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ARTICLE

Inhibitors of Oxidation and Matrix Metalloproteinases, Floridoside, and D-Isofloridoside from Marine Red Alga *Laurencia undulata*

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In the exploration of abundant marine biological resources, edible red alga *Laurencia undulata* led to two bioactive isolates: floridoside (**1**) and D-isofloridoside (**2**). For the first time, the antioxidant properties of both derivatives (**1** and **2**) were characterized via free radical scavenging using the ESR technique, reactive oxygen species (ROS) inhibition, membrane protein oxidation, myeloperoxidase (MPO) inhibition, gene expression levels of glutathione (GSH) and superoxide dismutase (SOD), and protein expression of MMP-2 and MMP-9. The results demonstrate that floridoside and D-isofloridoside possess significant antioxidant capacity and are potential inhibitors of MMP-2 and MMP-9. These results clarified that these components may be responsible for the relative activities of crude extract from this genus, which is used as folk medicine. Furthermore, the structure−activity relationships were also suggested. Both isomers could be effective candidates for applications in food and pharmaceutical fields as natural marine antioxidants.

KEYWORDS: Marine alga; *Laurencia undulata*; floridoside and D-isofloridoside; antioxidation; MMP-2 and MMP-9

INTRODUCTION

Marine algae are a rich source of new secondary metabolites with a wide variety of biological activities, such as antioxidant, anti-inflammatory, anti-HIV, etc. (*1*−*3*). *Laurencia undulata*, which is one of the highest-potential species for secondary metabolites from the genus *Laurencia* based on the recent investigation, is an edible marine red alga. The research of this species, however, is rare to date because there has been only anti-asthmatic activity available (*4*). This encouraged us to continue our study by isolating bioactive compounds from *L. undulata* with significant pharmaceutical potential.

For the first time, floridoside and D-isofloridoside were isolated from methanol extract of *L. undulata* by column chromatography and high-performance liquid chromatography (HPLC) techniques. Floridoside is reported to be an intracellular osmotic regulator and activator (*5*, *6*). There are no other bioactivities provided for floridoside or D-isofloridoside. According to the antitumor promoting activity of galactoglycerols oriented from the same substrate (*7*), floridoside and D-isofloridoside were tested by focusing on their potential inhibition against oxidation, MMP-2, and MMP-9.

Oxidative stress can induce many kinds of human diseases such as stroke, neurodegeneration, cancer, etc.; therefore, searching for effective antioxidants is an intense and continuous process. Considerable laboratory evidence indicates that antioxidants may slow or possibly prevent the development of cancers. Free radical-induced oxidative stress is found to be the causative factor in the pathogenesis of ischemic brain injury from chemical, cell culture, and animal studies (*8*, *9*). Free radicals (FRs) are mainly produced during cellular metabolism and mitochondrial energy production, and they are commonly used as effective checking points in basic research. In addition, FR magnetic signals and levels can be quantitatively monitored and determined by the ESR technique (*10*). Normally, reactive oxygen species (ROS) are effectively eliminated by the antioxidant defense systems such as antioxidant enzymes and nonenzymatic factors (*11*). Matrix metalloproteinases (MMPs) have been implicated in the degradation of the extracellular matrix in normal and pathological tissue remodeling. Previous studies reported a close relationship between the activation of MMP-2 and MMP-9 expression and oxidative stress, and these expression levels can be lowered by the use of antioxidants (*12*, *13*).

In this study, we describe the structural assignments of floridoside and D-isofloridoside in detail mainly by assessing NMR spectra. Moreover, this is a continuation of our previous study which involved scanning the effect of antioxidants derived from this genus in cellular systems. The bioactive effects of floridoside and D-isofloridoside were assessed by antioxidative properties and MMP-2 and MMP-9 inhibition.

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MATERIALS AND METHODS

Materials. The red seaweed *L. undulata* was collected along the Cheju Island Coast of South Korea in October 2007. The fresh sample was washed thoroughly with tap water to remove salt, sand, and epiphytes, dried to a constant weight in the air, and ground into a powder with a laboratory beater. The sample powder was stored at −20 °C until it was used.

Chemicals. Extraction of *L. undulata* was performed using the extraction unit (Dongwon Scientific Co.). Sephadex LH-20 (Sigma, St. Louis, MO) and thin-layer chromatography (TLC) plates (Kieselgel 60 F254, 0.25 mm, Merck) were used for column chromatography and analytical TLC, respectively. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan), using the DMSO-d_6 solvent peak (2.50 ppm in ^{1}H NMR and 39.5 ppm in ^{13}C NMR) as an internal reference standard. MS spectra were recorded on a JEOL JMS-700 spectrometer (JEOL). The ESR spectra were recorded on a JES-TE100 ESR spectrometer (JEOL). Silica gel 60 (230−400 mesh, Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), $FeSO_4$, H_2O_2, 2,2-azobis-(2-amidinopropane) hydrochloride (AAPH), (*R*)-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), dichlorofluorescin diacetate (DCFH-DA), acid-phenol-guanidine thiocyanate reagent except for TRIzol reagent (Promega Corp.) for preparation of high-quality RNA, and all reagents related to synthesis of cDNA were purchased from Sigma Chemical Co. M-MLV 5× reaction buffer was from Promega Corp. Human fetal lung fibroblast cell line MRC-5, mouse macrophage cell line RAW264.7, human leukemic cell line HL-60, and human fibrosarcoma cell line HT-1080 were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, and a 100 μg/mL penicillin/streptomycin/amphotericin mixture for cell culture were obtained from Gibco BRL, Life Technologies. Active MMP-2 (PF023) and active MMP-9 (PF024) were purchased from Calbiochem (Cambridge, MA). All other reagents were of the commercially available analytical grade.

Extraction and Isolation. The air-dried and finely ground powder (500 g) was successively extracted with 5 L of MeOH three times at room temperature. The resulting extract (136 g) was suspended in water followed by solvent partitioning with *n*-hexane, dichloromethane, EtOAc, and *n*-BuOH. The EtOAc fraction (30 g) was subjected to column chromatography over silica gel, eluting successively with a hexane/ethyl acetate gradient solvent system [10:1, 5:1, and 1:1 (v/v); each 4 L], dichloromethane (5 L), and mixtures of dichloromethane and methanol [10:1, 5:1, and 1:1 (v/v); each 4 L] to give 12 fractions based on TLC analysis. Fraction 8 was subjected to further purification by Sephadex LH-20 and finally purified using HPLC monitored with a RI detector to afford compound **1** (78 mg) and compound **2** (63 mg), respectively.

