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Evaluation of Inhibitory Effect of Phlorotannins from *Ecklonia cava* on Triglyceride Accumulation in Adipocyte

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ABSTRACT: In the present study, a methanolic extract of *Ecklonia cava* and its solvent-partitioned fractions were evaluated for their antiadipogenic effect in 3T3-L1 adipocytes. One of them, the *n*-BuOH fraction, effectively reduced lipid accumulation and glucose consumption. In addition, the presence of the *n*-BuOH fraction in adipocytes suppressed the regulations of adipogenic transcription factors, PPARγ and SREBP1c, and adipogenic specific genes, FABP4, FABP1, FAS, LPL, HSL, and ACS1. Further purification of *n*-BuOH fractions led to the isolation of six phlorotannins (**1**−**6**). The six phlorotannins effectively suppressed triglyceride accumulation. Comparative analysis showed that lipid accumulation in adipocytes was dramatically attenuated in the presence of eckstolonol (**4**).

KEYWORDS: *Ecklonia cava, antiadipogenic components, phlorotannin, 3T3-L1 cells*

INTRODUCTION

Seaweeds have been recognized as a valuable source for biologically active materials and for new food ingredients. Many recent studies have reported on their biological activities such as antioxidant, anti-inflammation, antiallergy, antiadipogenesis, apoptosis induction, and so on.[1−7] Brown alga *Ecklonia cava* (Laminariaceae) is one of the most abundant edible seaweeds in Asian countries such as Korea, Japan, and China, and its bioactivities have been reported in a broad range.[2−6,8−10]

In our continuing search for bioactive components found in many types of seaweeds, *E. cava* was collected off the shore of Busan, Korea, and was then air-dried and extracted with methanol (MeOH). The crude extracts were partitioned into four solvent fractions, *n*-hexane, 85% aqueous (aq) MeOH, *n*-BuOH, and H_2O. Their antiadipogenic effects were evaluated by measuring inhibition on adipogenic differentiation of 3T3-L1 preadipocytes. The six known compounds (**1**−**6**) were isolated from the most active *n*-BuOH fraction of *E. cava*, and their chemical structures were determined by comparison with NMR spectral data reported in the literature.[11−15] In this paper, we report the isolation of six phlorotannins (**1**−**6**) and their antiadipogenic activity through measuring lipid accumulation in adipocytes.

MATERIALS AND METHODS

Extraction, Fractionation, and Isolation. *E. cava* was purchased from local markets in Kijang, Busan, South Korea, in 2010. A voucher specimen has been deposited in the author's laboratory. The collected samples were air-dried, ground to a powder, and extracted with MeOH three times. The combined crude extracts (177.43 g) were concentrated under reduced pressure, and then the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was further partitioned between 85% aq MeOH and *n*-hexane. The aqueous layer was also repartitioned with *n*-BuOH and H_2O. The resulting four fractions were evaporated to dryness in vacuo, yielding the *n*-hexane (12.9 g), 85% aq MeOH (5.7 g), *n*-BuOH (60.32 g), and H_2O (98.5 g) fractions, respectively.

A portion of the *n*-BuOH (60.3 g) fraction was subjected to silica gel flash column chromatography using stepwise gradient mixtures of $CHCl_3$ and MeOH (100% $CHCl_3$; 5, 10, 20, 30, 40, 50, and 70% MeOH in $CHCl_3$; and 100% MeOH) and 90% MeOH in H_2O as eluents to give 10 subfractions, named nfc1−nfc10, respectively. Among them, nfc5 and nfc6 were subjected to silica gel flash column chromatography using stepwise gradient mixtures of $CHCl_3$ and MeOH (100% $CHCl_3$; 10, 15, 20, 25, 30, and 35% MeOH in $CHCl_3$; and 100% MeOH) to give eight subfractions, respectively.

The fourth subfraction (1 g) was further separated by LH 20 Sephadex (bead size 25−100 μm, Sigma) column chromatography with 30 or 40% MeOH in $CHCl_3$ to afford compounds **1** (47.5 mg), **2** (3.4 mg), **3** (70.9 mg), **4** (2.0 mg), and **5** (38.5 mg). The fifth subfraction (2.5 g) was further separated by LH 20 Sephadex (bead size 25−100 μm, Sigma) column chromatography with 40% MeOH in $CHCl_3$ and 100% MeOH to afford compound **6** (24.1 mg). The basic scheme for whole isolation and chemical structures of these compounds **1**−**6** are illustrated in Figure 1.

Structural Identification. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian NMR 300 spectrometer (300 MHz for ^{1}H and 75.5 MHz for ^{13}C). Chemical shifts (δ in ppm) were referenced to the residual solvent peak. The used solvent was CD_3OD (Cambridge Isotope Laboratories, Inc., USA, deuterium degree = 99.95%). The heteronuclear multiple-quantum correlation (HMQC) and hetero-nuclear multiple-bond correlation (HMBC) spectra were recorded using pulsed field gradients. The fast atom bombardment mass spectrometry (FAB-MS) spectra were obtained with a Concept-1S mass spectrometer (Kratos Co.).

