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Isolation and Identification of Phlorotannins from *Ecklonia stolonifera* with Antioxidant and Hepatoprotective Properties in Tacrine-Treated HepG2 Cells

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ABSTRACT: Four kinds of phlorotannins having antioxidant activity were isolated from the ethyl acetate fraction of *Ecklonia stolonifera* ethanolic extract. The structures of the phlorotannins were determined on the basis of spectroscopic evidence, including 1D and 2D nuclear magnetic resonance. The isolated phlorotannins showed potential radical-scavenging activities against 2,2-diphenyl-1-picrylhydrazyl and suppressed the intracellular reactive oxygen species in tacrine-treated HepG2 cells. Among them, eckol and 2-phloroeckol showed hepatoprotective activity in tacrine-treated HepG2 cells; however, phlorofucofuroeckol B and 6,6′-bieckol did not show the activity, even though having high antioxidant activity. Both eckol and 2-phloroeckol inhibited the expression of Fas-mediated cell-death proteins, including tBid, caspase-3, and poly(ADP-ribose) polymerase, and suppressed the release of cytochrome *c* from mitochondria to cytosol in a dose-dependent manner in tacrine-treated HepG2 cells. These results suggest that eckol and 2-phloroeckol are the principal hepatoprotective constituents of the ethyl acetate fraction of *E. stolonifera* ethanolic extract.

KEYWORDS: *2-phloroeckol, eckol, phlorofucofuroeckol B, 6,6′-bieckol, antioxidant, Ecklonia stolonifera, hepatoprotection*

INTRODUCTION

Oxidative stress induced by excessive reactive oxygen species (ROS) during the metabolism of drugs provokes cellular damage by targeting various macromolecules, such as proteins, lipids, and nucleic acids.[1] Enhanced ROS production induces apoptosis via the intrinsic apoptotic pathway resulting from mitochondrial dysfunction and the extrinsic pathway involved in cell surface death receptors, such as Fas (CD95) and TNFR1.[2] Fas activation results in Fas receptor clustering at the plasma membrane and activates caspase-8, which subsequently triggers the proteolytic activation of other caspases, including caspase-3, -6, and -9.[2,3] The activated caspase-8 also cleaves the BH3 interacting domain death agonist (Bid) to form truncated Bid (tBid), which induces mitochondrial damage, cell shrinkage, and nuclear condensation.[4] Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates and is thus relevant to prevent chronic diseases such as cancer, heart disease, and inflammation.[5] Among dietary antioxidants, polyphenols are most abundant and exert antioxidant properties through various mechanisms of actions including the scavenging of free radicals and activation of signal proteins related to cellular antioxidation, thereby preventing damage to intracellular macromolecules and eventually cell death.[6] Recent studies have shown that polyphenols are promising compounds to ameliorate oxidative stress and consequently attenuate hepatotoxicity in HepG2 cells and mice.[7−10]

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine hydrochloride) exhibits hepatotoxicity with oxidative stress, which was evidenced by enhanced ROS production and glutathione (GSH) depletion.[11] Tacrine-induced hepatotoxicity using HepG2 cells is a well-known in vitro system used to screen the hepatoprotective activity of plant extracts and liver-protective drugs.[12,13] In this study, HepG2 cells were used to assess the hepatoprotective and antioxidant activities of the isolated compounds on tacrine-induced hepatotoxicity.

Marine algal polyphenols, phlorotannins, which are known only in brown algae, are restricted to polymers of phloroglucinol (1,3,5-trihydroxybenzene).[14] Phlorotannins have been shown to possess diverse biological activities, including antioxidation,[15] antimelanogenesis,[16] and chemoprevention.[12] Among them, triphlorethol-A and eckol isolated from *Ecklonia cava* have been reported to protect cells against oxidative damage by reducing ROS or enhancing the cellular antioxidant activity.[17,18] We have recently isolated phlorotannins from *Ecklonia stolonifera* Okamura and demonstrated antioxidant and anti-inflammatory properties of the compounds.[19,20] In the present study, four kinds of phlorotannins showing high antioxidant activity were isolated from *E. stolonifera* through silica, gel, and reverse-phase column chromatographies based on antioxidant-guided separation. The objectives of this study are to investigate the antioxidant or hepatoprotective properties of phlorotannins isolated from *E. stolonifera* on tacrine-treated HepG2 cells. The findings demonstrate that the phlorotannins are likely the principal constituent for the antioxidant or hepatoprotective activities in

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E. stolonifera extract and may be used as a source of nutraceuticals for antioxidant or hepatoprotective effects.

MATERIALS AND METHODS

Plant Materials. *E. stolonifera* was collected along the coast of Busan, South Korea, in August 2010, with a voucher specimen deposited in our laboratory (H. R. Kim). The samples were dried in an air dryer (50 °C) for 72 h. A dried sample was ground with a hammer mill and stored at −20 °C until used.

Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2′,7′-dichlorofluorescein diacetate (DCF-DA), dimethyl sulfoxide (DMSO), and tacrine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay Kit was obtained from Promega (Madison, WI, USA). Minimum essential medium (MEM), penicillin−streptomycin mixture, 0.25% trypsin−ethylenediaminetetraacetate (EDTA), fetal bovine serum (FBS), sodium pyruvate, and nonessential amino acids were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). All solvents were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Polyclonal antibodies against poly(ADP-ribose) polymerase (PARP), Bid, cytochrome *c*, *β*-actin, and horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fas and cleaved caspase-3 (active form) were purchased from Millipore (Billerica, MA, USA), and tBid was purchased from Cell Signaling Technology (Danvers, MA, USA). The enhanced chemiluminescence (ECL) detection kit was purchased from GE Healthcare (Piscataway, NJ, USA). MitoTracker red CMXRos was purchased from Molecular Probes Inc. (Grand Island, NY, USA). Catalase and glutathione *S*-transferase (GST) assay kit were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Extraction and Isolation of Phlorotannins. The extractions and separations of antioxidant compounds were performed according to the method of Kim et al.[19] The dried powder (3 kg) of *E. stolonifera* was refluxed with 96% ethyl alcohol (EtOH, 3 × 9 L) for 3 h. The concentrated extract was suspended in water and partitioned with *n*-hexane, ethyl acetate (EtOAc), *n*-butanol solvents in sequence and yielded the *n*-hexane (93 g), EtOAc (78 g), *n*-butanol (87 g), and water (430 g) fractions. The EtOAc fraction, which exhibited the highest antioxidant activity on DPPH radical scavenging activity, was dissolved in methanol and applied to a silica gel (70−230 mesh, Merck) column (100 × 10 cm), eluted with a stepwise mixture of EtOAc and methanol (50:1−5:1, v/v, each 2 L). Fraction 6 eluted with a mixture of EtOAc and methanol (25:1, v/v) showed high antioxidant activity in DPPH radical scavenging activity and ws pooled and dried (32 g).

