Enzyme-treated *Ecklonia cava* extract inhibits adipogenesis through the downregulation of C/EBPα in 3T3-L1 adipocytes

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Abstract. In this study, we examined the inhibitory effects of enzyme- treated *Ecklonia cava* (EEc) extract on the adipogenesis of 3T3-L1 adipocytes. The components of *Ecklonia cava (E. cava)* were first separated and purified using the digestive enzymes pectinase (Rapidase® X-Press L) and cellulase (Rohament® CL). We found that the EEc extract contained three distinct phlorotannins: eckol, dieckol and phlorofucofuroeckol-A. Among the phlorotannins, dieckol was the most abundant in the EEc extract at 16 mg/g. Then we examined the inhibitory effects of EEc extract treatment on differentiation-related transcription factors and on adipogenesis-related gene expression *in vitro* using 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes were used to determine the concentrations of the EEc extract and *Garcinia cambogia* (Gar) extract that did not result in cytotoxicity. Glucose utilization and triglyceride (TG) accumulation in the EEc-treated adipocytes were similarly inhibited by 50 μg/ml EEc and 200 μg/ml Gar, and these results were confirmed by Oil Red O staining. Protein expression of adipogenesis differentiation-related transcription factors following treatment with the EEc extract was also examined. Only the expression of CCAAT/enhancer-binding protein (C/EBP)α was decreased, while there was no effect on the expression of C/EBPβ, C/EBPδ, and peroxisome proliferator-activated receptor γ (PPARγ). Treatment with the EEc extract decreased the expression levels of adipogenesis-related genes, in particular sterol regulatory element binding protein-1c (SREBP-1c), adipocyte fatty acid binding protein (A-FABP), fatty acid synthase (FAS) and adiponectin. These results suggest that EEc extract treatment has an inhibitory effect on adipogenesis, specifically by affecting the activation of the C/EBPα signaling pathway and the resulting adipogenesis-related gene expression.

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Introduction

Obesity is the most common metabolic disease worldwide and is a major public health issue, particularly in developed countries (1). Obesity occurs by a complex interaction between genetic and environmental factors (2,3), and is characterized by excessive fat cell size (hypertrophic obesity) or cell number (hyperplasic obesity). It is often associated with a high-calorie diet, type 2 diabetes, high blood pressure, cardiovascular disease, and/or metabolic complications (4-9). In addition to the associated morbidity, many metabolic complications, including type 2 diabetes, insulin resistance, hyperlipidemia, hypertension, stroke, coronary heart disease and cancer, have been linked to obesity (10-12). These complications result in higher mortality rates in obese patients compared to lean patients.

Adipogenesis, the process of pre-adipocyte differentiation into adipocytes, is the result of excess energy intake and lack of activity. Adipogenesis contributes to the deposition of excess fat in adipocytes during differentiation from pre-adipocytes. Multiple processes regulate adipogenesis, including pre-adipocyte proliferation, differentiation, and fatty acid oxidation and synthesis, and these processes are controlled by a number of factors. Adipogenesis includes concerted transcriptional and cellular events, including growth arrest, re-entry into the cell cycle for mitotic clonal expansion, and the initiation of transcription during differentiation (13-15). A number of genes have been shown to be involved in the development of obesity, including peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP)α, C/EBPβ and C/EBPδ (16-19). The expression levels of adipocyte-related proteins such as sterol regulatory element binding protein-1c (SREBP-1c), adiponectin, fatty acid synthase (FAS), adipocyte fatty acid binding protein-4 (A-FABP4), glucose transporter (GLUT4), lipoprotein lipase (LPL) and stearoyl-CoA desaturase-1 (SCD-1) (20-24) are also induced.

