae*[a]

		EC_{50} (μM $\pm$ SD)[b]		
	radicals	6,6′-bieckol	diphlorethohydroxycarmalol	phloroglucinol
1	DPPH	9.1 ± 0.4	10.5 ± 0.5	not determined
2	hydroxyl	28.7 ± 1.1	27.1 ± 0.9	408.5 ± 3.7
3	alkyl	17.3 ± 1.0	18.8 ± 1.2	103.5 ± 1.9
4	superoxide	15.4 ± 0.9	16.7 ± 0.6	124.7 ± 2.4

[a] Each value is expressed as the mean $\pm$ SD ($n = 3$). [b] EC_{50} value was defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%, respectively. The EC_{50} value was obtained by interpolation from linear regression analysis.

activity, the difference of the scavenging activity between 6,6′-bieckol and diphlorethohydroxycarmalol was not significant ($p > 0.05$).

Hydroxyl radical (HO•) is the most reactive ROS, which can react rapidly with almost all biological molecules and may be involved in the pathology of many human diseases. Hydroxyl radical was generated by the Fenton system; HO• are identified because of their ability to form nitroxide adducts from the commonly used DMPO spin trap. This spin trap has a greater ability of trapping oxygen-centered radicals than other nitrone spin traps (*39*). The adduct DMPO−OH radical exhibits a characteristic ESR response which can be detected by an ESR spectrometer. The capacity of phlorotannins to inhibit hydroxyl radical generated by the Fenton reaction was determined and is illustrated in **Figure 2B**. Both 6,6′-bieckol and diphlorethohydroxycarmalol exhibited significant potency ($p < 0.05$) to scavenging hydroxyl radical at a low concentration of 160 μM; the EC_{50}'s of 6,6′-bieckol and diphlorethohydroxycarmalol were 28.7 and 27.1 μM, respectively. The EC_{50} of both 6,6′-bieckol and diphlorethohydroxycarmalol was comparative to the EC_{50} of EGCG (30.6 μM), which suggested that both 6,6′-bieckol and diphlorethohydroxycarmalol were high hydroxyl radical scavengers. On the other hand, phloroglucinol showed the weakest scavenging activity toward hydroxyl radical; only 67.5% of the hydroxyl radical was scavenged by phloroglucinol at 800 μM.

The ability of phlorotannins to scavenge alkyl radical was tested with the ESR technique. Alkyl radical was generated by the decomposition of AAPH, a water-soluble peroxyl radical initiator, then the alkyl radical was trapped by 4-POBN, and the forming spin adducts were measured by ESR as described in the Materials and Methods. The scavenging activity was observed as a dose-dependent manner of all tested samples (**Figure 2C**). All phlorotannins showed the alkyl radical scavenging activity, and the scavenging activities of 6,6′-bieckol, diphlorethohydroxycarmalol, and phloroglucinol were 88.1%, 84.6%, and 42.6% at the concentration of 80 μM, respectively. The EC_{50}'s of 6,6′-bieckol, diphlorethohydroxycarmalol, phloroglucinol, and EGCG were 17.3, 20.4, 20.8, and 110.8 μM, respectively. Alkyl radical scavenging activity of those phlorotannins and EGCG followed the following order: 6,6′-bieckol > diphlorethohydroxycarmalol > EGCG > phloroglucinol, but the difference between 6,6′-bieckol, diphlorethohydroxycarmalol, and EGCG was not significant ($p > 0.05$).

Superoxide radical is formed at the beginning of oxidation reactions in the body; although it is a weak oxidant, it can transform to powerful and dangerous hydroxyl radical in the presence of metal such as iron and copper, thus inducing cell damage and ROS-related diseases. Superoxide radical was generated by UV irradiation of a riboflavin/EDTA system. In this study, the superoxide scavenging activity of phlorotannins from *I. okamurae* was measured by ESR spectrometer. **Figure 2D** shows the superoxide radical scavenging activity of different concentrations of phlorotannins and EGCG. Both of them had strong superoxide radical scavenging activity in a dose-dependent manner, but EGCG, used as a positive control in this study, exhibited the highest superoxide radical scavenging activity. The results were found statistically significant ($p < 0.05$) with the control. EC_{50}'s of 6,6′-bieckol, diphlorethohydroxycarmalol, EGCG, and phloroglucinol were 14.7, 16.2, 9.5, and 128.6 μM, respectively. The superoxide radical scavenging activity of those phlorotannins and EGCG followed the following order: EGCG > 6,6′-bieckol > diphlorethohydroxycarmalol > phloroglucinol. Although phloroglucinol showed very poor scavenging activities against DPPH and hydroxyl radicals, it possessed potent scavenging activities against alkyl and superoxide radicals.