An aliquot of fraction 6 from the silica gel column was subjected to preparative size exclusion column of Asahipak GS-310 (500 × 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 6 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 subfractions (GS1−GS5). The GS4 fraction (2.02 g) showing high antioxidant activity was chromatographed over a Shim-pack PREP-ODS column (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 the GS4 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% of solvent B for 60 min and re-equilibration of the column with 20% solvent B for 10 min. The flow rate was 7.0 mL/min, and detection was performed at 245 nm. The fraction gave six subfractions (GS4-ODS1−GS4-ODS6). GS4-ODS1 (85 mg), GS4-ODS4 (550 mg), GS4-ODS5 (55 mg), and GS4-ODS6 (63 mg) were purified by the same HPLC system with Luna RP-18 [Luna C18(2), 5 μm, 250 × 10 mm, Phenomenex, Torrance, CA, USA] and with the same mobile phase systems at a flow rate of 3.0 mL/min. Isolated fractions GS4-ODS1 (2-phloroeckol), GS4-ODS4 (eckol), GS4-ODS5 (phlorofucofuroeckol B), and GS4-ODS6 (6,6′-bieckol) were used in this study.

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

DPPH Radical-Scavenging Assay. DPPH radical-scavenging activities of isolated compounds were tested according to the modified method of Nanjo et al.[21] Twenty microliters of sample solution (or DMSO as control) was mixed with 40 μL of DPPH (100 μM) in a 96-well microplate and incubated at room temperature for 30 min. The absorbance at 520 nm was measured with a microplate reader (Glomax Multi Detection System, Promega). The values of the 50% effective concentration (EC_{50}) were determined as the mean effective concentration to exert half of antioxidant and were calculated by three different concentrations of sample.

Cell Viability and Cytoprotective Assay. HepG2 (human hepatocarcinoma) cell line was purchased from American Type Culture Collection (HB-8065; Manassas, VA, USA). The cells were maintained in MEM containing 10% FBS at 37 °C in a CO_2 incubator, in a humidified atmosphere with 5% CO_2. The cell viability was determined by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay using a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay Kit according to the manufacturer's manual. Briefly, cells were inoculated at a density of 2.5 × 10^4 cells/well into 96-well plate and cultured at 37 °C for 24 h. The culture media were replaced by 200 μL of MEM containing serial dilutions of tacrine (0−0.75 mM) or isolated phlorotannins (0−200 μM), and the cells were incubated for 24 h. The cytoprotective assay was measured with a cell viability assay kit after cotreatment of 0.3 mM tacrine and various concentrations of isolated phlorotannins (0−200 μM) for 24 h. The culture media were removed and replaced by 95 μL of fresh culture media and 5 μL of MTS solution. After 1 h, the absorbance at 490 nm was measured using a microplate reader.

Measurement of Intracellular ROS. The intracellular ROS scavenging activity of the sample was measured using the oxidant-sensitive fluorescent probe DCF-DA. HepG2 cells plated in a 12-well culture plate at a density of 1 × 10^6 per well were incubated with various concentrations of phlorotannins in the absence or presence of tacrine (0.3 mM) 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 DCF-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 Co., Sunnyvale, CA, USA).

Effect of 2-Phloroeckol and Eckol on Enzymatic Antioxidant Activities. HepG2 cells cultured in a 12-well plate (1 × 10^6 cells/well) were treated with the indicated concentrations of phlorotannins for 2 h. The cells were harvested by 50 mM potassium phosphate buffer (pH 7.0, 1 mM EDTA) containing 0.5% NP-40 and then centrifuged at 10000*g* for 15 min. The supernatant was transferred and stored at −70 °C until required. GST and catalase activities were measured using the GST and catalase assay kit according to the manufacturer's protocol.

Preparation of Cytosolic and Mitochondrial Fraction. Cytosolic and mitochondrial fractions were prepared as described previously with optimal modification of Emanuele et al.[22] Briefly, HepG2 cells plated in a 6-well culture plate at a density of 5 × 10^6 per well were incubated with various concentrations of phlorotannins in the absence or presence of tacrine (0.3 mM) for 24 h. Harvested cells were washed twice with PBS and harvested by centrifugation (500*g* for

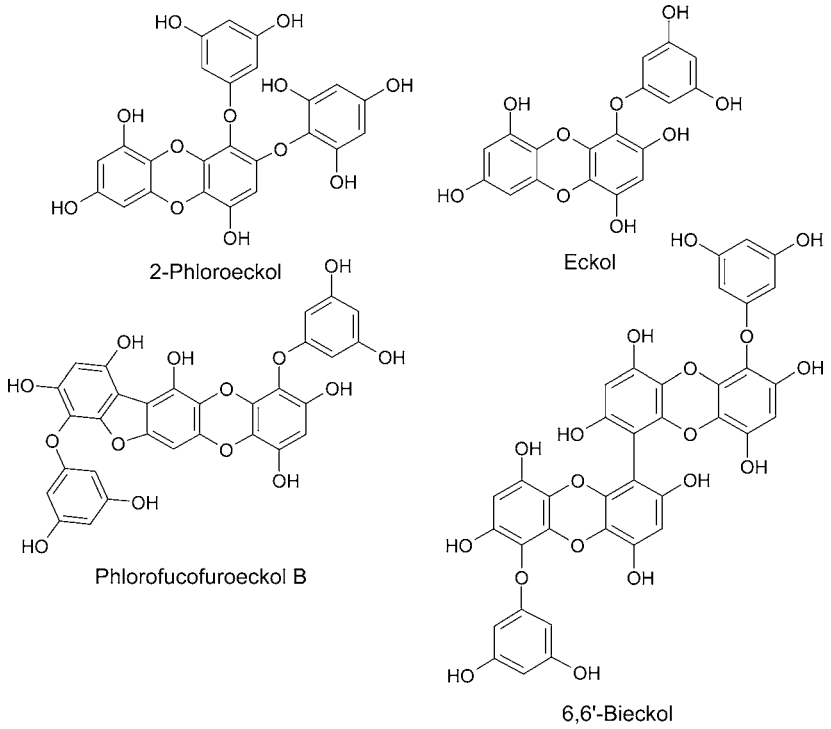

Figure 1. Chemical structures of eckol, 2-phloroeckol, phlorofucofuroeckol B, and 6,6′-bieckol isolated from *E. stolonifera*.