Cell Culture and Adipocyte Differentiation. Mouse 3T3-L1 preadipocytes were seeded in 6-well plates at a density of 2×10^5 cells/well to confluence in Dulbecco's modified Eagle's medium

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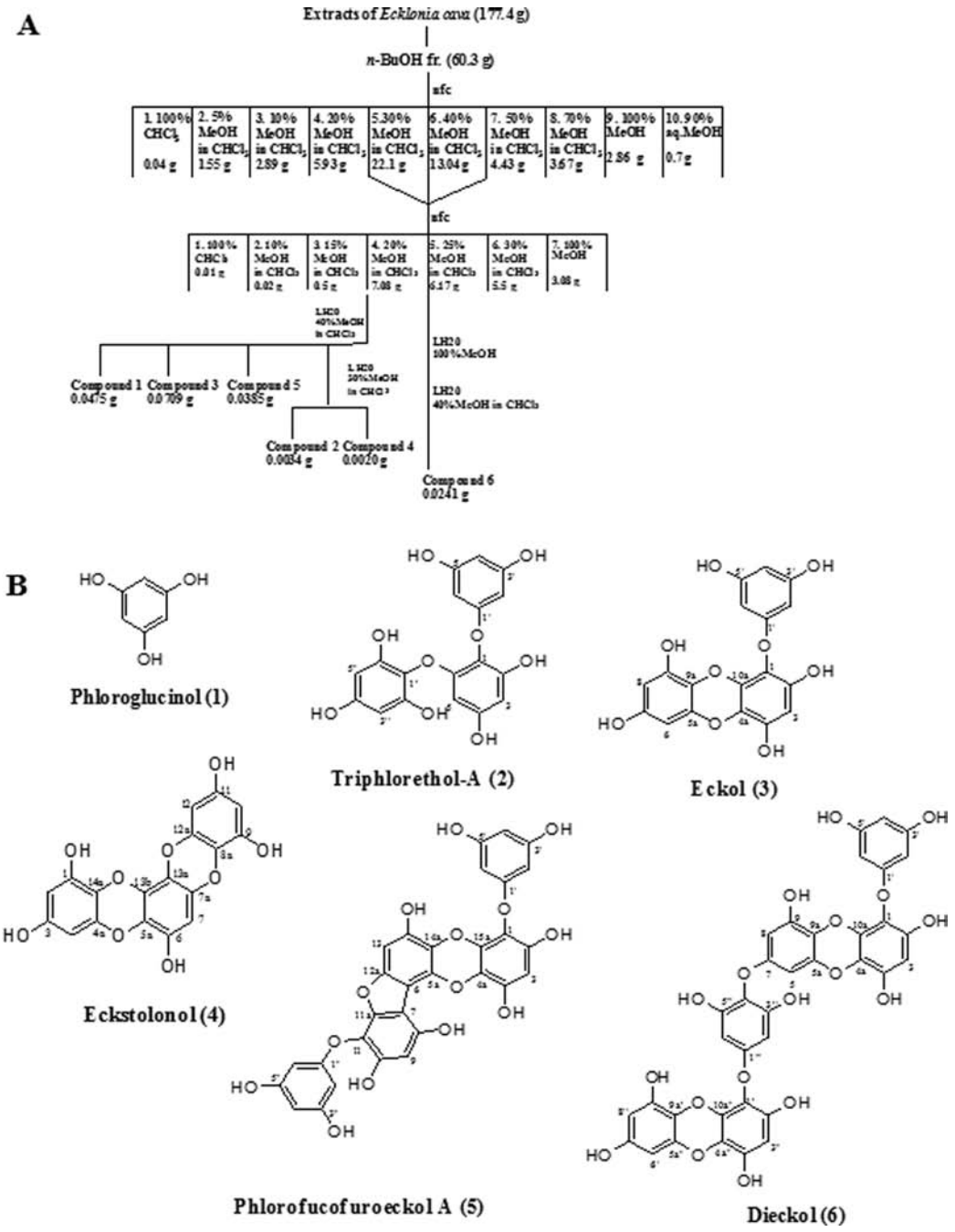

Figure 1. Basic scheme for whole isolation process (A) and chemical structures (B) of phlorotannins from *E. cava*.

(DMEM) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. At 1 day postconfluence (designated "day 0"), cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (5 μg/mL) in DMEM containing 10% FBS. After 48 h (day 2), the induction medium was removed and replaced with DMEM containing 10% FBS supplemented with insulin (5 μg/mL). This medium was changed every 2 days. Solvent-partitioned fractions and isolated compounds were administered to the culture medium from day 6 to day 8 to investigate the effect on not only lipid accumulation but also the hydrolysis of triglyceride.