Compound **1**: ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 4.81 (1H, d, J = 2.6 Hz, H-1), 4.58 (1H, m, HO-6), 4.56 (1H, m, HO-4), 4.47 (1H, m, HO-2′), 4.38 (1H, m, HO-3), 4.35 (1H, m, HO-2), 4.44 (1H, m, HO-1′), 3.76 (1H, t, J = 2.6 Hz, H-5), 3.69 (1H, brs, H-2), 3.51 (2H, m, H-3, H-4), 3.47 (6H, m, H-1′, H-3′, H-6), 3.41 (1H, m, H-2′); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 99.4 (C-1), 80.6 (C-2′), 71.3 (C-5), 69.7 (C-3), 68.9 (C-4), 68.8 (C-2), 61.0 (C-6), 60.8 (C-3′), 60.6 (C-1′) (see **Table 1**); LREIMS m/z 255.20 (0.09) $[M + H]^+$, 163.10 (11.34) $[M - C_3H_3O_3]^+$, 91.05 (38.57) $[M - C_6H_{11}O_4]^+$.

Compound **2**: ^{1}H NMR (400 MHz, DMSO-d_6) δ_H 4.86 (1H, d, J = 2.8 Hz, H-1), 4.58 (1H, m, HO-6), 4.57 (1H, m, HO-4), 4.47 (1H, m, HO-3′), 4.37 (1H, m, HO-3), 4.36 (1H, m, HO-2), 4.45 (1H, m, HO-1′), 3.78 (1H, t, J = 2.6 Hz, H-5), 3.71 (1H, brs, H-2), 3.63 (2H, m, H-3′), 3.74 (2H, m, H-3, H-4), 3.66 (2H, m, H-6), 3.49 (3H, m, H-1′, H-2′); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 101.3 (C-1), 72.4 (C-2′), 71.8 (C-1′), 71.1 (C-5), 70.3 (C-3), 70.1 (C-4), 69.0 (C-2), 62.5 (C-6), 62.3 (C-3′) (see **Table 1**); LREIMS m/z 255.20 (0.09) $[M + H]^+$, 163.10 (13.42) $[M - C_3H_3O_3]^+$, 91.05 (36.74) $[M - C_6H_{11}O_4]^+$.

Free Radical Scavenging Effects of Floridoside and D-Isofloridoside. The scavenging activities (I) of the tested floridoside and D-isofloridoside on DPPH, hydroxyl, alkyl, and superoxide anion species were expressed as $I(\%) = (A_c - A_s)/A_c \times 100$, where A_c and A_s are the relative peak heights of radical signals with and without the sample, respectively. The percentage of scavenging activity was plotted versus the sample concentration to yield the IC_{50} value.

Table 1. One-Dimensional NMR Data for Floridoside and D-Isofloridoside in DMSO-d_6[a]

	floridoside		D-isofloridoside	
position	δ_H (mult, J)	δ_C (mult)	δ_H (mult, J)	δ_C (mult)
1	4.81 (1H, d, J = 2.6 Hz)	99.4 (d)	4.86 (1H, d, J = 2.8 Hz)	101.3 (d)
2	3.69 (1H, brs)	68.8 (d)	3.71 (1H, brs)	69.0 (d)
3	3.51 (1H, m)	69.7 (d)	3.74 (1H, m)	70.3 (d)
4	3.51 (1H, m)	68.9 (d)	3.82 (1H, m)	70.1 (d)
5	3.76 (1H, t, J = 6.2 Hz)	71.3 (d)	3.78 (1H, t, J = 6.2 Hz)	71.1 (d)
6	3.47 (2H, m)	61.0 (d)	3.66 (2H, m)	62.5 (d)
1′	3.47 (2H, m)	60.6 (t)	3.49 (2H, m)	71.8 (t)
2′	3.41 (1H, m)	80.6 (d)	3.49 (1H, m)	72.4 (d)
3′	3.47 (2H, m)	60.8 (t)	3.63 (2H, m)	62.3 (t)
2-OH	4.35 (1H, m)		4.36 (1H, m)	
3-OH	4.38 (1H, m)		4.37 (1H, m)	
4-OH	4.56 (1H, m)		4.57 (1H, m)	
6-OH	4.58 (1H, m)		4.58 (1H, m)	
1′-OH	4.44 (1H, m)			
2′-OH			4.45 (1H, m)	
3′-OH	4.47 (1H, m)		4.47 (1H, m)	

[a] Recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C and ^{13}C DEPT.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Effects of Floridoside and D-Isofloridoside. DPPH radical scavenging activity was evaluated using the method described by Nanjo et al. (*14*). A 25 μL solution of floridoside and D-isofloridoside in 10% DMSO was added to 25 μL of DPPH (60 μM) in a methanol solution. After being mixed vigorously for 10 s, the solution was transferred into a 100 μL quartz capillary tube, and then the spin adduct was measured on an ESR spectrometer exactly 2 min later. The instrumental parameters were as follows: magnetic field, 6.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; and sweep time, 30 s.

Hydroxyl Radical Scavenging Effects of Floridoside and D-Isofloridoside. Hydroxyl radicals were generated by an iron-catalyzed Fenton Haber−Weiss reaction, and the generated hydroxyl radicals were rapidly reacted with nitrone spin trap DMPO (*15*). The resulting DMPO−OH adducts could be detected with an ESR spectrometer. The floridoside and D-isofloridoside (in 10% DMSO) solutions (15 μL) were mixed with DMPO (0.3 M, 15 μL), $FeSO_4$ (10 mM, 15 μL), and H_2O_2 (10 mM, 15 μL) in a phosphate buffer solution (pH 7.4) and then transferred to a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The instrumental parameters were as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 200; and sweep time, 30 s.

Alkyl Radical Scavenging Effects of Floridoside and D-Isofloridoside. Alkyl radicals generated by AAPH were measured according to the previous report of Hiramoto et al. (*16*). Briefly, 15 μL of 40 mM AAPH was mixed with 15 μL of phosphate-buffered saline (PBS), 20 μL of 40 mM 4-POBN, and 15 μL of the floridoside and D-isofloridoside solutions. 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.

Superoxide Anion Radical Scavenging Effects of Floridoside and D-Isofloridoside. Superoxide anion radicals are known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (*17*). The reaction mixtures containing 0.8 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO (15 μL), and the indicated concentrations of floridoside and D-isofloridoside were irradiated for 1 min under an UV lamp at 365 nm. The reaction mixture was transferred to a 100 μL quartz capillary tube of the ESR spectrometer for measurement. The instrumental parameters were as follows: magnetic field, 336.5 ± 5 mT; microware power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; scan width, 10 mT.