5 min). Cell pellets were resuspended in 50 μL of mitochondrial isolation buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose) containing protease inhibitor cocktail by gentle pipetting and centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was collected, and the pellet was resuspended with the same volume of mitochondrial isolation buffer to obtain cytosolic supernatant by centrifugation at 14400*g* for 10 min at 4 °C. The combined supernatant was used as cytosolic fraction. The pellet was gently resuspended in 50 μL of mitochondrial isolation buffer and was used as mitochondria-containing fraction.

Western Immunoblot. HepG2 cells plated in a 6-well culture plate at a density of 5×10^6 per well were cotreated with 0.3 mM tacrine and various concentrations of 2-phloroeckol and eckol for 24 h. Control and sample-treated HepG2 cells were washed twice with cold PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM phenylmethanesulfonyl fluoride) on ice for 1 h. After centrifugation at 18000*g* for 10 min, the protein concentrations in supernatants were determined.

Aliquots of protein (40 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dried milk in Tris-buffered saline with Tween-20 (TBST) buffer for 1 h, followed by incubation 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 immune complexes were detected using an ECL detection kit. Densitometric analysis of the data obtained from at least three independent experiments was performed using a cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software (ATTO Co., Tokyo, Japan).

Confocal Microscopic Analysis. Release of cytochrome *c* from mitochondria was measured using confocal imaging of cells double-labeled with MitoTracker red CMXRos and a cytochrome *c* antibody. The cells were maintained on glass coverslips (SPL Lifesciences Co., Gyeonggi-do, Korea) in a 24-well plate (5×10^5) for 24 h. After experimental treatment, cells were incubated with 100 nM MitoTracker red CMXRos for 30 min at 37 °C, washed with PBS, and fixed with 4% paraformaldehyde in PBS at 37 °C for 30 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min at 4 °C, followed by a 2 h incubation at room temperature in blocking solution (2% normal goat serum, 0.1% Triton X-100 in PBS, pH 7.4) containing cytochrome *c* antibody. After washing, cells were incubated for 2 h in PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100). Cell images were captured using an LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). The excitation and emission wavelengths for MitoTracker red CMXRos were 510 and 590 nm, respectively, and those for fluorescein isothiocyanate were 488 and 510 nm, respectively.

Statistical Analysis. Data were expressed as the mean ± SD from at least three separate experiments unless otherwise indicated. Data were analyzed using one-way ANOVA, followed by each pair of Student's *t* tests for multiple comparisons. Differences were considered to be significant if $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL, USA).

RESULTS

Isolation and Structural Determination of Phlorotannins. In the previous study, we found strong antioxidant activity in the EtOAc fraction of *E. stolonifera*.[19] The antioxidant activity of the fraction was attributed to a rich polyphenolic compound content, including phlorotannins. The EtOAc fraction of the *E. stolonifera* led to the isolation of four kinds of phlorotannins (Figure 1). Their chemical structures were verified by 1D (^{1}H and ^{13}C NMR) and 2D (HMQC and HMBC) spectroscopic analyses and by comparisons to published spectral data. The chemical structures of compounds were identified as eckol,[12] 2-phloroeckol,[23] phlorofucofuroeckol B,[24] and 6,6′-bieckol.[15] The chemical structures (Figure 1) and spectral data were as follows.

2-Phloroeckol: $C_{24}H_{16}O_{12}$ (MW = 496); ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.80 (H-3), 5.82 (H-6), 5.96 (H-8), 5.86 (H-2′), 5.84 (H-4′), 5.86 (6′), 5.84 (H-3″), 5.84 (5″); ^{13}C NMR (100 MHz, DMSO-d_6) δ 123.0 (C-1), 148.1 (C-2), 96.4 (C-3), 142.0 (C-4), 124.7 (C-4a), 142.9 (C-5a), 94.3 (C-6), 153.5 (C-

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7), 99.1 (C-8), 146.5 (C-9), 123.0 (9a), 137.5 (C-10a), 160.7 (C-1′), 94.5 (C-2′), 159.2 (C-3′), 96.8 (C-4′), 159.2 (5′), 94.5 (6′), 122.4 (C-1″), 151.6 (C-2″), 95.3 (C-3″), 155.1 (C-4″), 95.3 (5″), 151.6 (6″).

Eckol: $C_{18}H_{12}O_9$ (MW = 372); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (H-2), 6.13 (H-3), 9.42 (H-4), 5.78 (H-6), 9.15 (H-7), 5.95 (H-8), 9.48 (H-9), 5.71 (H-2′), 9.12 (H-3′), 5.79 (H-4′), 9.12 (H-5′), 5.71 (H-6′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 122.9 (C-1), 145.6 (C-2), 97.9 (C-3), 141.5 (C-4), 122.9 (C-4a), 142.2 (C-5a), 93.6 (C-6), 152.6 (C-7), 98.3 (C-8), 122.3 (C-9a), 136.8 (C-10a), 160.0 (C-1′), 93.5 (C-2′), 158.4 (C-3′), 96.0 (C-4′), 158.4 (C-5′), 93.5 (C-6′).

Phlorofucofuroeckol B: $C_{30}H_{18}O_{14}$ (MW = 602); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.19 (H-3), 6.75 (H-6), 6.48 (H-10), 5.76 (H-2′), 5.82 (H-4′), 5.76 (6′), 5.71 (H-2″), 5.82 (H-4″), 5.71 (6″); ^{13}C NMR (100 MHz, DMSO-d_6) δ 122.2 (C-1), 146.3 (C-2), 98.6 (C-3), 142.0 (C-4), 123.0 (C-4a), 141.4 (C-5a), 91.5 (C-6), 150.3 (C-6a), 149.2 (C-7a), 120.4 (C-8), 150.4 (C-9), 98.6 (C-10), 145.5 (C-11), 104.9 (C-12), 108.0 (C-13), 137.2 (C-14), 125.8 (C-14a), 136.7 (C-15a), 160.3 (C-1′), 93.7 (C-2′), 93.7 (6′), 158.9 (C-3′), 96.5 (C-4′), 158.9 (5′), 159.9 (C-1″), 93.5 (C-2″), 159.0 (C-3″), 96.3 (C-4″), 159.0 (5″), 93.5 (6″).