Determination of Oil-Red O Staining. For Oil-Red O staining, cells were fixed with 10% fresh formaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and stained with filtered Oil-Red O solution (60% isopropanol and 40% water) for at least 1 h. After staining, the Oil-Red O staining solution was removed, and the plates were washed with distilled water or PBS and dried. Images of lipid droplets in 3T3-L1 adipocytes were collected by an Olympus microscope (Tokyo, Japan). Finally, the dye retained in the cells was eluted with isopropyl alcohol and quantified by measuring optical absorbance at 500 nm using a microplate reader (Bio-Tec Instrument, USA).

Glucose Consumption. The glucose level in the cultured medium was determined using a commercial glucose assay kit (GLZYME, Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was isolated from 3T3-L1 adipocytes treated with/without crude extracts and solvent-partitioned fractions using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA). For synthesis of cDNA, RNA (2 μg) was added to RNase-free water and oligo (dT), denaturated at 70 °C for 5 min. and cooled

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immediately. RNA was reverse transcribed in a master mix containing 1× RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 units M-MLV reverse transcriptase, and 40 units RNase inhibitor at 42 °C for 60 min and at 72 °C for 5 min using an automatic Whatman thermocycler (Biometra, UK). The target cDNA was amplified using the following sense and antisense primers: forward 5′-TTT-TCA-AGG-GTG-CCA-GTT-TC-3′ and reverse 5′-AAT-CCT-TGG-CCC-TCT-GAG-AT-3′ for PPARγ; forward 5′-TGT-TGG-CAT-CCT-GCT-ATC-TG-3′ and reverse 5′-AGG-GAA-AGC-TTT-GGG-GTC-TA-3′ for SREBP1c; forward 5′-TCA-CCT-GGA-AGA-CAG-CTC-CT-3′ and reverse 5′-AAT-CCC-CAT-TTA-CGC-TGA-TG-3′ for FABP4; forward 5′-TGC-CTC-TGC-CTT-GAT-CTT-TT-3′ and reverse 5′-GGA-ACC-GTG-GAT-GAA-CCT-AA-3′ for FATP1; forward 5′-TTG-CTG-GCA-CTA-CAG-AAT-GC-3′ and reverse 5′-AAC-AGC-CTC-AGA-GCG-ACA-AT-3′ for FAS; forward 5′-TCC-AAG-GAA-GCC-TTT-GAG-AA-3′ and reverse 5′-CCA-TCC-TCA-GTC-CCA-GAA-AA-3′ for LPL; forward 5′-GAG-GGA-CAC-ACA-CAC-ACC-TG-3′ and reverse 5′-CCC-TTT-CGC-AGC-AAC-TTT-AG-3′ for HSL; forward 5′-CAA-CCC-AGA-ACC-ATG-GAA-GT-3′ and reverse 5′-CTG-ACT-GCA-TGG-AGA-GGT-CA-3′ for ACS1; forward 5′-CGG-AGT-CAA-CGG-ATT-TGG-DCG-TAT-3′ and reverse 5′-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3′ for GAPDH. The amplification cycles were carried out at 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 45 s. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide visualized by UV light using AlphaEase gel image analysis software (Alpha Innotech, Santa Clara, CA, USA).

Statistical Analysis. The data are presented as the mean ± SD. Differences between the means of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 (SAS Institute, Cary, NC, USA) with Duncan's multiple-range tests. The significance of differences was defined at the $p < 0.05$ level.

RESULTS

Effects of Solvent-Partitioned Fractions on Intracellular Lipid Accumulation. The MeOH extracts of *E. cava* were solvent-partitioned to yield four fractions, *n*-hexane, 85% aq MeOH, *n*-BuOH, and H_2O fractions. Their effects on the induction of terminal differentiation markers were estimated at the end of adipocyte differentiation (day 8). Treatment with MeOH extracts and solvent-partitioned fractions up to a concentration of 1 mg/mL did not induce significant toxic effect. Therefore, all experiments were performed within the range of this concentration. Lipid accumulation in 3T3-L1 adipocytes can be quantified by Oil-Red O staining because the absorbance value of the eluted Oil-Red O solution in adipocytes indicates a quantitative analysis of neutral lipid content related to lipid droplet accumulation in the cytoplasm.[16−18] Therefore, the adipocytes treated with/without the crude extracts and solvent-partitioned fractions were stained with Oil-Red O staining solution and followed by comparing the absorbance values of the eluted Oil-Red O solution (Figure 2). Crude extracts and all solvent-partitioned fractions significantly inhibited the lipid accumulation levels ($p < 0.05$). Among them, the reduction was the most effective in the presence of the *n*-BuOH fraction. As the other major marker of adipogenesis, lipid accumulation was quantified by measuring glucose consumption in the cultured medium (Figure 3). Glucose consumption was reduced by treatment with the crude extract and all solvent-partitioned fractions.