Cell Culture and Cytotoxicity (MTT) Assays in Vitro. MRC-5, RAW264.7, and HT-1080 cell lines were cultured in DMEM medium. The suspension-dependent HL-60 cell line was cultured in Roswell Park

Memorial Institute medium (RPMI). All the cell lines were supplemented with 10% heated inactivated FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under a humidified atmosphere with 95% air and 5% CO_2. Cell viability or cytotoxicity was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (a tetrazolium dye) assay (*18*). Various concentrations of floridoside and D-isofloridoside were cultured in microtiter 96-well plates (1.5×10^3 cells/well) with serum-free medium for 24 h. Floridoside and D-isofloridoside were dissolved in 10% DMSO and sterilized by being filtered through a 0.22 μm filter membrane. The final concentration of DMSO in culture media was kept below 0.1%. Sequentially, 20 μL of the MTT dye solution was added to each well. After incubation for 4 h, 200 μL of the DMSO solution was added to dissolve the formazan crystals and the absorbance was read using a GENios microplate reader (TECAN Austria Gmbh) at a wavelength of 540 nm by measuring the optical density (OD) of each well. The viabilities of cells treated with various concentrations of floridoside and D-isofloridoside were calculated using the equation cell viability (%) = $OD_s/OD_b \times 100\%$, where OD_s is the absorbance value of the tested samples of floridoside and D-isofloridoside and OD_b is the absorbance value of the blank.

Measurement of Intracellular ROS Levels. Intracellular ROS levels were measured by detecting the fluorescence intensity of the oxidant-sensitive DCFH-DA (*19*). RAW264.7 cells growing in fluorescence microtiter 96-well plates were labeled with 20 μM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated for 20 min in the dark at room temperature. Then the cells were treated with different concentrations of the tested samples and incubated for an additional 1 h. After the cells had been washed with PBS three times, 0.3 mM H_2O_2 (100 μL) was added. The intensity of the fluorescence signal was detected time-dependently with an excitation wavelength (Ex) of 485 nm and an emission wavelength (Em) of 535 nm using a GENios microplate reader. Dose-dependent and time-dependent effects of treatments were group plotted and compared with the fluorescence intensity of control and blank groups.

Membrane Protein Oxidation Assay. The level of oxidation of cellular proteins was reduced from carbonyl moieties generated by ROS attack, and then the level of damaged membrane protein was measured by determining the protein content (*20*). The lysate was aliquoted into microtubes (0.5 mL) and incubated with the indicated concentrations of floridoside and D-isofloridoside 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 remaining pellet was resuspended in 150 μL of 0.2% 2,4-dinitrophenylhydrazine in 2 mM HCl and allowed to stand at room temperature for 40 min. The reaction mixture was vortexed in 10 min intervals to facilitate the reaction with proteins. The protein was precipitated again with 20% trichloroacetic acid, and the pellet was washed three times with an 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 450 nm. A blank was prepared with a parallel procedure using 2 mM HCl alone instead of 2,4-dinitrophenylhydrazine reagent. The carbonyl group content was expressed in nanomoles per milligram of protein, using a molar absorption coefficient of 22000 M^{-1} cm^{-1}.

Myeloperoxidase (MPO) Activity Assay. MPO, an indicator of polymorphonuclear leukocyte accumulation, was measured by the *o*-dianisidine method with modifications (*21*). HL-60 cells were suspended in RPMI-1640 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-R (0.05 μg/mL) at 37 °C for 30 min. 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 was added to the cells. The amount of released MPO was spectrophotometrically measured at a wavelength of 460 nm, and the inhibition percents of floridoside and D-isofloridoside on MPO activity were calculated using the equation MPO inhibition activity (%) = $[1 - (A_s - A_b)/(A_c - A_b)] \times 100\%$, where A_s is the absorbance value of floridoside and D-isofloridoside, A_b is the absorbance value of the blank, and A_c is the absorbance value of the control. Cells in the control and blank were grown in the absence of floridoside and D-isofloridoside, and a blank was made without the stimulation with TNF-α.

DNA Extraction and Polymerase Chain Reaction (PCR) Analysis. Total RNA from the RAW264.7 cell line cultured in 10 cm dishes for 24 h was isolated using Trizol reagent according to the supplier's protocol and measured at 260 nm. In brief, 1 μg of total RNA was used to synthesize first-strand cDNAs with Moloney murine leukemia virus reverse transcriptase (M-MLV RT), which is cDNA synthesis with long mRNA templates (> 5 kb) (*22*). Then, the first-strand cDNA was diluted with water in a ratio of 1:9, and the aliquots were processed to amplify the genes related to oxidative defense mechanisms of GSH and SOD. The sequences of the GSH have been published as follows: GSH sense, 5′-GGGTGTTCTTCTTTGTGATCC-3′; and GSH antisense, 5′-TCA-CAGGAGGAATCTTCATC-3′. The SOD primers were based on the reported human sequence as follows: SOD sense, 5′-GTA ATG GAC CAG TGA AGG TGT G-3′; and SOD antisense, 5′-CAA TTA CAC CAC AAG CCA AAC G-3′. Finally, the cDNA amplification was determined with Top-*Taq* polymerase (Core-Bio-System) and primers (GSH and SOD) via the PCR technique. The cycling conditions were as follows: initial denaturation at 94 °C for 4 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 4 min, and final extension at 72 °C for 5 min. Tubes were heated at 95 °C for 3 min and snap-cooled on ice for at least 3 min. Samples were injected onto a 1.5% agarose gel for 30 min at 100 V followed by dying with ethidium bromide for 30 min, and then the sample was subjected to UV light at 305 nm so an image could be recorded (Gel Image Analysis System).

Inhibition Effects of Floridoside and D-Isofloridoside on MMPs. Inhibition effects of floridoside and D-isofloridoside against MMP-2 and MMP-9 were determined by zymography as described previously (*23*). Cells of the HT-1080 cell line in serum-free DMEM were seeded in 24-well plates at a density of 2×10^5 cells/well and pretreated with different concentrations of the tested samples for 1 h. Expression of MMPs was stimulated by treatment with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL), and the cells were incubated for 36 h. After incubation, conditioned media were collected and their protein contents were determined by the Bradford protein determination method. A cell-conditioned medium was subjected to substrate gel electrophoresis. Equal amounts of protein-containing conditioned media were applied under nonreducing conditions on 10% polyacrylamide gels containing 1.5 mg/mL gelatin. After electrophoresis, polyacrylamide gels were washed with 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 at room temperature to remove sodium dodecyl sulfate. Gels were then incubated overnight at 37 °C in a developing buffer containing 10 mM $CaCl_2$, 50 mM Tris-HCl, and 150 mM NaCl to digest gelatin by MMPs. Areas of gelatin hydrolyzed by MMPs were visualized as clear zones against a blue background by Coomassie Blue staining, and the intensities of the bands were estimated by densitometry (Multi Gauge version 3.0, Fujifilm Life Science, Tokyo, Japan).