A number of drugs have been developed for the treatment of obesity that target appetite regulation, fat absorption and fat oxidation (25,26). A number of these drugs have been withdrawn from the market because of low efficacy and side effects, thus only a few drugs remain (27,28). For example, the anti-obesity drugs orlistat (Xenical®) and sibutramine are commonly prescribed, although they are associated with

significant side effects such as bladder pain, diarrhea, fever, loss of appetite, nasal congestion and difficulty in sleeping (orlistat), and headache, insomnia, increased appetite, asthenia, nausea and anorexia (sibutramine) (29-31). Thus, there is clearly a need to develop safer and more effective anti-obesity drugs.

Brown algae may represent a renewable natural material for use as novel therapeutic agents because they are rich in bioactive substances, including sulfated polysaccharides, proteins, dietary fibers and carotenoids (32-40). *Ecklonia cava (E. cava)* is an edible marine brown algal species that mainly inhabits coastal Japan and Korea (41). It has rarely been used for food but has been widely utilized in the aquaculture of abalone and seashells. Studies of *E. cava* have reported its antioxidant (42-46), anti-inflammatory (47-50), anticancer (51,52), and antibacterial (53,54) properties.

Recently, studies have examined the physiological activity of *E. cava* because of its polyphenol components, which include phlorotannins (55-61). *E. cava* polyphenols exhibit antioxidant (55,56) and anticancer (51,52,59-61) properties as well as contribute to hair growth (57,58). Polyphenol extracts of *E. cava* have the potential to treat Alzheimer's disease (62) and the polyphenolic extract Seanol affects lipid and glucose metabolism (63).

Previous studies have investigated the anti-obesity properties of *E. cava* extracts using zebrafish, mice and cell cultures (64-70). In the present study, enzyme-treated *Ecklonia cava* (EEc) extract was prepared using the digestive enzymes pectinase (Rapidase® X-Press L) and cellulase (Rohament® CL) for separation and purification of the effective components in *E. cava*. We examined the inhibitory effects of the EEc extract on adipocyte differentiation and adipogenesis-related gene expression *in vitro* using 3T3-L1 adipocytes. *Garcinia cambogia* (Gar) extracts are used as natural supplements and are known to suppress appetite and lower body fat by blocking the lipid synthesis pathway (71-73). A Gar extract was used as a positive control.

Materials and methods

Preparation of enzyme-treated E. cava extract. E. cava was purchased in 2013 from Taekyug-nongsan (Jeju-do, Korea). *E. cava* chips of approximately 5 cm were prepared by cutting the leaves and removing the stem and roots of the algae. The extract was prepared by placing 30 kg *E. cava* chips in 750 liters of distilled water with the added enzymes (300 g pectinase, Rapidase X-Press L and 300 g cellulase, Rohament CL). The suspension was stirred for 24 h at 50°C, centrifuged at 3,000 x g at 4°C for 20 min, vacuum filtered, and then three volumes of 60% ethanol were added. After 18 h, the solution was filtered and concentrated using rotary evaporation to 6° Bx. The concentrated solution was made into a powder using a spray dryer. The final extract weighed 3.56 kg, representing a yield of 10.7% (EEc; JY202-MM130126R). *Garcinia cambogia* powder extract was purchased from ES Ingredient Co., Ltd. (Gyeonggi-do, Korea).

Cell culture. 3T3-L1 mouse fibroblast cells (CL-173) were obtained from the American Type Culture Collection (ATCC) (Rockvile, MD, USA), and were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum (NBCS; Gibco, Life Technologies Corp., Auckland, New Zealand) containing 50 μg/ml penicillin, 25 μg/ml amphotericin B and 50 μg/ml streptomycin. At 70% confluency, the cells were harvested by trypsinization and seeded in 6-well plates in pre-adipocyte expansion medium (DMEM supplemented with 10% NBCS). When 100% confluency was reached, the cells were fed differentiation medium [DMEM supplemented with 10% fetal bovine serum (FBS) containing 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μg/ml insulin] for 48 h. The medium was then replaced with adipocyte maintenance medium (DMEM supplemented with 10 μg/ml insulin) and changed every 48 h for 8 days.