6,6′-Bieckol: $C_{36}H_{22}O_{18}$ (MW = 742); ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.12 (H-1), 6.08 (H-2), 9.05 (H-3), 8.61 (H-7), 6.04 (H-8), 9.25 (H-9), 5.74 (H-2′), 9.13 (H-3′), 5.8 (H-4′), 9.13 (H-5′), 5.74 (H-6′), 9.12 (H-2″), 6.08 (H-3″), 9.05 (H-4″), 8.61 (H-7″), 6.04 (H-8″), 9.25 (H-9″), 5.74 (H-2‴), 9.13 (H-3‴), 5.8 (H-4‴), 9.13 (H-5‴), 5.74 (H-6‴); ^{13}C NMR (100 MHz, DMSO-d_6) δ 123.5 (C-1), 145.4 (C-2), 97.7(C-3), 141.4 (C-4) 121.9 (C-4a), 141.3 (C-5a), 99.7 (C-6), 151.3 (C-7), 97.8 (C-8), 144.5 (C-9), 122.7 (C-9a), 137.2 (C-10a), 160.4 (C-1′), 93.7 (C-2′), 158.8 (C-3′), 96.1 (C-4′), 158.8 (C-5′), 93.7 (C-6′).

Cytotoxicity of Phlorotannins. The cytotoxicities of isolated phlorotannins and tacrine were measured using MTS assays on HepG2 cells prior to testing of phlorotannins for their suppression of ROS production and hepatoprotective activities. As shown in Figure 2A, 2-phloroeckol, eckol, and 6,6′-bieckol did not show cytotoxicities in HepG2 cells up to 200 μM; however, phlorofucofuroeckol B showed cytotoxicity of approximately 18% at 50 μM concentration (Figure 2B). The cytotoxicity of tacrine was also measured using the MTS assay. Compared with untreated cells, HepG2 cells treated with tacrine (0−0.75 mM) resulted in a dramatic decrease of cell viability in a dose-dependent manner (Figure 2C). The value of the 50% inhibitory concentration (IC_{50}) of tacrine on cell death was estimated to be 0.30 ± 0.05 mM, which was used for the hepatoprotection assay of the isolated phlorotannins.

Antioxidant Activity of Phlorotannins. The isolated phlorotannins from *E. stolonifera* were assessed for their antioxidant activity by measuring their ability to scavenge free radicals with DPPH. The antioxidant activities of the isolated compounds were 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 2-phloroeckol, eckol, phlorofucofuroeckol B, and 6,6′-bieckol were estimated to be 35.2 ± 0.4, 10.6 ± 0.4, 4.9 ± 0.2, and 9.5 ± 0.2 μM, respectively. Eckol and 6,6′-bieckol showed an antioxidant activity similar to that of L-ascorbic acid as a positive control. Additionally, the antioxidant activities of phlorotannins were determined in cellular systems to investigate whether isolated phlorotannins can affect the free radical mediated oxidation in

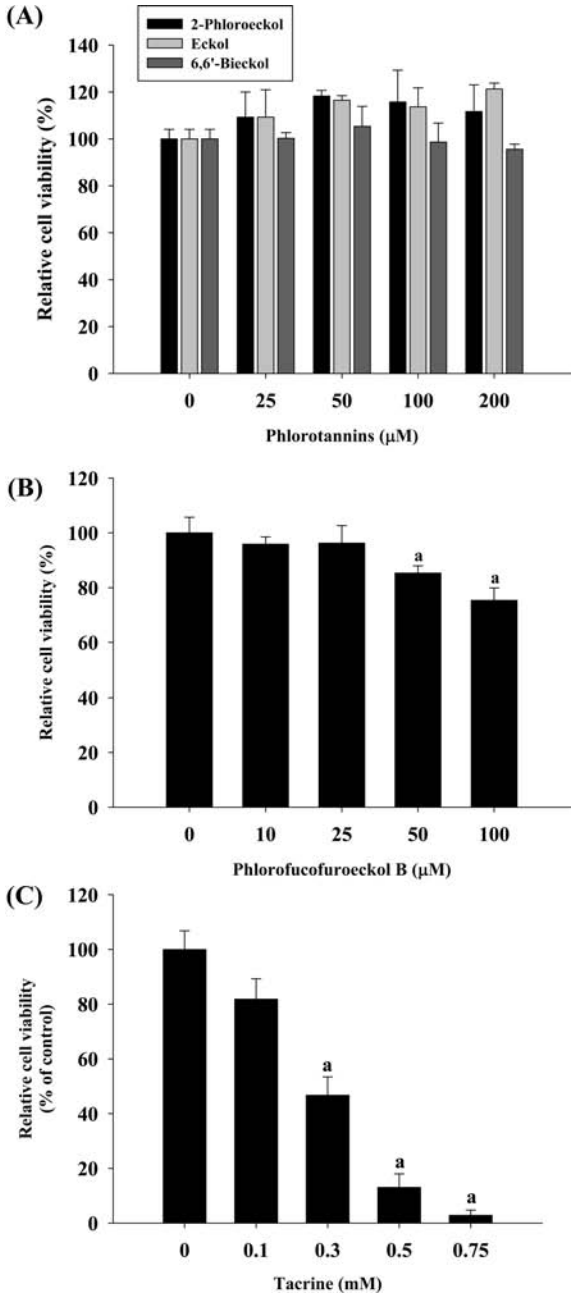

Figure 2. Cytotoxic effect of phlorotannins and tacrine in HepG2 cells. Cells were incubated for 24 h with eckol, 2-phloroeckol, and 6,6′-bieckol (A), phlorofucofuroeckol B (B), and tacrine (C) at indicated concentrations. Cell viability was measured by MTS assay. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences from the control group.

Table 1. DPPH Radical-Scavenging Activity of Phlorotannins Isolated from *E. stolonifera*

compound	EC_{50}[a] (μM)
2-phloroeckol	35.2 ± 0.4
eckol	10.6 ± 0.4
phlorofucofuroeckol B	4.9 ± 0.2
6,6′-bieckol	9.5 ± 0.2
L-ascorbic acid	10.3 ± 0.5

[a]Values are the mean ± SD of three independent experiments.

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cellular systems or not. For this purpose, tacrine was used as a model compound for the generation of intracellular ROS. Figure 3A shows the effect of 2-phloroeckol, eckol, and 6,6′-