Statistical Analysis. The data are shown as the means of three replicate determinations with the standard deviation (SD), and statistical comparisons were made with the Student's *t* test. The values were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Structural Elucidation of Floridoside and D-Isofloridoside. The LREIMS of compound **1** was consistent with a molecular formula of $C_9H_{18}O_8$, showing an ion peak at *m*/*z* 255.20 $[M + H]^+$. The calculated unsaturated degree was one corresponding to one ring based on the ^1H and ^{13}C NMR spectral data. In the ^1H NMR spectrum, 18 hydrogen atoms were observed for compound **1**. The ^1H NMR spectrum displayed characteristics of a typical saccharide moiety, illustrating chemical shifts of monosaccharide from 3 to 5 ppm, including 3.47, 3.51, 3.69, 3.76, and 4.81 ppm, 4.35, 4.38, 4.56, and 4.58 ppm signals from hydroxyl groups, and chemical shifts of the glycerol moiety (δ_H) at 3.41, 3.47, 4.44, and 4.47 ppm. The ^{13}C and ^{13}C DEPT spectra suggested nine carbon signals consisting of there methylenes (60.6, 60.8, and 61.0 ppm), six methines (99.4, 80.6, 71.3, 69.7, 68.9, and 68.8 ppm), and no quaternary carbon atoms. The

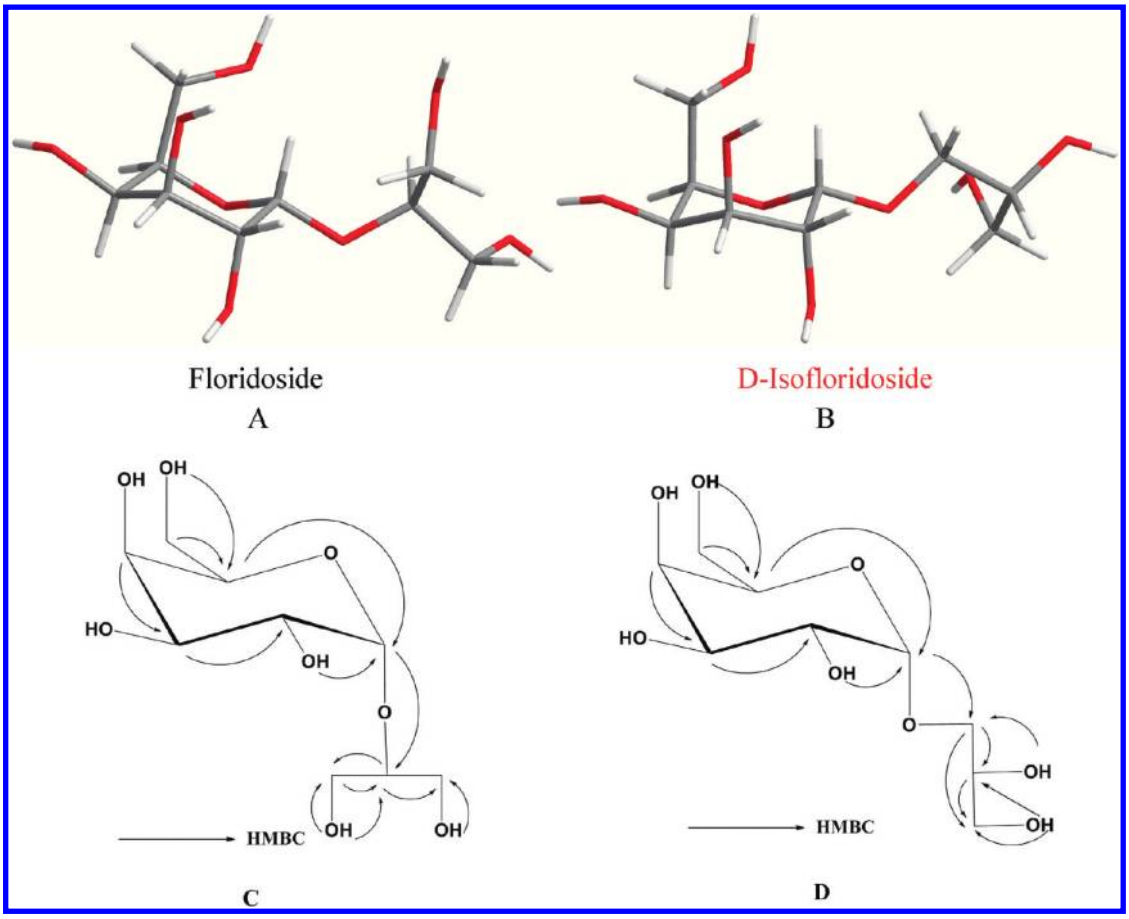

Figure 1. (**A**) Chemical structures of floridoside [α-D-galactopyranosyl-(1−2)-L-glycerol] and (**B**) D-isofloridoside [α-D-galactopyranosyl-(1−1)-D-glycerol]. (**C**) HMBC correlations of floridoside. (**D**) HMBC correlations of D-Isofloridoside.

fragment ion peaks in the mass spectrum provided more evidence of these partial moieties of compound **1**, such as *m*/*z* 163.10 (monosaccharide) $[M - C_3H_3O_3]^+$ and 91.05 (glycerol) $[M - C_6H_{11}O_4]^+$. The one-bond linkages between protons and carbons were clearly supported by HMQC analysis except for hydroxyl protons. The long correlations between protons and carbons led to the assignments of two moieties based on HMBC data, such as among 3.47 and 80.6, 3.41, and 60.6 and 60.8. The correlation between 4.81 and 80.6 indicated the link between glycoside group and glycerol residue (see **Table 1**). On the basis of the comprehensive spectral analysis and data published previously, compound **1** was elucidated as a known [α-D-galactopyranosyl-(1−2)-glycerol] (floridoside) as shown in **Figure 1A**.

The LREIMS of compound **2** showed the consistent spectrum with compound **1** indicating the same moieties. The ^1H and ^{13}C NMR spectra of compound **2** indicated that the substituted position of the glycerol moiety was C-1. The connection with the terminal carbon in the monosaccharide part was clearly assigned according to the correlation between H-1′ and C-1 in the HMBC spectrum. Compound **2** was elucidated as a known isomer of compound **1** as shown in **Figure 1B**, named D-Isofloridoside on the basis of one- and two-dimensional NMR spectra and published literature data (*24*).

Free Radical Savenging Activity by ESR. Antioxidant activities of floridoside and D-isofloridoside were assessed by the scavenging effect on DPPH, hydroxyl, alkyl, and superoxide anion free radicals using the ESR spin trapping technique. The violet-colored free stable radical DPPH is used as an ESR standard and as a colorimetric reagent for redox processes because it can be reduced to a nonradical colorless DPPH-H by the reaction occurring when accepting an electron or hydrogen atom in the presence of a hydrogen-donating antioxidant (*25*). The DPPH scavenging activities of floridoside and D-isofloridoside are presented in **Figure 2A,B**. The results indicate statistically significant scavenging activities at various concentrations (100, 25, 10, and 1 μM) of floridoside and D-isofloridoside, and the scavenging activities of floridoside and D-isofloridoside were 85.4 and 84.3%, respectively, at 100 μM. The IC_{50} values of floridoside and D-isofloridoside were 39.3 and 41.8 μM, respectively (**Table 2**).