Cell viability assay. Cell viability was estimated using a Cyto X cell viability assay kit (LPS solution, Daejeon, Korea). Cells were seeded in 96-well plates at $2x10^4$ cells/well in 100 μl medium and allowed to attach for 24 h. Attached cells were treated with 12.5, 50, or 200 μg/ml EEc extract or Gar extract in serum-free medium (SFM) for 24 h. Cyto X solution was added to the cells and incubation was carried out for 1 h, and the absorbance of each well was measured at 450 nm using a FilterMAX F5 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

Glucose utilization assay. Cells were seeded in 6-well plates and differentiation was induced. Following differentiation, the cells were treated with 12.5, 50 or 200 μg/ml EEc extract or Gar extract for 24 h. Glucose utilization was performed with a glucose assay kit (ASAN glucose kit; Asan Pharm Co., Gyeonggi-do, Korea), as per the manufacturer's instructions. Briefly, glucose in the cell medium of each well was allowed to react with the glucose assay reagent for 5 min; then the contents were transferred to a 96-well plate, and the absorbance was measured at 500 nm using a microplate reader (Gen 5; Epoch BioTek Instrument, Inc., Winooski, VT, USA).

Triglyceride (TG) accumulation assay. Following differentiation, the cells were washed with phosphate-buffered saline (PBS), harvested in ice-cold PBS, and sonicated for 1 min. To measure TG content, TG assay reagent was added to the cell lysate according to the manufacturer's instructions (Cleantech TG-S; Asan Pharm Co.). Briefly, each cell lysate (30 μl) was reacted with the TG assay reagent solution for 10 min, transferred to a 96-well plate, and then the absorbance was measured at 550 nm.

Oil Red O staining. Following differentiation, the cells were washed twice with PBS and fixed with 10% formaldehyde for 1 h at room temperature. After washing with PBS, the cells were stained with Oil Red O working solution [0.5 g Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) in 60% isopropanol] for 1 h. After the staining solution was removed, the lipid droplets were washed with water and dried. Stained oil droplets in 3T3-L1 cells were imaged with a light microscope (Eclipse TS100-F; Nikon, Tokyo, Japan). To quantify the Oil Red O uptake, cells in each well were extracted with 1 ml 100% isopropanol for 10 min, transferred to a 96-well plate, and then the absorbance was measured at 540 nm using a microplate reader.
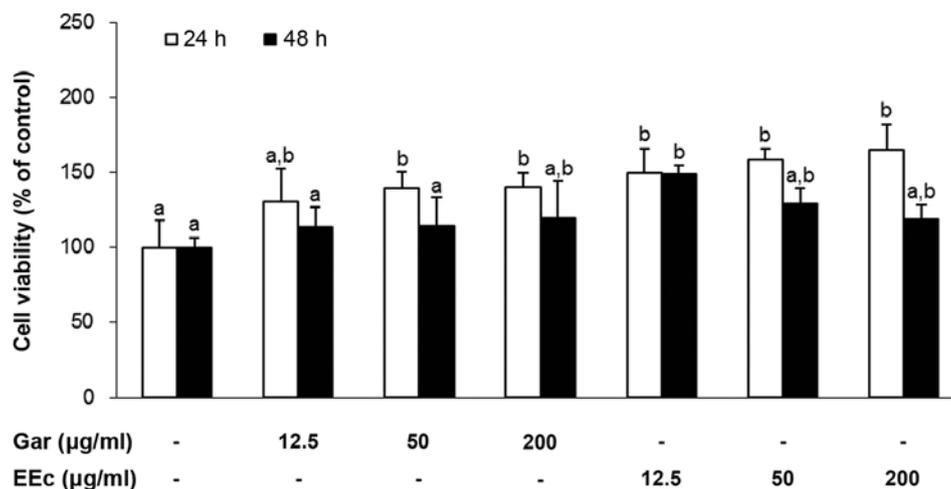
Figure 1. Treatment with Gar and EEc extracts does not cause cytoxicity in the 3T3-L1 pre-adipocytes. Effects of the treatment with the Gar and EEc extracts on cell viability were determined in 3T3-L1 pre-adipocytes by the Cyto X assay. Cells were treated with various concentrations of the Gar or EEc extracts. Data are presented as the means ± standard deviation (P<0.05) from three independent experiments. Gar, *Garcinia cambogia* extract; EEc, enzyme-treated *Ecklonia cava* extract. The different letters at all concentrations represent significant differences (P<0.05) as determined by Duncan's multiple range test.