Effects of Solvent-Partitioned Fractions on Adipogenic-Specific Gene Expression. The effects of crude extracts and solvent-partitioned fractions on the expression of adipogenic transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ), and differentiation-dependent factor 1/sterol regulatory element-binding protein (SREBP1c) were compared by RT-PCR analysis (Figure 4). Among them,

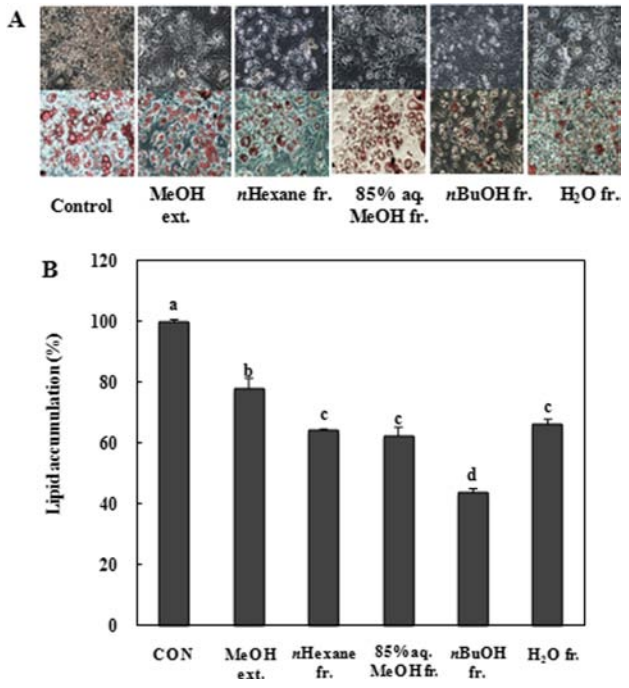

Figure 2. Effects of crude extraction and its solvent fraction from *E. cava* on intracellular lipid accumulation in 3T3-L1 adipocytes. (A) Observation of lipid droplets stained with Oil-Red O staining and (B) absorbance value of the eluted Oil-Red O solution. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days in 6-well plates, and the cells were treated with crude extract and its solvent fractions at the end of the differentiation period for 2 days (from day 6 to day 8). The lipid droplets were fixed with 10% fresh formaldehyde and stained with Oil-Red O staining solution. The dye retained in the cells was eluted with isopropanol and quantified by measuring optical absorbance at 500 nm. Con, fully differentiated control adipocytes (0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 5 μg/mL insulin). Means with the different letters (a−d) are significantly different ($p < 0.05$) by Duncan's multiple-range test.

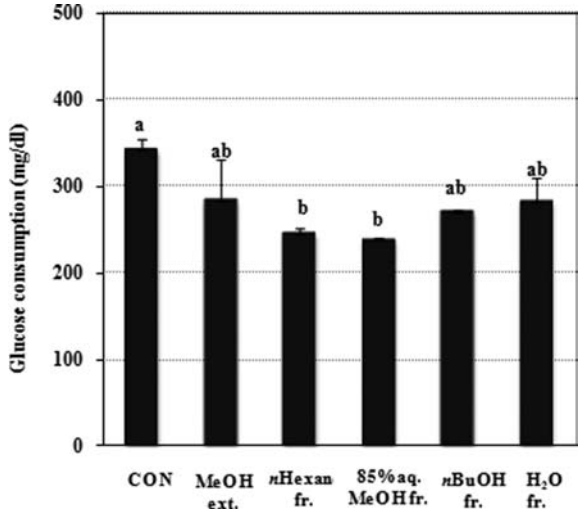

Figure 3. Effects of crude extract and its solvent fraction from *E. cava* on glucose consumption. Means with the different letters (a, b) are significantly different ($p < 0.05$) by Duncan's multiple-range test.

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the *n*-BuOH fraction effectively reduced the regulation of PPARγ and SREBP1c.