The hydroxyl radical, $^{\bullet}OH$, is the neutral form of the hydroxide ion. It plays an important role in radical chemistry as a consequence. In this study, the capacity of floridoside and D-isofloridoside to inhibit hydroxyl radical generated by the Fenton reaction was measured, and the results are illustrated in **Figure 2A,B**. Hydroxyl radicals generated by the Fenton system were identified by their ability to form nitroxide adducts from the commonly used DMPO spin trap. This spin trap has a greater ability to trap oxygen-centered radicals than other nitrone spin traps (*26*). The DMPO−OH radical adduct exhibits a characteristic ESR response which can be detected by an ESR spectrometer. Floridoside and D-isofloridoside exhibited significant potency ($p < 0.05$) in scavenging hydroxyl radical at various concentrations (100, 25, 10, and 1 μM) at scavenging percentages of 95% (**1**) and 98% (**2**), 72% (**1**) and 75% (**2**), 36% (**1**) and 36% (**2**), and 13% (**1**) and 21% (**2**), respectively. Both floridoside and D-isofloridoside exhibited significant potency ($p < 0.05$) to scavenge hydroxyl radical at a low concentration with IC_{50} values of 27.4 and 22.7 μM, respectively (**Table 2**).

The scavenging activities of floridoside and D-isofloridoside on the alkyl radical were tested with an ESR spectrometer. Alkyl

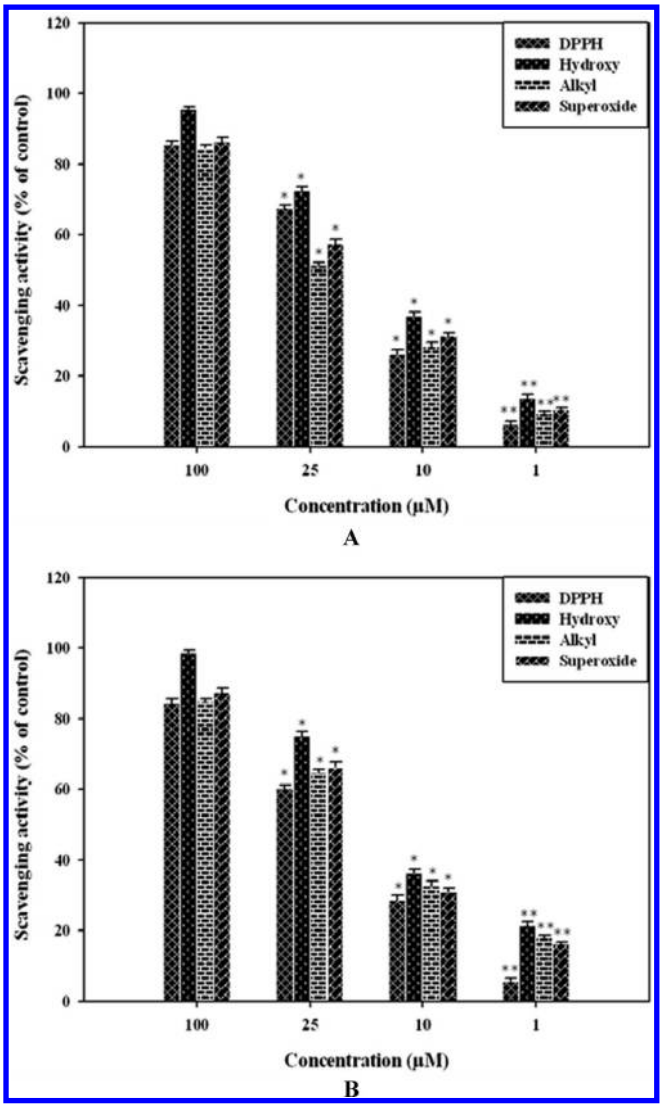

Figure 2. (**A** and **B**) Free radical scavenging activities of floridoside and D-isofloridoside from *L. undulata* by ESR, respectively. Different bars indicate the different radicals, including DPPH, hydroxyl, alkyl, and superoxide radicals. Values are expressed as the means ± SD of three replicate experiments. One asterisk means $p < 0.05$; two asterisks mean $p < 0.01$.

Table 2. IC_{50} Values of Floridoside and D-Isofloridoside from *L. undulata*

		IC_{50}[a] ± SD (μM)	
	radical	floridoside	D-isofloridoside
1	DPPH	39.3 ± 1.4	41.8 ± 0.8
2	hydroxyl	27.4 ± 0.7	22.7 ± 0.6
3	alkyl	43.7 ± 1.4	32.3 ± 1.6
4	superoxide	39.4 ± 1.1	33.6 ± 1.6

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

radical is a univalent radical consisting of carbon and hydrogen atoms, arranged in a chain. Alkyl radical was generated by the decomposition of AAPH, a water-soluble peroxyl radical initiator, and then the alkyl radical was trapped by 4-POBN, at 37 °C for 30 min with a decrease in the magnitude of the ESR signals with the close increasing (*27*). The scavenging activity was observed in a dose-dependent manner with floridoside and D-isofloridoside (**Figure 2A,B**). Floridoside and D-isofloridoside exhibited alkyl radical scavenging activities, and the scavenging activities were 84.7 and 91.4% at 100 μM, respectively. Both of them had strong alkyl radical scavenging activity as determined in a dose-dependent manner. The results were statistically significant ($p < 0.05$) compared with the control. At various tested concentrations of floridoside and D-isofloridoside, the results showed strong activity with IC_{50} values of 43.7 and 32.3 μM, respectively (**Table 2**).

The electron spin resonance spectrum of superoxide anion radical adsorbed on floridoside and D-isofloridoside was detected directly at room temperature. Superoxide anion radical was generated during the oxidation reactions that occur in the body; it is a weak oxidant, but it can transform to a powerful and dangerous hydroxyl radical in the presence of metals such as iron and copper, subsequently inducing cell damage and ROS-related diseases. In this study, superoxide anion radical was generated in a riboflavin/EDTA system by UV irradiation (*28*). The superoxide anion radical scavenging activity of floridoside and D-isofloridoside are presented in **Figure 2A,B**. Floridoside and D-isofloridoside followed a dose-dependent manner in scavenging superoxide anion radical. Strong scavanging activities were observed at various concentrations of floridoside and D-isofloridoside with IC_{50} values of 39.4 and 33.6 μM, respectively (**Table 2**).