Previous studies have suggested that the radical scavenging activities of polyphenols such as flavonoids are ascribed to the phenolic hydroxyl groups attached to the benzene ring, and the structure−activity relationship of flavonoids was studied using various antioxidant assays (*40, 41*). Although the structure−activity relationship of phlorotannins is not clear, it may be that phenolic hydroxyl groups attached to the eckol skeleton play an important role in the radical scavenging activities (*42*). In the present study, the radical scavenging activities of 6,6′-bieckol and diphlorethohydroxycarmalol were much higher than those of phloroglucinol, which implied that the amount of hydroxyl groups attached to the benzene ring indeed plays an important role in the radical scavenging activities. Because the radical scavenging activities between 6,6′-bieckol and diphlorethohydroxycarmalol were not very different, more phlorotannins with different benzene rings and amounts of hydroxyl groups are needed to study the structure−activity of phlorotannins by different antioxidant assays.

Effects of Phlorotannins on Free Radical Meditated Oxidation in Cellular Systems. Phlorotannins, mainly derived from brown algae, have many bioactivities such as antioxidant, antiallergic, antitumor, antibacterial, enzyme inhibition, and so on. The antioxidant activities of phlorotannins were studied widely, but to our best knowledge, very few studies about the antioxidant activities of phlorotannins in cellular systems have been conducted. In view of this, the antioxidant activities of phlorotannins from *I. okamurae* were also determined in cellular systems to investigate whether phlorotannins can affect the free radical-meditated oxidation in cellular systems or not. Two cell lines, RAW 264.7 for the study of direct ROS scavenging effects and membrane protein oxidation inhibitory effects and HL-60 for the study of MPO inhibitory effects of phlorotannins, were employed in the present study. These cells are usually used to study ROS-mediated cellular events since they can produce high amount of ROS following stimulation (*43*).

Cytotoxic effects of phlorotannins were investigated by MTT assay on MRC-5, RAW 264.7, and HL-60 cells. The results (**Figure 3**) showed that no phlorotannins had cytotoxic effects on those cells under the test concentrations. Even at the highest concentration of 50 μM, only about 5% of the cells were