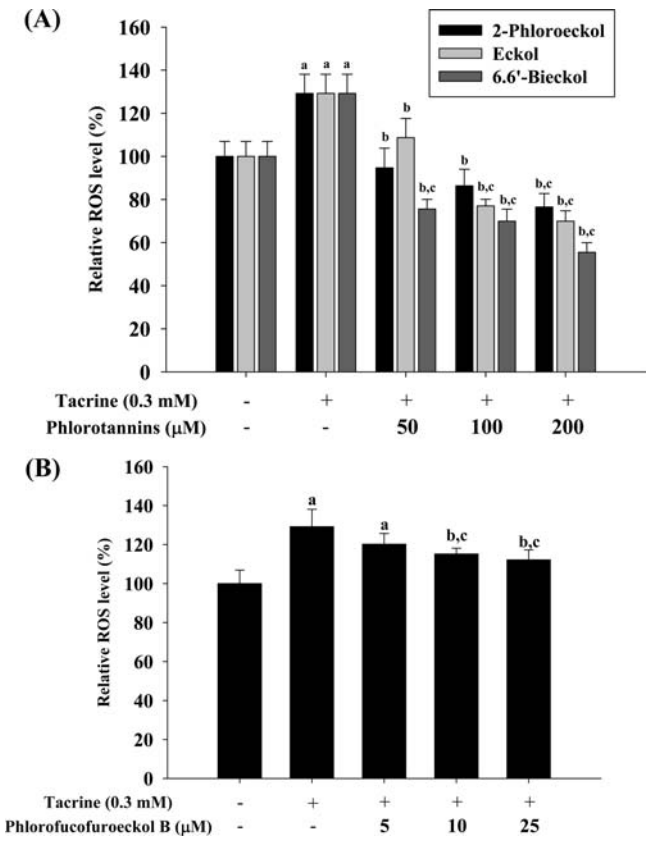

Figure 3. Effect of phlorotannins on tacrine-induced ROS production in HepG2 cells. Cells were cotreated with tacrine (0.3 mM) and indicated concentrations of eckol, 2-phloroeckol, and 6,6′-bieckol (A) and phlorofucofuroeckol B (B) and incubated for 2 h. ROS levels were measured by DCF-DA with fluorescent analysis. Control values were obtained in the absence of tacrine and phlorotannins. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences between control and tacrine-treated group; b, $P < 0.05$ indicates significant differences from tacrine-treated group; c, $P < 0.05$ indicates significant differences between control and phlorotannin-treated group.

bieckol on ROS production in tacrine-treated HepG2 cells. Treatment of cells with 2-phloroeckol, eckol, and 6,6′-bieckol significantly inhibited the production of ROS in a dose-dependent manner ($P < 0.05$). Furthermore, ROS levels in the treatment of 50−200 μM 6,6′-bieckol, 100 and 200 μM eckol, and 200 μM 2-phloroeckol were significantly lower than those in the control group ($P < 0.05$). Inhibition patterns of ROS production by three phlorotannins were similar to those of DPPH radical-scavenging activity. Phlorofucofuroeckol B treatment significantly reduced ROS levels induced by tacrine treatment in HepG2 cells (Figure 3B); however, its reduced levels at concentrations of 10 and 25 μM phlorofucofuroeckol B were higher than control cells.

Effect of Phlorotannins on Tacrine-Induced Hepatotoxicity. To examine the hepatoprotective effect of the isolated phlorotannins on tacrine-treated HepG2 cells, the cells were cotreated with 0.3 mM tacrine and the indicated concentrations of phlorotannins for 24 h. As shown in Figure 4A, 2-phloroeckol and eckol treatment over 25 μM concentration

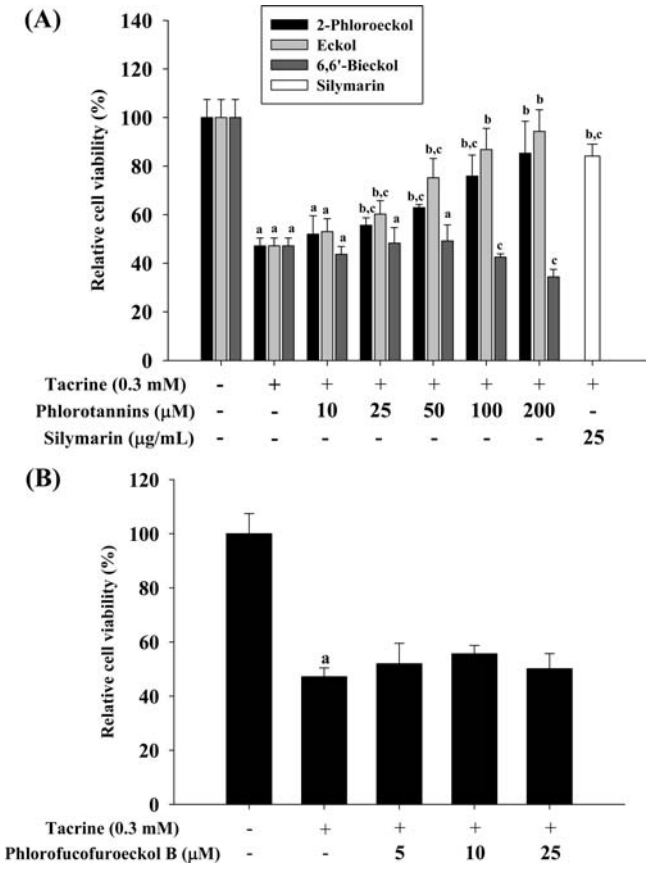

Figure 4. Hepatoprotective effect of eckol, 2-phloroeckol, phlorofucofuroeckol B, and 6,6′-bieckol on tacrine-treated HepG2 cells. Cells were cotreated with tacrine (0.3 mM) and indicated concentrations of eckol, 2-phloroeckol, and 6,6′-bieckol (A) and phlorofucofuroeckol B (B) for 24 h. After incubation, cell viability was measured by MTS assay. Control values were obtained in the absence of tacrine and phlorotannins. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences from control group; b, $P < 0.05$ indicates significant differences from tacrine-treated group; c, $P < 0.05$ indicates significant differences between control and sample-treated group.

significantly increased cell viability in tacrine-treated HepG2 cells in a dose-dependent manner ($P < 0.05$), and cell viabilities were shown to be comparable to the control level by treatment with 100 and 200 μM eckol and 200 μM 2-phloroeckol. However, 6,6′-bieckol and phlorofucofuroeckol B did not recover cell viability (Figure 4), whereas these compounds showed antioxidant activities (Figure 3). The EC_{50} values of 2-phloroeckol and eckol for the cell viability in tacrine-treated HepG2 cells were estimated to be 95 ± 6.5 (47.1 μg/mL) and 49 ± 4.5 μM (18.2 μg/mL), respectively, which were higher than that of silymarin (13.6 μg/mL).