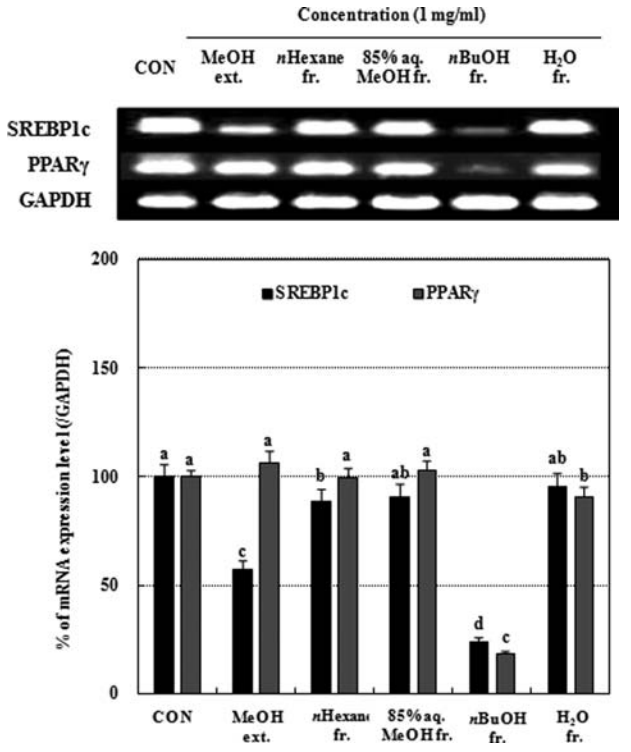

Figure 4. Effect of crude extraction and its solvent fraction from *E. cava* on the regulation of adipogenic transcription factors. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days in 6-well plates, and the cells were treated with crude extract and its solvent fractions at the end of the differentiation period for 2 days (from day 6 to day 8). The expression levels of adipogenic transcription factors such as PPARγ and SREBP1 were determined by RT-PCR analysis. Means with the different letters (a–d) are significantly different in each sample ($p < 0.05$) by Duncan's multiple-range test.

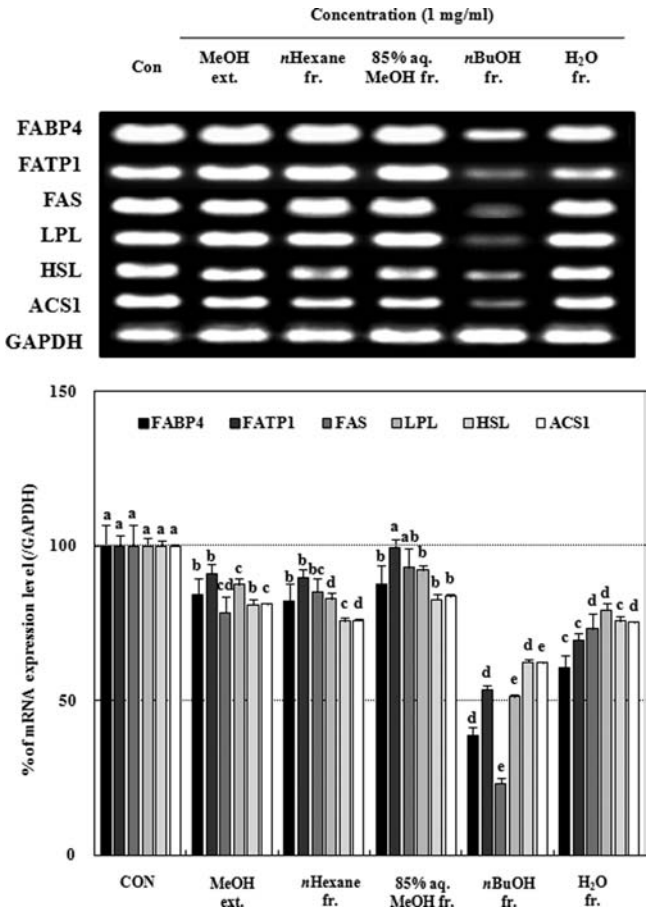

Figure 5. Effect of crude extract and its solvent fraction from *E. cava* on the regulation of adipogenic-specific genes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days in 6-well plates, and the cells were treated with crude extraction and its solvent fractions at the end of the differentiation period for 2 days (from day 6 to day 8). The expression levels of adipogenic-specific genes such as FABP4, FATP1, FAS, LPL, HSL, and ACS1 were determined by RT-PCR analysis. Means with the different letters (a–e) are significantly different in each sample ($p < 0.05$) by Duncan's multiple-range test.

We further investigated whether crude extracts and each solvent-partitioned fraction regulate the promoters of adipogenic target genes such as adipocyte fatty acid binding protein (FABP)-4, fatty acid transport protein (FATP)-1, fatty acid synthase (FAS), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), and acyl-CoA synthetase 1 (ACS1) (Figure 5). Comparative analysis indicates that the presence of the *n*-BuOH fraction in 3T3-L1 adipocytes effectively down-regulated the gene expression of FABP4, FATP1, FAS, LPL, HSL, and ACS1.

Structural Identification of Chemical Components from *E. cava*. Six phlorotannins, phloroglucinol (**1**), triphlorethol-A (**2**), eckol (**3**), eckstolonol (**4**), phlorofucofuroeckol A (**5**), and dieckol (**6**), were isolated from the *n*-BuOH fraction as shown in Figure 1. Their chemical structures were determined by a combination of 2D NMR spectroscopy such as ^{1}H gDQCOSY, TOCSY, NOESY, gHMQC, and gHMBC and by comparison with the data reported in the literature[11−15] (Figure 1B).