Western blot analysis. Following differentiation, the cells were incubated for 24 h in SFM containing 12.5, 50, or 200 μg/ml Gar or EEc extract. Then the cells were washed with PBS and lysed with extraction buffer (20 mM Tris, 150 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate, 100 μM ammonium molybdate, 1 mM β-glycerophosphate, 0.1% NP-40, and 0.1% SDS, pH 8.0) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μM sodium orthovanadate and 1 mM PMSF). The extracts were centrifuged at 9,750 x g for 10 min, and the supernatant was used for western blot analysis.

Total protein (40 μg) was electrophoresed on an SDS-PAGE gel and transferred to a polyvinylidene fluoride transfer membrane (Millipore Corp., Billerica, MA, USA). Membranes were blocked with 1% bovine serum albumin (BSA) in TBS-T (5 mM Tris-HCl, 20 mM sodium chloride, pH 7.4, and 0.1% Tween-20) and incubated with primary antibodies (1:1,000) in 1% BSA in TBS-T with gentle shaking overnight at 4˚C. Membranes were washed twice for 15 min in TBS-T, and incubated with the corresponding HRP-conjugated secondary antibodies (1:10,000) for 2 h at room temperature and washed again. The immunoreactive bands were detected using an enhanced chemiluminescence substrate (Advansta, Menlo Park, CA, USA) and visualized using the GeneSys imaging system (SynGene Synoptics, Ltd., London, UK). The following primary antibodies were used: anti-C/EBPα (sc-9314, anti-goat), anti-C/EBPβ (sc-150, anti-rabbit), anti-C/EBPδ (sc-151, anti-rabbit), anti-PPARγ (sc-1984, anti-goat), anti-SREBP-1c (sc-366, anti-rabbit), anti-A-FABP (sc-18661, anti-goat), anti-FAS (sc-55580, anti-mouse), anti-GLUT4 (sc-1606, anti-rabbit), anti-adiponectin (sc-26497, anti-goat), anti-leptin (sc-842, anti-rabbit), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778, anti-rabbit) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The secondary antibodies used were HRP-conjugated anti-mouse IgG (sc-2031, Santa Cruz Biotechnology, Inc.), anti-rabbit (A-0545, Sigma-Aldrich), and anti-goat (A50-101P, Bethyl Laboratories Inc., Montgomery, TX, USA).

Statistical analysis. The results are presented as mean ± standard deviation of at least three independent experiments (P<0.05). Significant differences among multiple mean values were assessed by analysis of variance (ANOVA) followed by the Duncan's multiple range test using PASW statistics 18 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EEc extract treatment does not exert cytotoxic effects on 3T3-L1 pre-adipocytes. To determine the effects of Gar or EEc extract treatment on the viability of the 3T3-L1 pre-adipocytes, the cells were treated with 12.5, 50 or 200 μg/ml Gar or EEc extract for 24 or 48 h. As shown in Fig. 1, treatment with Gar or EEc extract had no significant effect on the viability of the 3T3-L1 pre-adipocytes at 48 h. However, treatment with 12.5, 50 or 200 μg/ml Gar extract significantly increased cell viability with values of 30.9, 39 and 39.9%, respectively at 24 h. Also, treatment with 12.5, 50, or 200 μg/ml EEc significantly increased cell viability at 49.8, 58.3 and 65.5%, respectively at 24 h, compared to the control group.