Effect of 2-Phloroeckol and Eckol on Enzymatic Antioxidant Activities. Because 2-phloroeckol and eckol showed hepatoprotective activities in tacrine-treated HepG2 cells, intracellular catalase (Figure 5A) and GST (Figure 5B) activities were measured to confirm whether enzymatic antioxidant activities were involved in the increased cell viability in tacrine-treated HepG2 cells. Tacrine treatment significantly decreased catalase (Figure 5A) and GST (Figure 5B) activities ($P < 0.05$) compared with the control group. 2-Phloroeckol and eckol significantly increased both catalase and GST activities in tacrine-treated HepG2 cells in a dose-

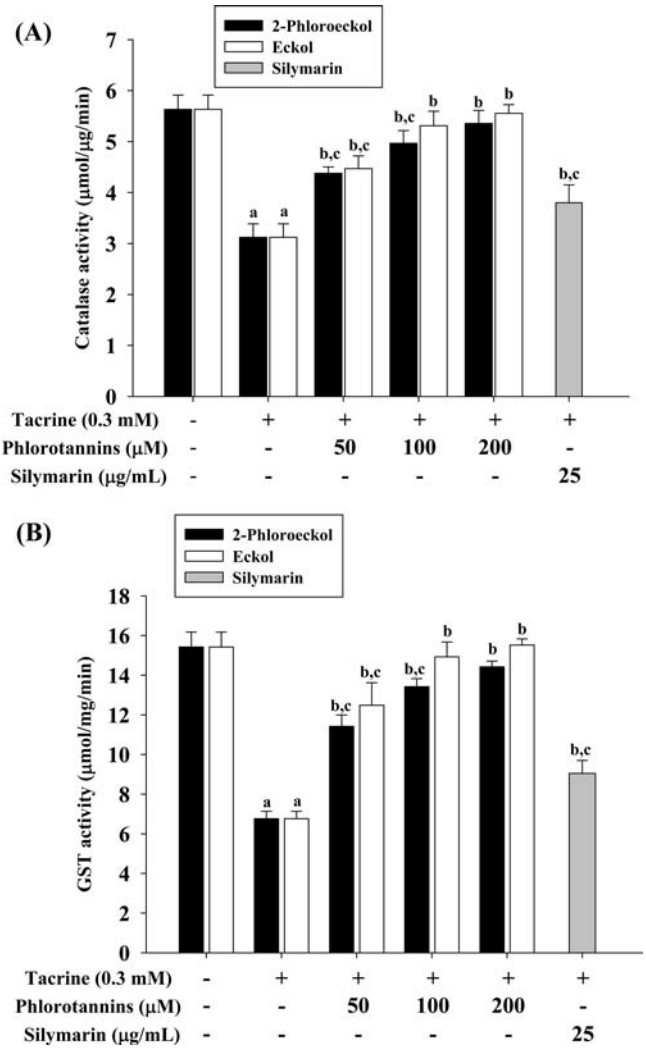

Figure 5. Effect of 2-phloroeckol and eckol on catalase and GST activities in tacrine-treated HepG2 cells. Cells were cotreated with 0.3 mM tacrine and indicated concentrations of 2-phloroeckol and eckol for 2 h. Whole cell protein was used for catalase (A) and GST (B) activity assay. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences from control group; b, $P < 0.05$ indicates significant differences from tacrine-treated group; c, $P < 0.05$ indicates significant differences between control and sample-treated group.

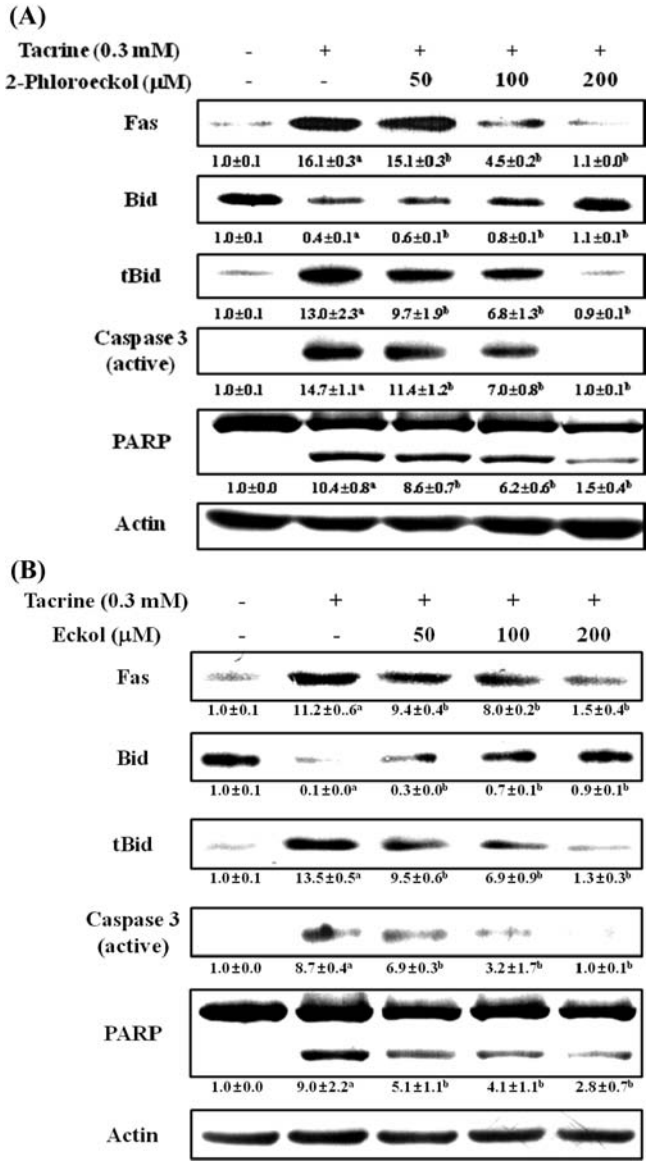

Figure 6. Effect of 2-phloroeckol and eckol on cell-death protein expression in tacrine-treated HepG2 cells. Cells were cotreated with 0.3 mM tacrine and indicated concentrations of 2-phloroeckol (A) and eckol (B) for 24 h. Whole cell proteins were prepared, and 40 μg of proteins was separated with SDS-PAGE to Western blot analysis. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences from control group; b, $P < 0.05$ indicates significant differences from tacrine-treated group.

dependent manner. Catalase and GST activities with treatment of 100 and 200 μM eckol and 200 μM 2-phloroeckol were found to be comparable to those with the control group. Furthermore, both catalase and GST activities after treatment with 50 μM eckol (18.6 μg/mL) and 2-phloroeckol (24.8 μg/mL) were higher than those with silymarin treatment (25 μg/mL).

Effects of 2-Phloroeckol and Eckol on the Suppression of Fas-Related Cell-Death Proteins. To evaluate the effect of 2-phloroeckol and eckol on the expression of Fas-related cell-death proteins in tacrine-treated HepG2 cells, we measured the expression of the proteins related to cell-death proteins, such as Fas, cleaved caspase-3, Bid, tBid, and PARP, in tacrine-treated HepG2 cells. As shown in Figure 6, the stimulation of HepG2 cells with tacrine led to a significant decrease of Bid and increases of Fas, tBid, cleaved caspase-3, and PARP protein. Treatment of 2-phloroeckol (Figure 6A) and eckol (Figure 6B) inhibited cell-death protein expression in a dose-dependent manner, and levels of Bid and tBid were recovered to control level. In addition, both 2-phloroeckol and eckol inhibited activation of caspase-3 and degradation of PARP by tacrine in a dose-dependent manner, indicating hepatoprotective properties of 2-phloroeckol and eckol in tacrine-induced HepG2 cells.