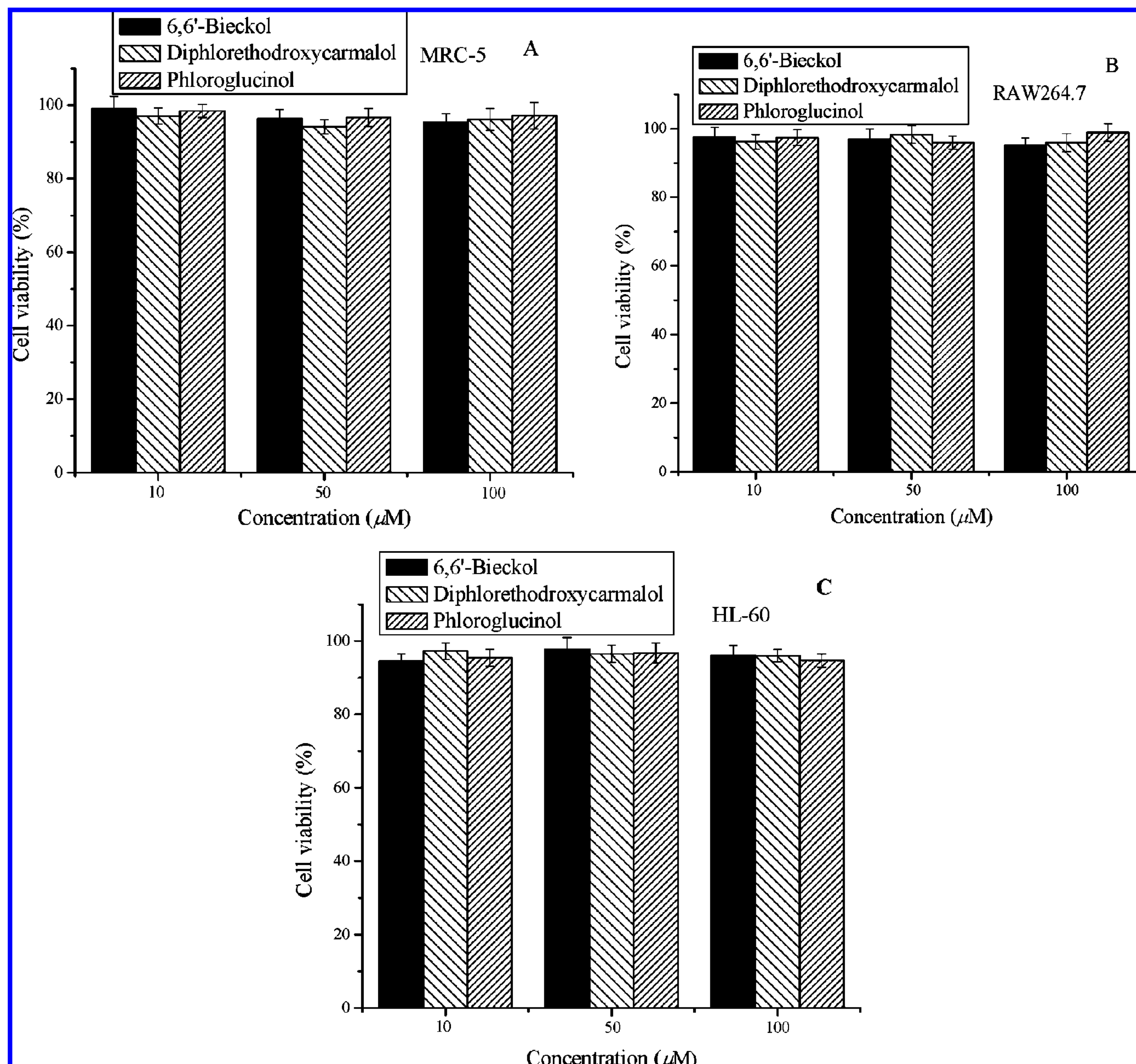

Figure 3. Cytotoxic effects of phlorotannins isolated from *I. okamurae* on MRC-5 cells (**A**), RAW246.7 cells (**B**), and HL-60 cells (**C**). Values are expressed as the mean ± SD of three replicate experiments.

decrement, and the cell viability of cells treated with various concentrations of phlorotannins was not significantly different from the control ($p > 0.05$).

In order to examine whether phlorotannins from *I. okamurae* affect the intracellular production of ROS, RAW264.7 cells were preincubated with 5, 10, and 50 μM phlorotannins for 1 h and labeled with fluorescence probe DCFH-DA as described in the Materials and Methods. When DCFH-DA enters viable cells, it can be deacetylated by intracellular esterases to form 2′, 7′-dichloro-dihydrofluorescein (DCFH) which can react quantitatively with ROS within the cell to produce 2′,7′-dichlorofluorescein (DCF), which is fluorescent (*44*). As shown in **Figure 4**, fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time course increment up to 210 min. It was found that preincubation with 6,6′-bieckol and diphlorethohydroxycarmalol decreased the DCF fluorescence dose- and time-dependently. Even after 30 min of incubation, 6,6′-bieckol and diphlorethohydroxycarmalol exerted a considerable radical scavenging activity at the concentration of 5 μM. More clearly, at the concentration of 50 μM, 6,6′-bieckol and diphlorethohydroxycarmalol could scavenge ROS significantly throughout the incubation time. After 210 min of incubation, 6,6′-bieckol and diphlorethohydroxycarmalol reduce the production of ROS by 78.9% and 77.2% at 50 μM, respectively. However, phloroglucinol could not reduce the production of ROS at all test concentrations. Because 6,6′-bieckol and diphlorethohydroxycarmalol were preincubated with the cells and removed after incubation, their activity only could take place intracellularly; our results demonstrated that the antioxidant activities of those phlorotannins are due to direct scavenging of cellular ROS, and thus they may be a potent antioxidant compound to inhibit cellular ROS formation.