Therefore, we selected the maximum dose of Gar and EEc (200 μg/ml) for subsequent experiments to rule out the possibility that EEc-dependent inhibition of adipogenesis may be a result of its cytotoxic effects on 3T3-L1 cells. In subsequent experiments, cells were treated with 12.5, 50 or 200 μg/ml Gar and EEc extract for 24 h without cytotoxicity.

EEc extract treatment inhibits glucose utilization and TG accumulation in the 3T3-L1 adipocytes. 3T3-L1 cells were induced to differentiate for 8 days and were then treated with various concentrations of EEc extract for 24 h. As shown in Fig. 2A, glucose utilization was assessed following EEc extract treatment. In the positive control [receiving 3-isobutyl-1-methylxanthine, dexamethasone and insulin (MDI)], glucose utilization was 156±7.0 mg/dl, while this was reduced to 84.4±17.9 mg/dl in the Gar group. Treatment with
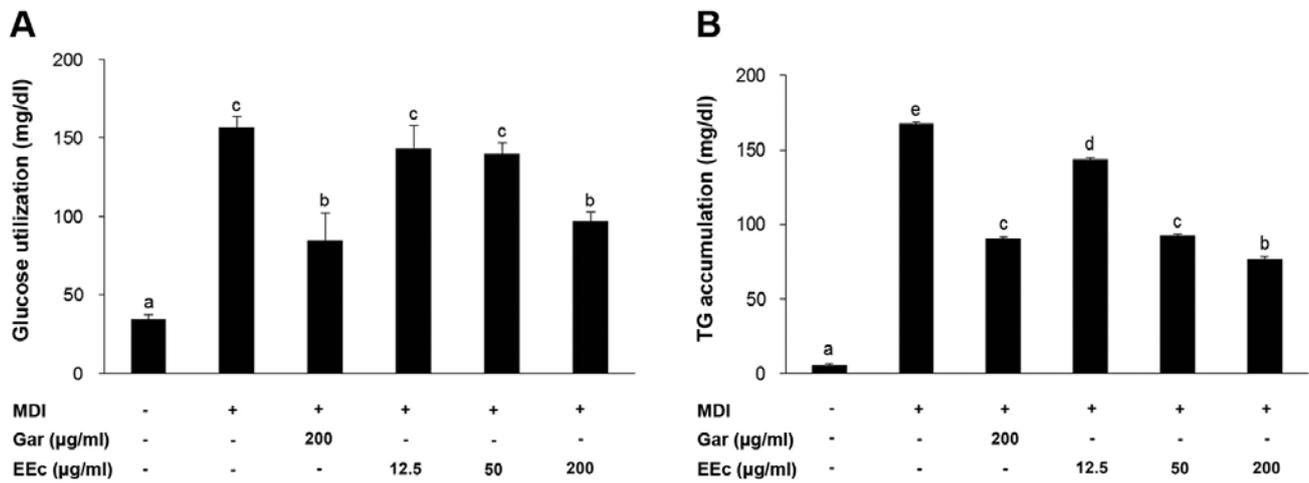
Figure 2. Treatment with the EEc extract inhibits glucose utilization and triglyceride accumulation in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were incubated with or without various concentration of the Gar and EEc extracts for 24 h. Glucose utilization in the culture medium was measured using glucose assay kit. (B) 3T3-L1 adipocytes were incubated with or without various concentration of the Gar and EEc extracts for 24 h. TG accumulation in the cell lysate was measured using TG assay kit. Data are presented as the means ± standard deviation (P<0.05) from three independent experiments. Gar, *Garcinia cambogia* extract; EEc, enzyme-treated *Ecklonia cava* extract; TG, triglyceride; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. The different letters at all concentrations represent significant differences (P<0.05) as determined by Duncan's multiple range test.