Effects of 2-Phloroeckol and Eckol on the Release of Cytochrome *c*. Because the isolated 2-phloroeckol and eckol showed hepatoprotective properties in tacrine-treated HepG2 cells, we further examined cytochrome *c* release from mitochondria to cytosol in tacrine-treated cells (Figure 7). In untreated control cells, cytochrome *c* immunoreactivity was colocalized with MitoTracker red fluorescence indicating that

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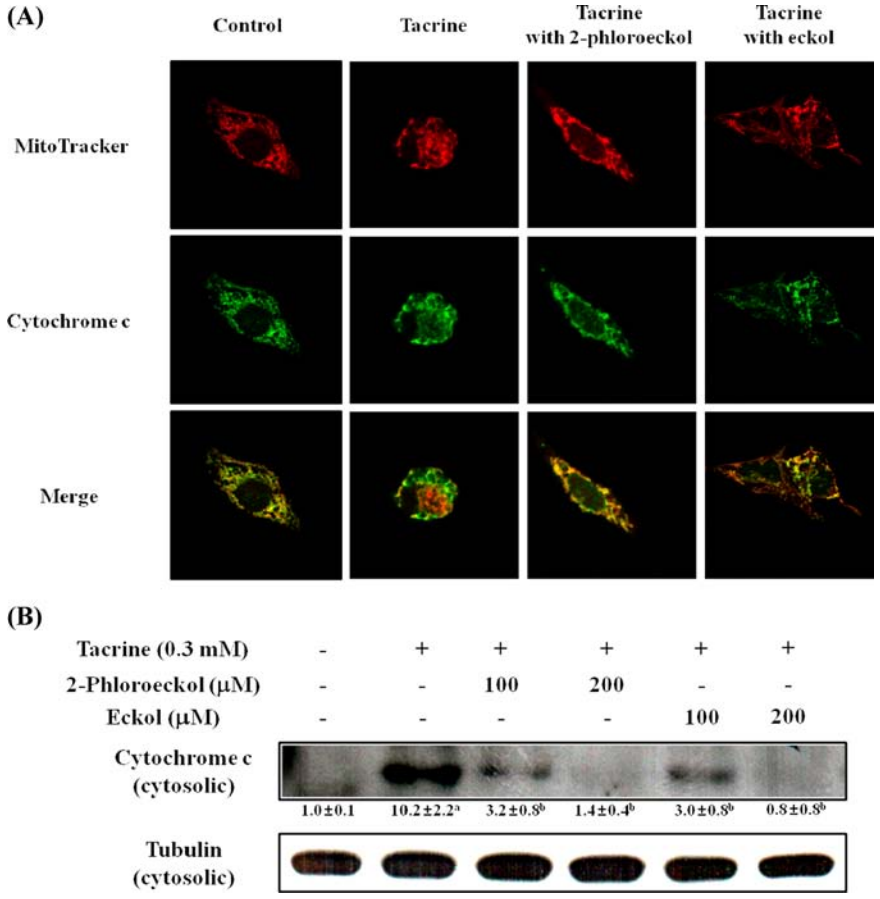

Figure 7. Effect of 2-phloroeckol and eckol on cytochrome *c* release in tacrine-treated HepG2 cells. (A) Control and treated cells were stained with MitoTracker red and cytochrome *c* antibody. MitoTracker red fluorescence (red), cytochrome *c* immunoreactivity (green), and merged images (yellow) were visualized by confocal microscope. (B) Cells treated with 0.3 mM tacrine and different concentrations of eckol or 2-phloroeckol were harvested, and the cytosolic fraction was separated with SDS-PAGE. Values are the mean ± SD of three independent experiments. a, $P < 0.05$ indicates significant differences from control group; b, $P < 0.05$ indicates significant differences from tacrine-treated group.

the cytochrome *c* was confined to mitochondria (Figure 7A). After exposure to tacrine, cytochrome *c* immunoreactivity in cells was diffused throughout the cytoplasm, and a decrease in mitochondria-associated cytochrome *c* immunoreactivity indicated that cytochrome *c* was released from mitochondria. However, cytochrome *c* immunoreactivities in cotreated cells with tacrine and 2-phloroeckol or eckol were colocalized with MitoTracker red fluorescence, indicating that the cytochrome *c* was not released from mitochondria. To confirm cytochrome *c* release from mitochondria to cytosol, we isolated mitochondrial and cytosolic fractions from control, tacrine-treated, and cotreated with tacrine and 2-phloroeckol or eckol cells and performed Western blotting. As shown in Figure 7B, increased release of cytochrome *c* in the cytosolic fraction by tacrine was suppressed by the 2-phloroeckol or eckol treatment in a dose-dependent manner. These results indicate that 2-phloroeckol and eckol have hepatoprotective properties via blocking cytochrome *c* release from mitochondria.

■ DISCUSSION

In previous studies, we isolated several antioxidative phlorotannins from *E. stolonifera*.[12,19,25] Among them, dioxinodehydroeckol and phlorofucofuroeckol A showed hepatoprotective effect on tacrine-treated HepG2 cells.[12] In this study, we isolated an additional four antioxidative phlorotannins: 2-phloroeckol, eckol, phlorofucofuroeckol B, and 6,6′-bieckol. These structures were identified by comprehensive spectroscopic analysis including NMR (^{1}H and ^{13}C), HMQC, and HMBC as well as comparison to previously published data. Among these phlorotannin derivatives, 2-phloroeckol was isolated for the first time from this species.