Protein oxidation by ROS plays an important role in the pathomechanism of many diseases. The attack by ROS against proteins results in the modification of amino acid side chains containing such functional groups as lysine, arginine, proline, and histidine, then generates carbonyl moieties (mainly aldehydes and ketones), which has been identified as an early marker

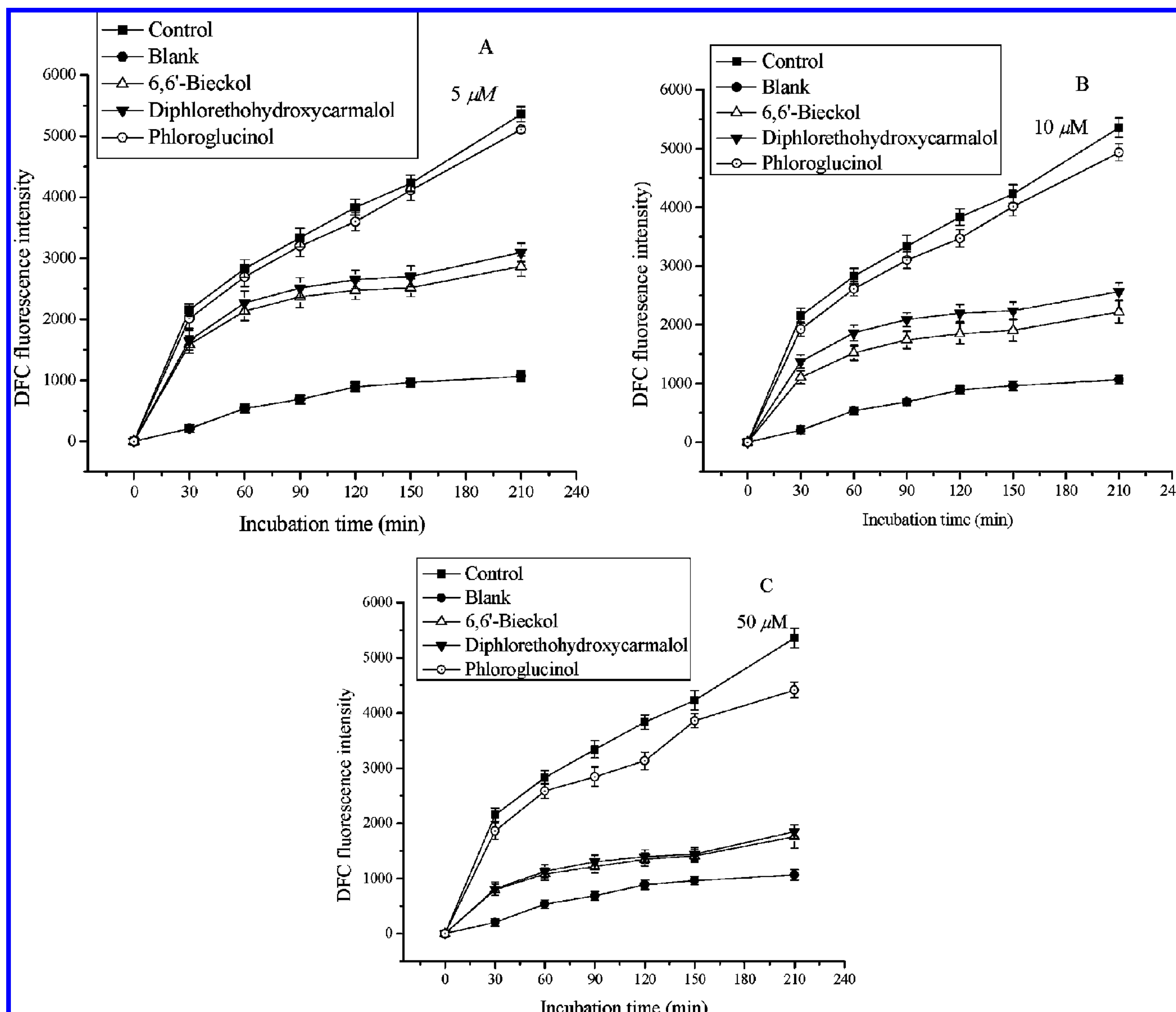

Figure 4. Intracellular radical scavenging activities of phlorotannins isolated from *I. okamurae* on RAW264.7 cells at the concentration of 5 (**A**), 10 (**B**), and 50 μM (**C**). ROS level is represented as DCF fluorescence. Values are expressed as the mean ± SD of three replicate experiments.

for protein oxidation and used as a measure of protein damage. Oxidative damage of membranes also results in increased membrane fluidity, compromised integrity, and inactivation of membrane-bound receptors and enzymes (*45, 46*). In the present study, inhibitory effects of phlorotannins on RAW264.7 cell membrane protein oxidation were investigated as described in the Materials and Methods. As shown in **Figure 5**, when mouse macrophage membranes were exposed to HO• generated by $Fe^{2+}-H_2O_2$ Fenton reaction, the extent of membrane protein oxidation increased as indicated by the increase of carbonyl group contents. About 12 times higher amount of carbonyl groups was observed in the oxidative stress induced control group compared to those of the blank group. Protein carbonyl groups in 6,6′-bieckol- and diphlorethohydroxycarmalol-treated groups were lower than those of the control group. Pretreatment with 6,6′-bieckol and diphlorethohydroxycarmalol for 30 min dose-dependently inhibited the oxidation of membrane protein. 