12.5, 50, or 200 μg/ml EEc extract also reduced glucose utilization in a dose-dependent manner, with values of 143.5±14.6, 139.8±7.3 and 96.7±6.2 mg/dl, respectively.

Next, we examined TG accumulation following EEc extract treatment of the 3T3-L1 adipocytes. We found that TG accumulation was 167.9±14.0 mg/dl in the MDI group while only 90.8±3.5 mg/dl in the Gar group (Fig. 2B). Treatment with 12.5, 50, or 200 μg/ml EEc extract resulted in significantly reduced TG accumulation in a dose-dependent manner with values of 143.5±8.7, 92.4±1.3 and 77.1±1.3 mg/dl, respectively. Consistent with these results, TG accumulation in the 3T3-L1 adipocytes treated with 200 μg/ml EEc extract was lower than that in the Gar group.

EEc extract treatment increases adipogenesis in the 3T3-L1 adipocytes. Following the induction of differentiation, 3T3-L1 pre-adipocytes underwent morphological changes, including a transition from spindle-like features to a round shape and accumulation of intracellular lipids. Lipid accumulation is a known indicator of adipogenesis, and thus Oil Red O staining was used to examine whether EEc treatment influenced lipid accumulation in the adipocytes. As shown in Fig. 3A, EEc treatment decreased the intracellular lipid content compared to that noted in the MDI group as observed in images of the differentiated 3T3-L1 adipocytes.

The inhibitory effect of EEc extract treatment on lipid accumulation was confirmed by lipid droplet quantification (Fig 3B). Intracellular lipid content was markedly decreased to 53.7±2.7 μg/ml when the 3T3-L1 adipocytes were treated with 200 μg/ml Gar extract (Fig. 3B). Treatment with 12.5, 50, or 200 μg/ml EEc extract reduced lipid content in the 3T3-L1 adipocytes by 52.2, 53.2 and 62.6%, respectively, compared to these values in the MDI group.

EEc extract treatment decreases the protein levels of differentiation-related transcription factors. Adipogenesis is accompanied by a change in the sequential activation of several pro-adipogenic transcription factors, including C/EBPα/β/δ and PPARγ. Thus, we examined whether the reduced lipid accumulation in adipocytes was due to downregulation of these transcription factors. Compared to the differentiated control cells, EEc extract treatment significantly decreased the expression of C/EBPα but not C/EBPβ/δ and PPARγ (Fig. 4). This suggests that EEc treatment inhibits adipogenesis by suppressing the expression of adipogenic transcription factors, particularly C/EBPα.

EEc extract treatment reduces the expression of adipogenesis-related proteins. To examine the effects of EEc extract treatment on adipocytes, the expression of adipogenesis-related proteins was determined in the EEc-treated and untreated 3T3-L1 cells. We also determined the expression of other adipogenesis-related proteins involved in lipogenic and fatty acid oxidation and glucose homeostasis pathways. We found that EEc extract treatment inhibited the expression of adipogenic differentiation-related transcription factors in the 3T3-L1 cells. As shown in Fig. 5, differentiated cells exhibited significantly increased expression of PPARγ target genes in the adipogenesis pathway, including A-FABP, SREBP-1c, FAS and adiponectin. In contrast, EEc extract treatment significantly decreased the protein levels of SREBP-1c, A-FABP, FAS and adiponectin compared to levels noted in the differentiated control cells. No significant differences were apparent in regards to GLUT4 and leptin expression.