Under physiological concentrations, ROS act as signaling molecules, mediating cell growth, migration, and differentiation, whereas excessive ROS cause damage to cells with different mechanisms and are involved in various pathological conditions, including aging, cancer, and neurodegenerative diseases.[5,26,27] Therefore, it is suggested that the use of antioxidant may help to prevent or alleviate diseases caused by mainly oxidative stress. Compounds derived from botanic sources, such as phenolic compounds, have enhanced cytoprotective properties through the removal of ROS by their enzymatic and/or nonenzymatic antioxidant activities.[8,9,28] Among antioxidant phlorotannins isolated from *E. cava*, cytoprotective mechanisms of eckol and triphlorethol-A were well demonstrated using lung fibroblast cells against oxidative damage, and cytoprotective effects were caused by enhancing the cellular antioxidant activity and/or the cellular signal pathway.[17,18,29] Phlorotannins having polyphenol structure are electron-rich compounds, which are prone to participation in electron-donation reactions to produce phenoxyl radical species. Phenoxyl radicals are stabilized by resonance delocalization of the unpaired electron to the ortho and para

positions of ring and by hydrogen bonding with an adjacent hydroxyl group. This intrinsic stability of phenolic structure might be related to the antioxidant activity of phlorotannins.[18] Four phlorotannins isolated in this study showed high DPPH radical-scavenging activities due to rich hydroxyl groups, suggesting that the isolated phlorotannins have cytoprotective properties against oxidative cellular damage. However, levels of ROS by phlorofucofuroeckol B treatment were not reduced to those of control, although the compound had potent inhibition on DPPH radical-scavenging activity. The precise mechanisms of compounds in the inhibition of intracellular ROS production are largely unknown, and further studies are required for better understanding of the mechanisms of actions. The pharmacological characteristics of phlorofucofuroeckol B found in previous studies, which include stimulus of signaling receptors in leukemia cells,[24] indicate the actions of phlorofucofuroeckol B may be related to cell-specific manner. In addition, because series of compounds have similar structural backbones, it is interesting to investigate structure−activity relationships by creating compound libraries in the future.

Various in vivo and in vitro studies have shown that the hepatoprotective effects of natural compounds could be associated with the inhibition of oxidative stress by enhancing the antioxidant defense system.[8,30−32] Enhanced ROS production and GSH depletion are a major mechanism involved in tacrine cytotoxicity; thus, application of antioxidants from a natural source is an appropriate strategy for the cytoprotection of HepG2 cells by alleviating oxidative stress.[7,11] Of interest, it has been previously reported that phlorofucofuroeckol A has a strong ability to inhibit ROS production in kidney homogenates and macrophages.[19,25] In this study we used a tacrine-treated HepG2 cell model to investigate the hepatoprotective activity of the phlorotannins, because tacrine induces cell damage through the excessive production of intracellular ROS.[11] The results of the present study demonstrated that four isolated phlorotannins showed high antioxidant activity; however, 2-phloroeckol and eckol had only a protective effect on tacrine-induced hepatotoxicity (Figure 4A). Catalase and GST are key components of the antioxidant defense system. Inhibition of these protective enzyme activities results in enhanced sensitivity to free radical induced cellular damage. Therefore, enhanced activities of these enzymes may protect HepG2 cells against tacrine-induced oxidative stress. Both compounds increased the activities of catalase and GST in tacrine-treated HepG2 cells, indicating the compounds' directly scavenging intracellular ROS led to cell protection against oxidative stress. The hepatoprotective properties of the isolated 2-phloroeckol and eckol may be in part due to their direct antioxidative activities associated with inhibition of intracellular ROS production. Furthermore, both compounds increased the activities of catalase and GST in tacrine-treated HepG2 cell, indicating the compounds stimulate the production of antioxidant enzymes responsible for the cell protection against oxidative stress. However, at present, why 6,6′-bieckol did not show hepatoprotective properties is unclear, even though the compound efficiently inhibits ROS production in the same cell.

The molecular mechanisms involved in cell death led by ROS formation under different conditions are involved in complicated and multiple processes, and numerous pieces of evidence from different cellular models suggest an action of ROS on intracellular signaling pathways involved in cell death/survival.[27] It has been shown that excessive ROS induces the activation of Fas receptor in the absence of ligand, leading cells to apoptotic death mediated by an activation of caspase-8, -10, and -3.[33] Active caspase-3 is translocated from the cytosol to the nucleus and cleaves nuclear proteins, such as lamin and PARP.[34] Also, activated caspase-8 cleaves Bid to form tBid, which induces mitochondrial damage and cell death.[4] The present study showed that tacrine induced the activation of caspase-3 through the up-regulation of Fas (Figure 5), which indicates that tacrine triggers cell death with an extrinsic apoptotic pathway. When 2-phloroeckol and eckol were cotreated with tacrine in HepG2 cells, both phlorotannins inhibited Fas and caspase-3 activation, Bid cleavage, and PARP fragmentation in a dose-dependent manner. Although little is known of the molecular mechanisms of phlorotannins on the regulation of cell-death signal, the present findings that treatment with 2-phloroeckol or eckol results in strong down-regulation of Fas-mediated apoptotic proteins in tacrine-treated HepG2 cells suggest that the phlorotannin-mediated hepatoprotection in tacrine-treated HepG2 cells is associated with the ability of phlorotannins to inhibit the Fas/FasL pathway activated by ROS production.

ROS produced by tacrine treatment attack membrane phospholipids and cause loss of mitochondrial membrane potential.[11,35,36] Moreover, the proapoptotic proteins belonging to the Bcl-2 family, such as tBid, are also activated by ROS. tBid triggers the release of cytochrome *c* from the mitochondria to the cytosol.[37] In the cytosol, released cytochrome *c* cleaves procaspase-3 and induces the activation of caspase-3. In this study, we confirmed that released cytochrome *c* from mitochondria by tacrine was markedly inhibited by cotreatment of 2-phloroeckol or eckol by confocal microscope and Western blotting (Figure 6), that suggests the compounds block the formation of ROS induced by tacrine and may prevent disruption of mitochondrial membrane integrity. Hence, the present findings that 2-phloroeckol and eckol inhibit cytochrome *c* release and Fas signal suggest the main mechanisms of both phlorotannin-mediated hepatoprotection in tacrine-treated HepG2 cells.

In conclusion, four phlorotannins, 2-phloroeckol, eckol, phlorofucofuroeckol B, and 6,6′-bieckol, were isolated from the brown macroalgae *E. stolonifera* by a series of column chromatographies. The four compounds exhibited strong DPPH radical-scavenging activity, and 2-phloroeckol, eckol, and 6,6′-bieckol showed potent inhibition of intracellular ROS production. Among them, 2-phloroeckol and eckol may be the main compounds in *E. stolonifera* for hepatoprotective activity in tacrine-treated HepG2 cells. The hepatoprotective properties of 2-phloroeckol and eckol result from down-expression of Fas and cytochrome *c* release in tacrine-treated HepG2 cells through their antioxidant activities. The brown marine macroalgae *E. stolonifera* may have potential in the development of functional foods for hepatoprotection and antioxidation.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

Bid, BH3 interacting domain death agonist; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetate; EtOAc, ethyl acetate; FBS, fetal bovine serum; GSH, glutathione; GST, glutathione *S*-transferase; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum correlation; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; MTS, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; NMR, nuclear magnetic resonance; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween-20.

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