6,6′-Bieckol and diphlorethohydroxycarmalol can effectively inhibit the protein oxidation by 51.8% and 59.1% of the control, respectively ($p < 0.05$). On the other hand, phloroglucinol showed a very weak inhibitory effect only at the highest concentration. The results suggested that 6,6′-bieckol and diphlorethohydroxycarmalol could protect proteins in RAW264.7 cells from ROS-derived oxidation.

Finally, we were interested in studying the effects of phlorotannins on the activity of MPO. MPO is a leukocyte-derived heme peroxidase which has long been considered as a microbial enzyme centrally linked to the unspecific immune defense system. MPO plays an important role in oxidant production by polymorphonuclear neutrophils (PMNs). It uses hydrogen peroxide (H_2O_2) and chloride to catalyze the production of hypochlorous acid (HOCl), which is the most powerful oxidant and contributes to both microbial killing and subsequent oxidative injury of host tissue triggering severe inflammatory disorders (*47, 48*). Thus, the search for compounds for the inhibition of MPO activity is an important approach to control ROS-mediated oxidation of biomolecules in neutrophils. In the present study, HL-60 cells were used because of their high expression of MPO after stimulation with TNF-α. The absorbance at 460 nm of the control was very high compared to those pretreated with 6,6′-bieckol and diphlorethohydroxycarmalol, which indicated an increased activity of MPO of the control (data of absorbance not shown). As illustrated in **Figure 6**, the activity of MPO was dose-dependently inhibited by pretreating

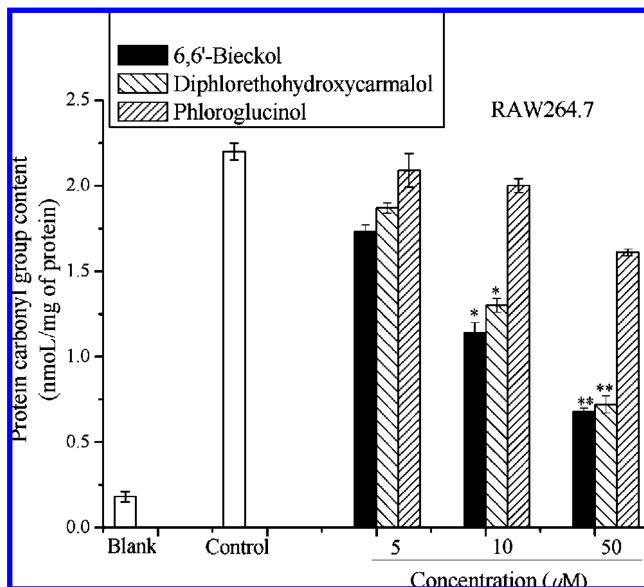

Figure 5. Inhibitory effects of phlorotannins isolated from *I. okamurae* on cell membrane protein oxidation in RAW264.7 cells. Values are expressed as the mean ± SD of three replicate experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$ compared with control.