*Triphlorethol-A (**2**):* colorless gum; LREIMS *m*/*z* 426 $[M]^+$, $C_{18}H_{14}O_9$; $[\alpha]_D^{20}$ +16.67° (*c* 0.12, MeOH); IR (NaCl) υ_{max} cm^{-1} 3400−3300, 2930, 1680, 1620, 1470, 1220; UV (MeOH) λ_{max} nm 237 (3.77); ^{1}H NMR (300 MHz, CD_3OD) δ 6.04 (1H, d, *J* = 2.8 Hz, H-5), 6.00 (1H, d, *J* = 2.2 Hz, H-2′/-6′), 5.92 (1H, t, *J* = 2.2 Hz, H-4′), 5.89 (1H, s, H-3″/-5″), 5.75 (1H, d, *J* = 2.8 Hz, H-3); ^{13}C NMR (75 MHz, CD_3OD) δ 162.1 (C-1′), 160.0 (C-3′/-5′), 156.1 (C-4″), 155.9 (C-4), 153.5 (C-6), 152.3 (C-2), 151.9 (C-2″/-6″), 125.4 (C-1), 124.4 (C-1″), 97.8 (C-3), 97.3 (C-4′), 96.0 (C-3″/-5″), 95.2 (C-2′/-6′), 94.8 (C-5).

*Eckol (**3**):* light brown powder; FAB-MS *m*/*z* 372 $[M]^+$, $C_{18}H_{12}O_9$; IR (KBr) υ_{max} cm^{-1} 3250, 1605; UV (MeOH) λ_{max} nm 230 (ε 32000), 290 (ε 3100); ^{1}H NMR (300 MHz, CD_3OD) δ 6.12 (1H, s, H-3), 5.93 (1H, s, H-6/-8), 5.92 (1H, s, H-2′/-4′/-6′); ^{13}C NMR (75 MHz, CD_3OD) δ 161.60 (C-1′), 159.88 (C-3′/-5′), 146.99 (C-2), 146.83 (C-9), 143.97 (C-5a), 143.09 (C-4), 138.30 (C-10a), 125.35 (C-1), 124.27 (C-4a), 124.61 (C-9a), 99.66 (C-8), 99.16 (C-3), 97.51 (C-4′), 95.60 (C-6), 95.18 (C-2′/6′).

*Eckstolonol (**4**):* colorless gum; LRFABMS *m*/*z* 431 $[M + Na]^+$, $C_{18}H_{10}O_9$; $[\alpha]_D^{25}$ +12.27° (*c* 0.73, MeOH); IR (NaCl) υ_{max} cm^{-1} 3400−3300, 2925, 1690, 1635, 1220; UV (MeOH) λ_{max} nm 224 (3.86); ^{1}H NMR (300 MHz, CD_3OD) δ 6.13 (1H, s, H-7), 6.00 (1H, d, *J* = 2.8 Hz, H-2), 5.99 (1H, d, *J* = 2.8 Hz, H-10), 5.94 (1H, d, *J* = 2.5 Hz, H-12), 5.87 (1H, d, *J* = 2.5 Hz,

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H-4); ^{13}C NMR (75 MHz, CD_3OD) δ 154.45 (C-3), 154.09 (C-11), 146.95 (C-1), 146.74 (C-9), 143.52 (C-4a), 143.26 (C-12a), 141.08 (C-6), 138.97 (C-7a), 132.85 (C-13b), 127.66 (C-5a), 124.73 (C-8a), 123.63 (C-13a), 124.32 (C-14a), 99.87 (C-2), 99.75 (C-10), 98.84 (C-7), 95.69 (C-4), 95.58 (C-12).

*Phlorofucofuroeckol A (**5**):* light brown powder; FAB-MS m/z 603 $[M]^+$, $C_{30}H_{18}O_{14}$; IR (KBr) υ_{max} cm^{-1} 3350, 3300, 1605, 1470, 1360, 1110, 1060, 880; UV (MeOH) λ_{max} nm 224 (sh), 224 (ε 58000), 292 (ε 6800), 304 (sh), 317 (ε 3100); ^{1}H NMR (300 MHz, CD_3OD) δ 6.62 (1H, s, H-13), 6.39 (1H, s, H-9), 6.25 (1H, s, H-3), 5.96 (2H, d, J = 2.0 Hz, H-2′/-6′), 5.93 (1H, t, J = 2.2 Hz, H-4″), 5.91 (1H, t, J = 2.0 Hz, H-4′), 5.87 (2H, d, J = 2.2 Hz, H-2″/6″); ^{13}C NMR (75 MHz, CD_3OD) δ 161.66 (C-1′), 161.63 (C-1″), 159.98 (C-3″/-5″), 159.96 (C-3′/-5′), 152.97 (C-12a), 151.51 (C-10), 150.96 (C-11a), 148.09 (C-2), 148.03 (C-8), 145.76 (C-14), 143.73 (C-4), 138.20 (C-15a), 135.15 (C-5a), 127.90 (C-14a), 124.86 (C-4a), 124.56 (C-1), 122.19 (C-11), 105.22 (C-7), 105.17 (C-6), 99.85 (C-9), 99.25 (C-3), 97.65 (C-4″), 97.53 (C-4′), 96.11 (C-13), 95.31 (C-2′/-6′), 95.27 (C-2″/-6″).