In this research, four kinds of typical free radicals, including DPPH, hydroxyl, superoxide anion, and alkyl radicals, were selected for the evaluation of the scavenging capacity of floridoside and D-isofloridoside. The results reveal that floridoside and D-isofloridoside had strong scavenging activity on hydroxyl and alkyl radicals. However, D-isofloridoside had stronger scavenging activity than floridoside on hydroxyl and alkyl radicals at 22.7 and 27.4 μM, respectively (**Table 2**). To the best of our knowledge, it is the first time the antioxidant activity of floridoside and D-isofloridoside had been evaluated by the ESR method.

Cell Viability of Floridoside and D-Isofloridoside. The cytotoxic effects of floridoside and D-isofloridoside at molecular and cellular levels were tested on MRC-5, RAW264.7, HL-60, and HT-1080 cultured cell lines. The results suggest that floridoside and D-isofloridoside displayed no cytotoxic effects at a concentration of 100 μM on these four tested cultured cell lines as shown in **Figure 3**. These results provide scientific evidence of the subsequent research in cultured cell systems.

Intracellular ROS Estimations. This study was conducted to examine the generation of intracellular ROS using RAW264.7 cells which were preincubated with floridoside and D-isofloridoside at concentrations of 100, 25, 10, and 1 μM for 1 h and labeled with fluorescence probe DCFH-DA as described in Materials and Methods. When DCFH-DA enters viable cells, it can be deacetylated by intracellular esterases to form 2′,7′-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell to produce 2′,7′-dichlorofluorescein (DCF), which is a fluorescent reagent (*29*). As shown in **Figure 4**, fluorescence emitted by DCF due to ROS-mediated oxidation of DCFH followed a time course increment up to 240 min. It was found that preincubation with floridoside and D-isofloridoside decreased the DCF fluorescence in a dose- and time-dependent manner. Even after incubation for 30 min, floridoside and D-isofloridoside exerted a considerable scavenging activity at the lowest concentration of 1 μM. Meanwhile, at the highest concentration of 100 μM, floridoside and D-isofloridoside were able to scavenge ROS significantly throughout the incubation time. After incubation for 210 min, floridoside and D-isofloridoside reduced the production of ROS by 68.8% at a concentration of 100 μM. The results revealed that the antioxidant activities of floridoside and D-isofloridoside are caused by direct scavenging

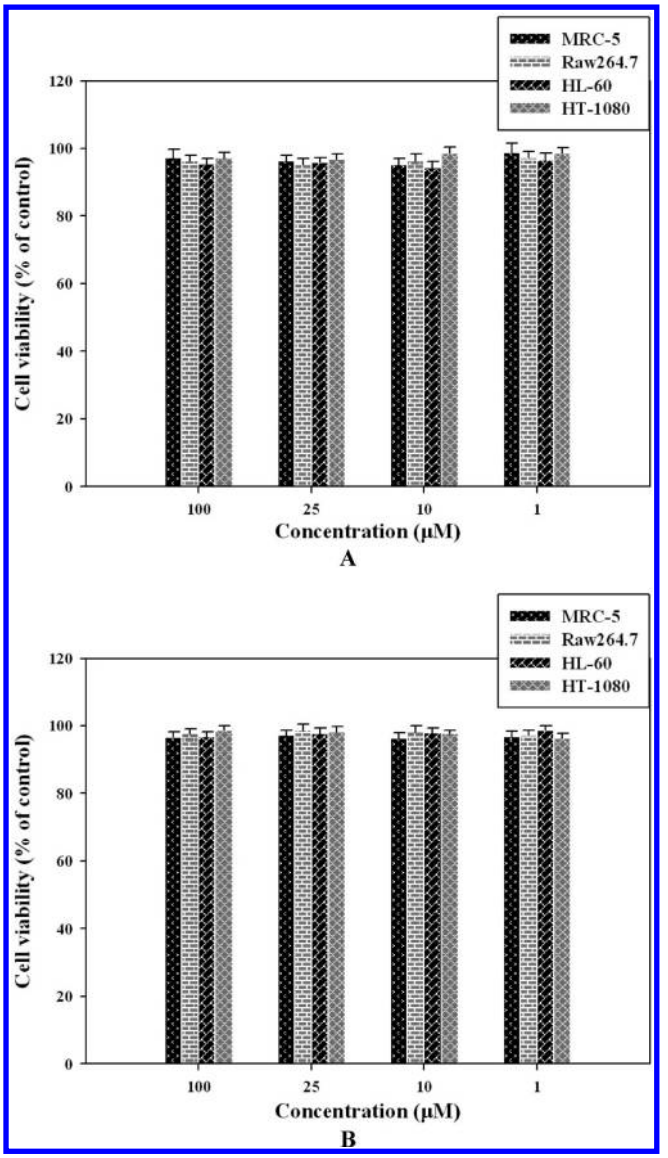

Figure 3. Cell viability determined by the MTT assay. (**A** and **B**) Cytotoxic effects of floridoside and D-isofloridoside, respectively, from *L. undulata* on MRC-5, RAW246.7, HL-60, and HT-1080 cell lines. Values are expressed as the means ± SD of three replicate experiments.