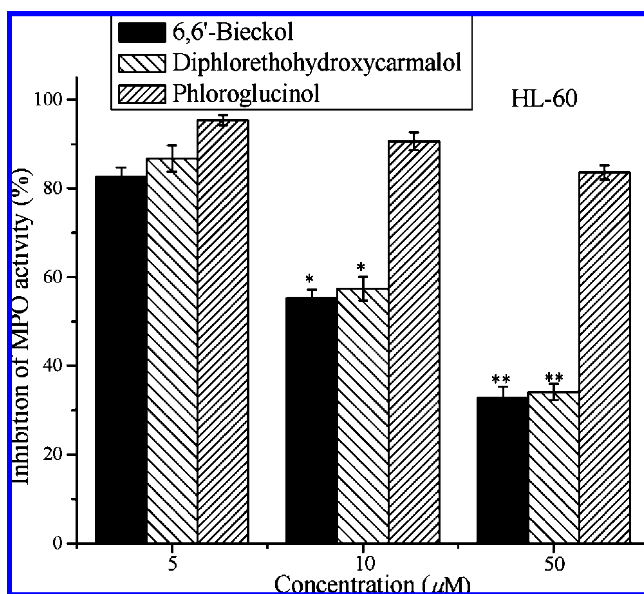

Figure 6. Inhibitory effects of phlorotannins isolated from *I. okamurae* on MPO activity in HL-60 cells. Values are expressed as the mean ± SD of three replicate experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$ compared with control.

with 6,6′-bieckol and diphlorethohydroxycarmalol; at the concentration of 10 and 50 μM, only 55.3%, 57.4% and 32.8%, 34.1% of the control were observed, from which it could be assumed that this is an indirect way of acting as a cellular antioxidant. The inhibitory effect on MPO activity between 6,6′-bieckol and diphlorethohydroxycarmalol was almost the same ($p < 0.05$). However, phloroglucinol exhibited very weak activity on the inhibition of MPO compared with the control.

In conclusion, three phlorotannins, including phloroglucinol, 6,6′-bieckol, and diphlorethohydroxycarmalol, were isolated from the brown alga *I. okamurae* by column chromatography. The structures of the phlorotannins were elucidated based on NMR and MS data. Of the isolated phlorotannins, diphlorethohydroxycarmalol and 6,6′-bieckol exhibited strong free radical scavenging activity against DPPH, hydroxyl, alkyl, and superoxide radicals as determined by ESR. Diphlorethohydroxycarmalol and 6,6′-bieckol scavenged intracellular ROS directly as manifested by DCFH-DA assay. Membrane protein oxidation and MPO activity were dose-dependently inhibited by pretreating with diphlorethohydroxycarmalol and 6,6′-bieckol. These results, which demonstrated the radical scavenging activity and antioxidant activity of phlorotannins isolated from *I. okamurae* in cellular systems, suggest that phlorotannins from marine resources might be proposed as a functional foodstuff, dietary supplement, or drug for the prevention or treatment of various ROS-related diseases.

ABBREVIATIONS USED

ESR, electron spin resonance spectrometry; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MRC-5, human fetal lung fibroblasts cell line; RAW264.7, mouse macrophages cell line; HL-60, human leukemic cell line; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; MPO, myeloperoxide; ROS, reactive oxygen species; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TLC, thin-layer chromatography; DMPO, 5,5-dimethyl-l-pyridine *N*-oxide; AAPH, 2,2-azobis-(2-amidinopropane)-hydrochloride; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; EGCG, epigallocatechin gallate; DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO, dimethyl sulfoxide; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline.

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