*Dieckol (**6**):* light brown powder; FAB-MS m/z 743 $[M]^+$, $C_{36}H_{22}O_{18}$; IR (KBr) υ_{max} cm^{-1} 3250, 1605; UV (MeOH) λ_{max} nm 235 (ε 34000), 292 (ε 3500); ^{1}H NMR (300 MHz, CD_3OD) δ 6.14 (1H, s, H-3″), 6.12 (1H, s, H-3), 6.08 (2H, s, H-2‴/-6‴), 6.06 (1H, d, J = 2.8 Hz, H-8), 6.05 (1H, d, J = 3.0 Hz, H-6″), 5.97 (1H, d, J = 2.8 Hz, H-8″), 5.94 (1H, d, J = 2.8 Hz, H-6), 5.92 (3H, s, C-2′/-4′/-6′); ^{13}C NMR (75 MHz, CD_3OD) δ 161.57 (C-1′), 159.81 (C-3′/-5′), 157.57 (C-1‴), 155.76 (C-7), 154.28 (C-7″), 152.16 (C-3‴/-5‴), 147.11 (C-2), 147.07 (C-9″), 146.90 (C-9), 146.69 (C-2″), 144.08 (C-5a″), 143.93 (C-5a), 143.18 (C-4″), 143.08 (C-4), 138.44 (C-10a), 138.26 (C-10a″), 126.26 (C-4‴), 126.00 (C-9a), 125.46 (C-4a), 125.40 (C-4a″), 124.68 (C-9a″), 124.44 (C-1″), 124.38 (C-1), 99.73 (C-8″), 99.59 (C-3), 99.34 (C-3″), 99.23 (C-8), 97.53 (C-4′), 96.08 (C-2‴/-6‴), 95.71 (C-6″), 95.64 (C-2′/-6′), 95.64 (C-), 95.23 (C-6).

Effect of Phlorotannins on Intracellular Lipid Accumulation. The antiadipogenic effect of phlorotannins isolated from *E. cava* was compared by measuring lipid accumulation levels, which were quantified by Oil-Red O staining (Figure 6). All phlorotannins suppressed significantly adipogenic differentiation; that is, the presence of phlorotannins during differentiation reduced the absorbance values at a concentration of 50 μM (p < 0.05). Among phlorotannins, eckstolonol (**4**) most effectively attenuated the lipid accumulation level during adipogenic differentiation.

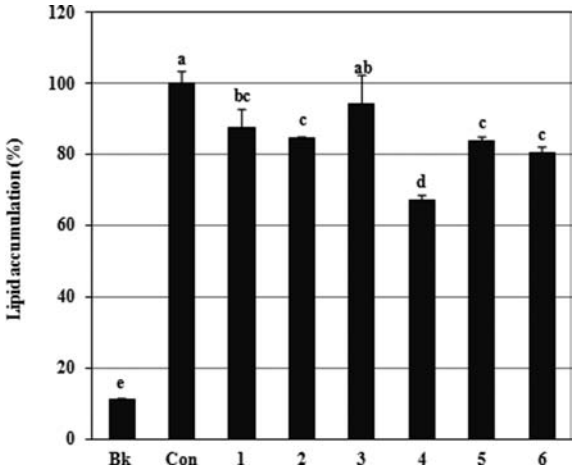

Figure 6. Effect of phlorotannins isolated from *E. cava* on intracellular lipid accumulation in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days in 6-well plates, and the cells were treated with phlorotannins at the end of the differentiation period for 2 days (from day 6 to day 8). The lipid droplets were fixed with 10% fresh formaldehyde and stained with Oil-Red O staining solution. The dye retained in the cells was eluted with isopropanol and quantified by measuring optical absorbance at 500 nm. Bk, preadipocytes; Con, fully differentiated control adipocytes (0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 5 μg/mL insulin); **1**, phloroglucinol; **2**, triphlorethol-A; **3**, eckol; **4**, eckstolonol; **5**, phlorofucofuroeckol A; **6**, dieckol. Means with the different letters (a–e) are significantly different (p < 0.05) by Duncan's multiple-range test.