Discussion

Studies on *E. cava* have examined its antioxidant (42-46), anti-inflammatory (47-50), anticancer (51,52) and antibacterial (53,54) properties. Recently, phlorotannins, the polyphenol component of *E. cava*, were examined with respect to their antioxidant (55,56), anticancer (51,52,59-61) and anti-obesity (64-70) properties, as well as their effects on hair growth (57,58) and Alzheimer's disease (62). Inhibition
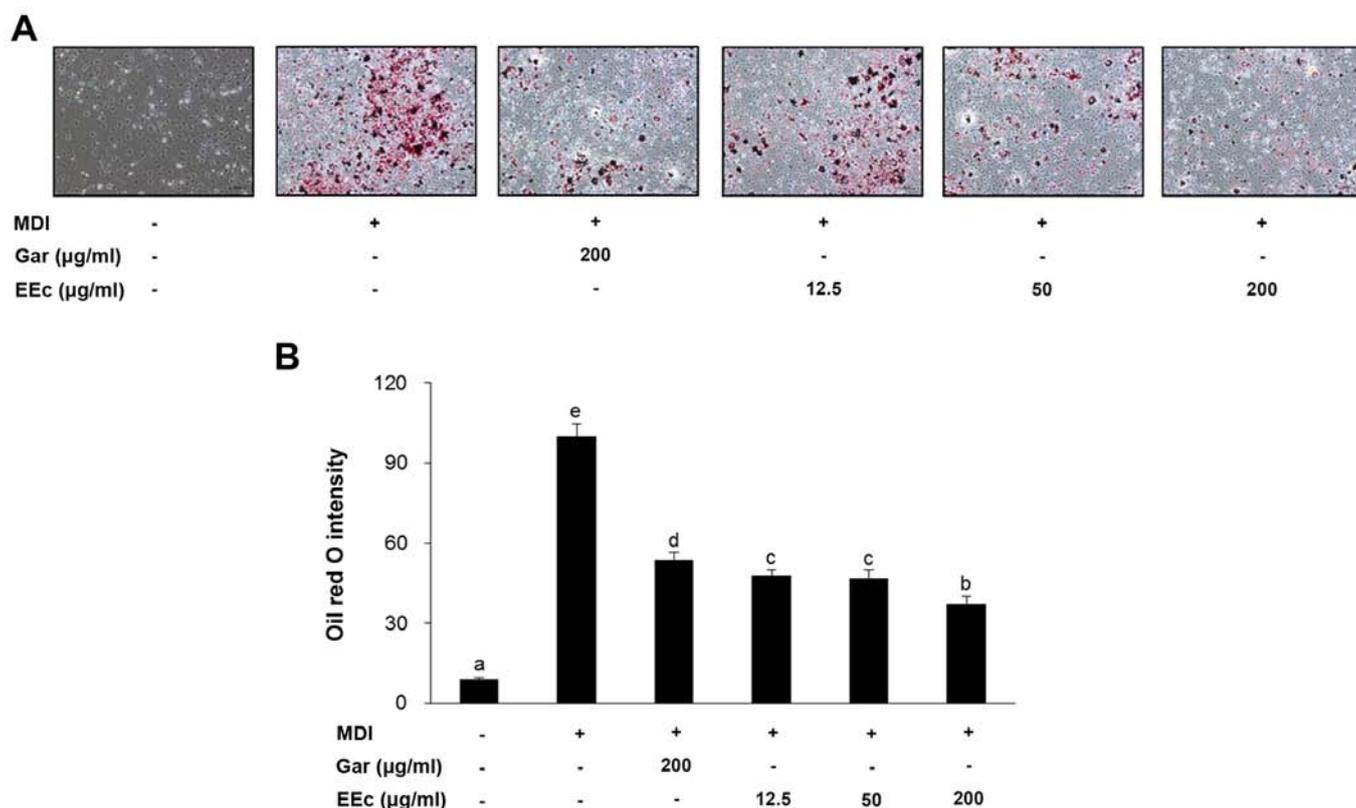
Figure 3. Treatment with the EEc extract inhibits lipid accumulation in the 3T3-L1 adipocytes. (A) 3T3-L1 pre-adipocytes were induced to differentiate in the absence or presence of the Gar and EEc extracts for 24 h. The morphological changes associated with cell differentiation were imaged following Oil Red O staining. (B) Stained lipids were extracted and quantified by measuring the absorbance at 540 nm. Data are presented as the means ± standard deviation (P<0.05) from three independent experiments. Gar, *Garcinia cambogia* extract; EEc, enzyme-treated *Ecklonia