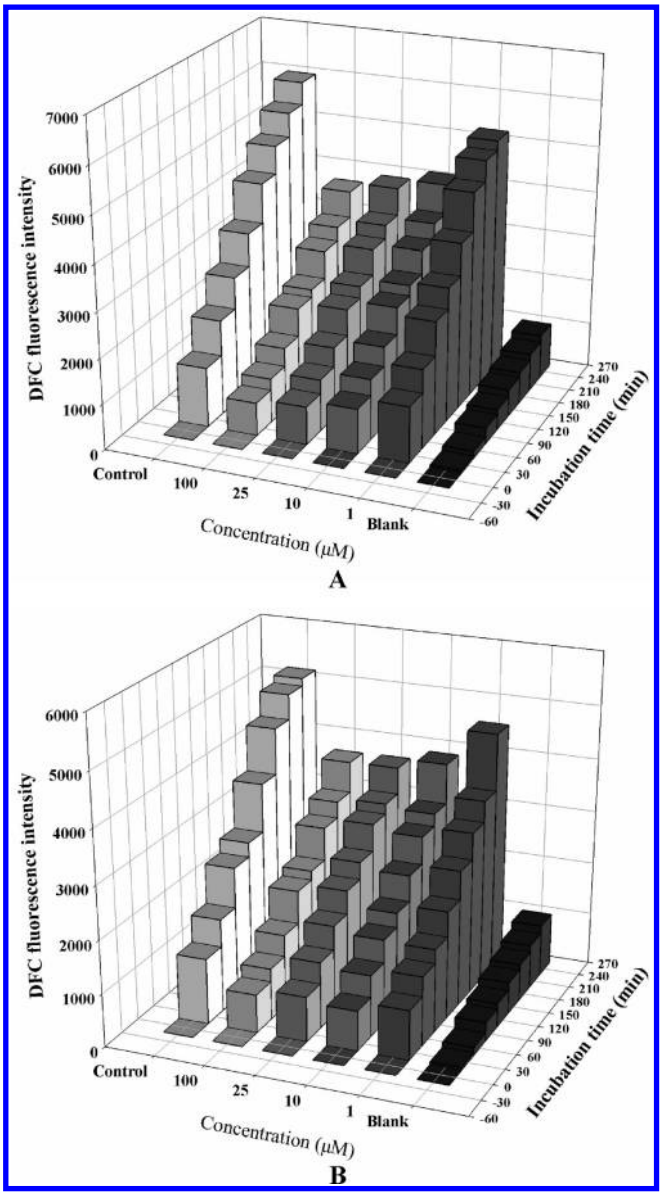

Figure 4. RAW264.7 cell lines were exposed to serial dilutions of floridoside and D-isofloridoside and then challenged by H_2O_2 to produce ROS. Intracellular radical scavenging activities of *L. undulata* at the different concentrations (100, 25, 10, and 1 μM). The ROS level is represented as DCF fluorescence. Values are expressed as the means ± SD of three replicate experiments.

of cellular ROS. Therefore, both floridoside and D-isofloridoside could be developed as potent antioxidant candidates for inhibiting cellular ROS formation.

Effect of Floridoside and D-Isofloridoside on Membrane Protein Oxidation. Oxidative damage of membranes is considered as the main factor in induction of many diseases. The amino acids, lysine, arginine, proline, and histidine, present in membrane proteins are highly susceptible to oxidative attack by ROS and result in accumulation of their carbonyl moieties (mainly aldehydes and ketones) (*30*). Effects of floridoside and D-isofloridoside on membrane protein oxidation were tested using a cultured RAW264.7 cell line as described in Materials and Methods. The results are depicted in **Figure 5A**, when mouse macrophage membranes were exposed to $HO^{\bullet}$ generated by the $Fe^{2+}-H_2O_2$ Fenton reaction, and the extent of membrane protein oxidation was increased by monitoring the increase in carbonyl group content. The contents of protein carbonyl groups in floridoside- and D-isofloridoside-treated groups were lower than those of the control group. Pretreatment with floridoside for 30 min dose-dependently inhibited the oxidation of the membrane protein which was indicated by protein carbonyl group contents of 0.53 (100 μM), 0.89 (25 μM), 1.1 (10 μM), and 1.60 (1 μM); D-isofloridoside pretreatment for 30 min dose-dependently inhibited the oxidation of membrane protein, indicated by protein carbonyl group contents of 0.43 (100 μM), 0.75 (25 μM), 1.1 (10 μM), and 1.42 (1 μM).

Effect of Floridoside and D-Isofloridoside on MPO. MPO is a leukocyte-derived heme peroxidase that has long been considered a microbial enzyme centrally linked to the unspecific immune defense system. MPO plays an important role in the production of oxidants in polymorphonuclear neutrophils (PMNs). To determine the tissue's MPO content, the widely used method is to quantify the degree of neutrophil infiltration. 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

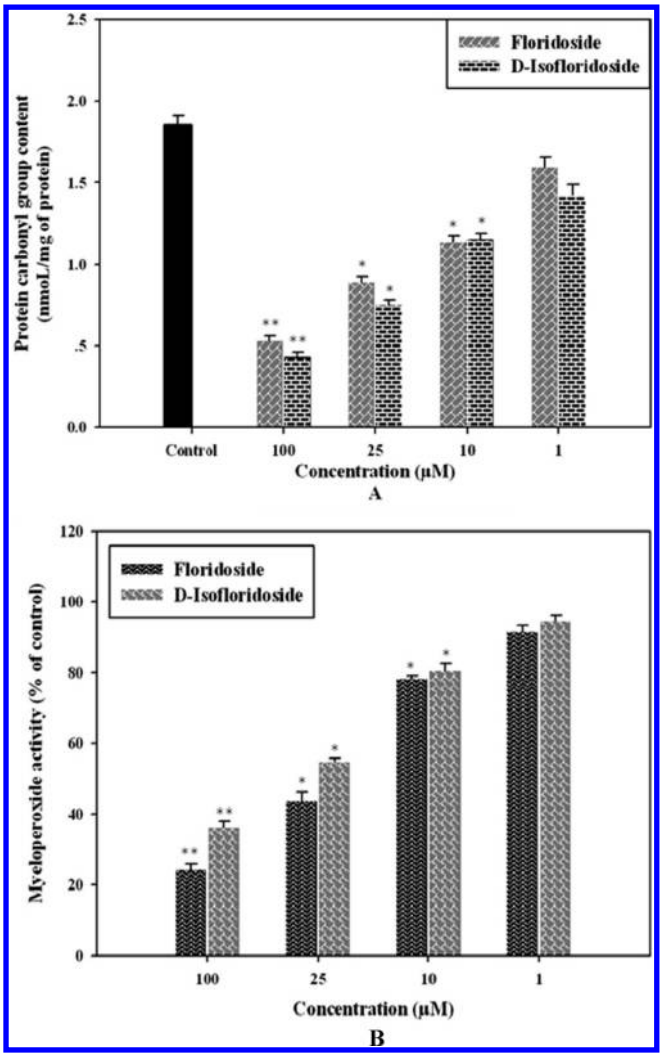

Figure 5. (**A**) Inhibitory effects of floridoside and D-isofloridoside from *L. undulata* on cell membrane protein oxidation in the RAW264.7 cell line. Values are expressed as the means ± SD of three replicate experiments. (**B**) Inhibitory effects of floridoside and D-isofloridoside from *L. undulata* on MPO activity in the HL-60 cell line. Values are expressed as the means ± SD of three replicate experiments. One asterisk means $p < 0.05$; two asterisks mean $p < 0.01$.