DISCUSSION

Obesity has been recognized as a significant factor contributing to health complications including the pathogenesis of various chronic diseases such as type-2 diabetes, hypertension, cancer, cardiac injury, and heart disease.[17−19] Recently a number of research teams have concentrated on exploring new components from natural resources for the purpose of assessing adipogenic differentiation and gene regulation in several stages associated with obesity.[20]

Adipocyte differentiation, known as adipogenesis, is the process of fat cell accumulation, which is accompanied by dramatic alteration in the cellular pattern of adipogenic-related gene expression and protein synthesis. It is well-known that adipogenesis and the subsequent fat accumulation are closely related to obesity.[21]

In the present study, we tried to explore the potent components from antiadipogenic active fraction of *E. cava* in 3T3-L1 adipocytes. As a primary screening, the effects of the MeOH extract from *E. cava* and all of its solvent-partitioned fractions on lipid accumulation in 3T3-L1 adipocytes were evaluated. The *n*-BuOH fraction gave the most noticeable effect on suppression of lipid accumulation (Figure 2), and the 85% aq MeOH fraction was the most effective in inhibition of glucose consumption (Figure 3).

Among solvent-partitioned fractions, the *n*-BuOH fraction applied to adipocytes significantly reduced the regulation of the key adipogenic marker PPARγ and SREBP1c, which are secreted in proportion to triacylglyceride accumulation (Figure 4). The transcription factors PPARγ and SREBP1c are able to accelerate expression of adipocyte specific genes, secretion of adipokines, and elevation of adipose cells.[22]

In addition, the presence of the *n*-BuOH fraction in adipocytes effectively reduced the expression levels of adipocyte-specific genes such as FABP4, FATP1, FAS, LPL, HSL, and ACS1 (Figure 5). The FABPs are members of the family of lipid-binding proteins[23,24] and facilitate the transportation of fatty acids between extra- and intracellular membranes in fatty acid metabolism.[25] Both FABP4 and FATP1 are associated with fatty acid utilization and fatty acid transport in cells.[26] Adipose tissue may be the major site for fat synthesis, and FAS acts as the key enzyme in adipocyte differentiation.[27] Hormone-sensitive lipase (HSL) mediates the hydrolysis of triglyceride into fatty acids and glycerol.[18] LPL catalyzes the hydrolysis of triglycerides, and ACS1 is prominent in the liver as well as adipocytes, likely providing acyl-CoA for triacylglycerol synthesis.[17,18] These results suggest that the *n*-BuOH fraction from *E. cava* effectively attenuates lipid

accumulation through inhibition of fatty acid synthesis but not hydrolysis of triglyceride.

On the basis of these results, further separation from the *n*-BuOH fraction led to the isolation of six phlorotannins, phloroglucinol (**1**), triphlorethol-A (**2**), eckol (**3**), eckstolonol (**4**), phlorofucofuroeckol A (**5**), and dieckol (**6**). The phlorotannins significantly suppressed lipid accumulation compared to the adipocytes. Notably the suppression was more effective in the presence of eckstolonol (**4**), rather than other phlorotannins. Recent studies have revealed that *E. cava* contains an array of polyphenolic components with unique linkages such as eckol, 7-phloroeckol, 6,6′-bieckol, dieckol, eckstolonol, phlorofucofuroeckol, and fucodiphloroethol G.[15,28,29] Several scientists have reported that the phlorotannins from *E. cava* possess a number of biological activities.[28,30−32] However, antiadipogenic activity of phlorotannins has rarely been reported, and the only reported phlorotannins are eckstolonol and 7-phloroeckol.[16,17] The *n*-BuOH fraction of *E. cava* has eckstolonol (**4**), a known antiadipogenic compound. Nevertheless, we can expect that other antiadipogenic phlorotannins besides eckstolonol (**4**) exist in the *n*-BuOH fraction because **4** was a very minor component (wt % of **4** in *n*-BuOH fraction: = 0.0033%). Further investigations on additional isolation of other phlorotannins and their antiadipogenic activities are in progress.

In conclusion, the present results demonstrate that crude MeOH extracts and solvent-partitioned fractions of *E. cava* have the potential to suppress adipogenic differentiation. Further purification was made from the *n*-BuOH fraction, which most effectively suppressed adipogenic differentiation, and then six phlorotannins (**1**−**6**) were obtained. Although the direct relationship between structure and bioactivity in the phloroglucinol ring system has not been reported until now, it has been well-known that bioactivity and aromatic hydroxyl functionalities of many polyphenolic compounds are closely related.[33,34] Therefore, the antiadipogenic activity of phlorotannins may be due to the hydroxyl group in the phloroglucinol ring system. More study should be encouraged to explore the structure−activity relationship on antiadipogenic activity of phlorotannins from *E. cava*.

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Notes

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