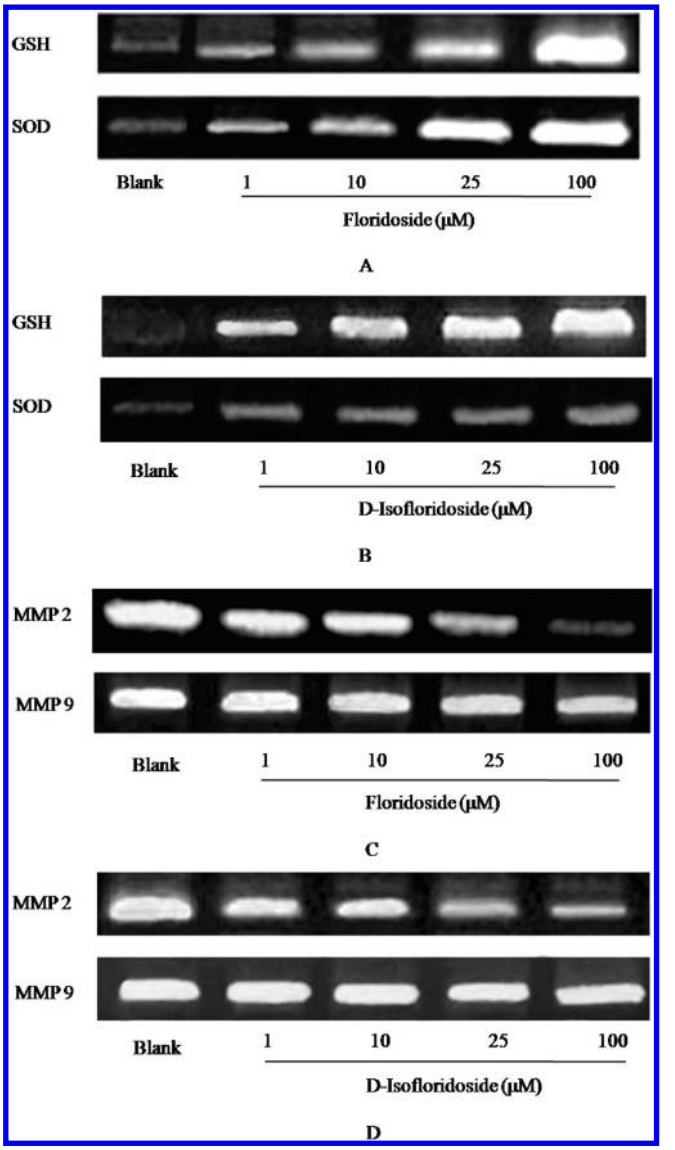

Figure 6. (**A** and **B**) mRNA expressions of genes of floridoside and D-isofloriodside, which are related to enzymatic antioxidant defense and DNA repair by PCR, and normalized by GSH and SOD. (**C** and **D**) Effect of floridodside and D-isofloriodside, respectively, on protein expression of MMP-2 and MMP-9 in the HT-1080 cell line determined by gelatin zymography.

disorders (*31*). We assessed MPO activity in HL-60 cultured cells preincubated with various concentrations of floridoside and D-isofloridoside for 30 min followed by stimulation with TNF-α for 30 min. The results are shown in **Figure 5B**; floridoside and D-isofloridoside could reduce MPO activity compared to the TNF-α-stimulated control group. The activity of MPO was dose-dependently inhibited by the pretreatment at different concentrations. The inhibitory activities of floridoside were 91.8% (100 μM), 78.4% (25 μM), 43.8% (10 μM), and 24.4% (1 μM), and the inhibitory activities of D-isofloridoside were 94.7% (100 μM), 80.7% (25 μM), 55.0% (10 μM), and 36.3% (1 μM). Accordingly, these results suggested that floridoside and D-isofloridoside could be used as cellular antioxidants via an indirect way in living cell systems.

Effects of Floridoside and D-Isofloridoside on GSH and SOD Expression in the Gene Level by RT-PCR. Free radicals and ROS generated in cells are effectively scavenged by the antioxidant defense system which consists of antioxidant enzymes such as GSH and SOD. When the activity of the antioxidant defense system decreases or the ROS production increases, an oxidative stress may occur (*16*). GSH is the most abundant low-molecular weight thiol compound in cells and plays an important role in antioxidant defense and detoxification. GSH provides primary defense against oxidative stress by its ability to scavenge free radicals or participates in the reduction of H_2O_2 catalyzed by GSH peroxidase. A decrease in the level of GSH can compromise cell defenses against oxidative damage and may lead to cell death (*32*). Treatment of high-concentration floridoside and D-isofloridoside at 100 μM resulted in a significant increase in GSH gene expression levels. Also, superoxide dismutase (SOD) catalyzes the destruction of superoxide radicals and hence protects oxygen-metabolizing cells from the harmful effects of these free radicals. Floridoside and D-isofloridoside treatment could lead to decreased oxidative stress and thus could be part of the mechanism for the defensive effect of floridoside and D-isofloridoside against oxidative stress.

To examine whether floridoside and D-isofloridoside can affect the expression of GSH and SOD at the gene level, RAW264.7

cells were treated with the measured floridoside and D-isofloridoside at various concentrations, and the effective trend of cDNA was measured by the PCR technique as illustrated in **Figure 6A**. The tested floridoside and D-isofloridoside exhibited dose-dependent activity to improve the expression on both GSH and SOD enzymes clearly. At the lowest concentration of 1 μM, an obvious band of cDNA can be observed in comparison to the nontreated blank group. The increased levels of regulation of GSH and SOD gene expression were observed in a concentration-dependent manner. Thus, the result demonstrated that the antioxidant effects of floridoside and D-isofloridoside were again proven at the gene level.

Inhibition Effect of Floridoside and D-Isofloridoside on MMP-9 and MMP-2 Activities. The inhibitory effects of floridoside and D-isofloridoside against MMP-2 and MMP-9 activities were evaluated. The cultured medium of HT-1080 cells was subjected to the MMP-2 and MMP-9 activity-based zymography in the presence of various concentrations of floridoside and D-isofloridoside. As shown in **Figure 6B**, treatment with floridoside and D-isofloridoside reduced significantly the expression level of MMP-2 and MMP-9 compared with that of the PMA-stimulated group. At different concentrations (1, 10, 25, and 100 μM), floridoside exhibited MMP-2 activity similar to that in nonstimulated cells with PMA, and a relatively lower level of expression of MMP-9 versus MMP-2. The similar expression of D-isofloridoside was also observed.

The structure−activity relationships (SARs) could be suggested according to the structural characteristics of floridoside and D-isofloridoside. These two glycosides are the isomers with a different linkage; the galactose group and glycerol residue can donate a hydrogeon ion easily, and then excited hydroxyl groups can attract electrons easily. Hence, it is why these isolates are more effective with respect to antioxidant activity. Meanwhile, small differences can also be found between these compounds due to the different connection positions.

This study demonstrates significant antioxidant character and inhibition of MMP-2 and MMP-9 by the tested floridoside and D-isofloridoside from *L. undulata*. On the basis of the results presented above, two compounds can effectively scavenge free radicals, where floridoside is more effective than D-isofloridoside. Although floridoside and D-isofloridoside inhibited MMP-2 expression, there was no obvious inhibition on MMP-9. Future research is needed for their practical application as antioxidants and/or inhibitors related to antioxidation and MMP-reduced diseases.

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