cava* extract; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. The different letters at all concentrations represent significant differences (P<0.05) as determined by Duncan's multiple range test.
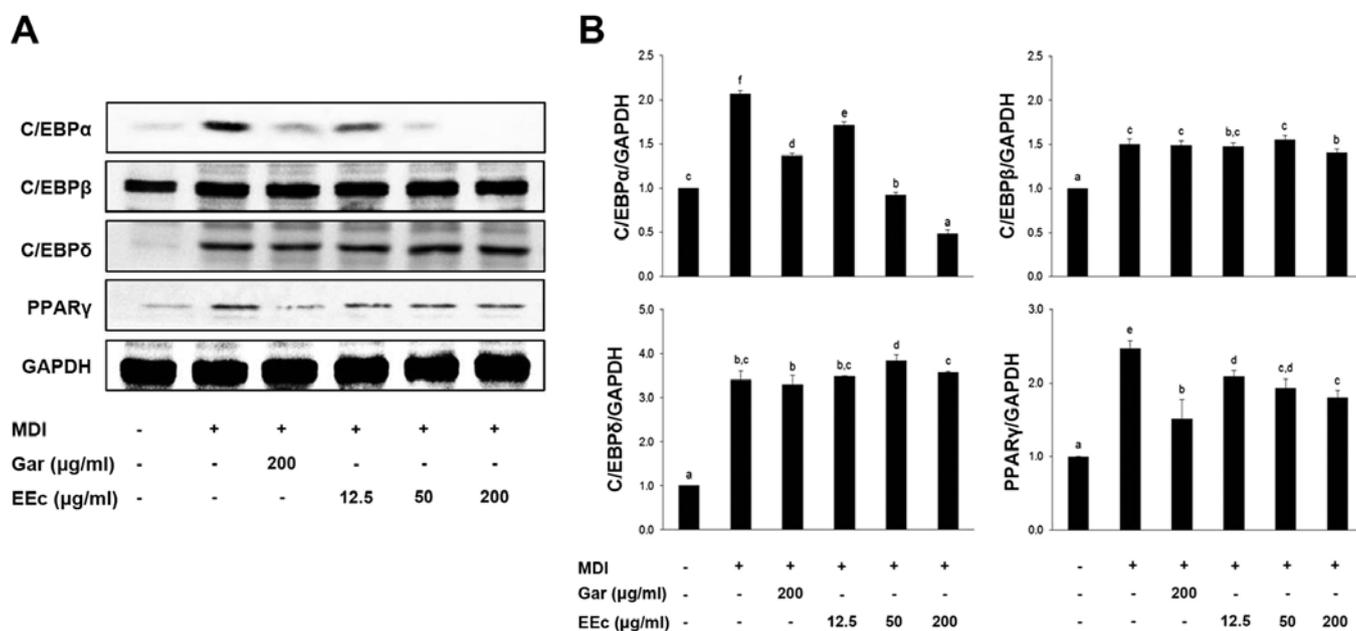
Figure 4. Treatment with the EEc extract inhibits the expression of differentiation-related transcription factors. (A) 3T3-L1 adipocytes were incubated with or without various concentrations of the EEc extract for 24 h. Protein levels of PPARγ, C/EBPα, C/EBPβ, and C/EBPδ were examined by western blotting as described in Materials and methods. (B) The bands were normalized to an internal control (GAPDH), and the relative ratio is graphed. Data are presented as the means ± standard deviation (P<0.05) from three independent experiments. C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor γ; Gar, *Garcinia cambogia* extract; EEc, enzyme-treated *Ecklonia cava* extract; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. The different letters at all concentrations represent significant differences (P<0.05) as determined by Duncan's multiple range test.
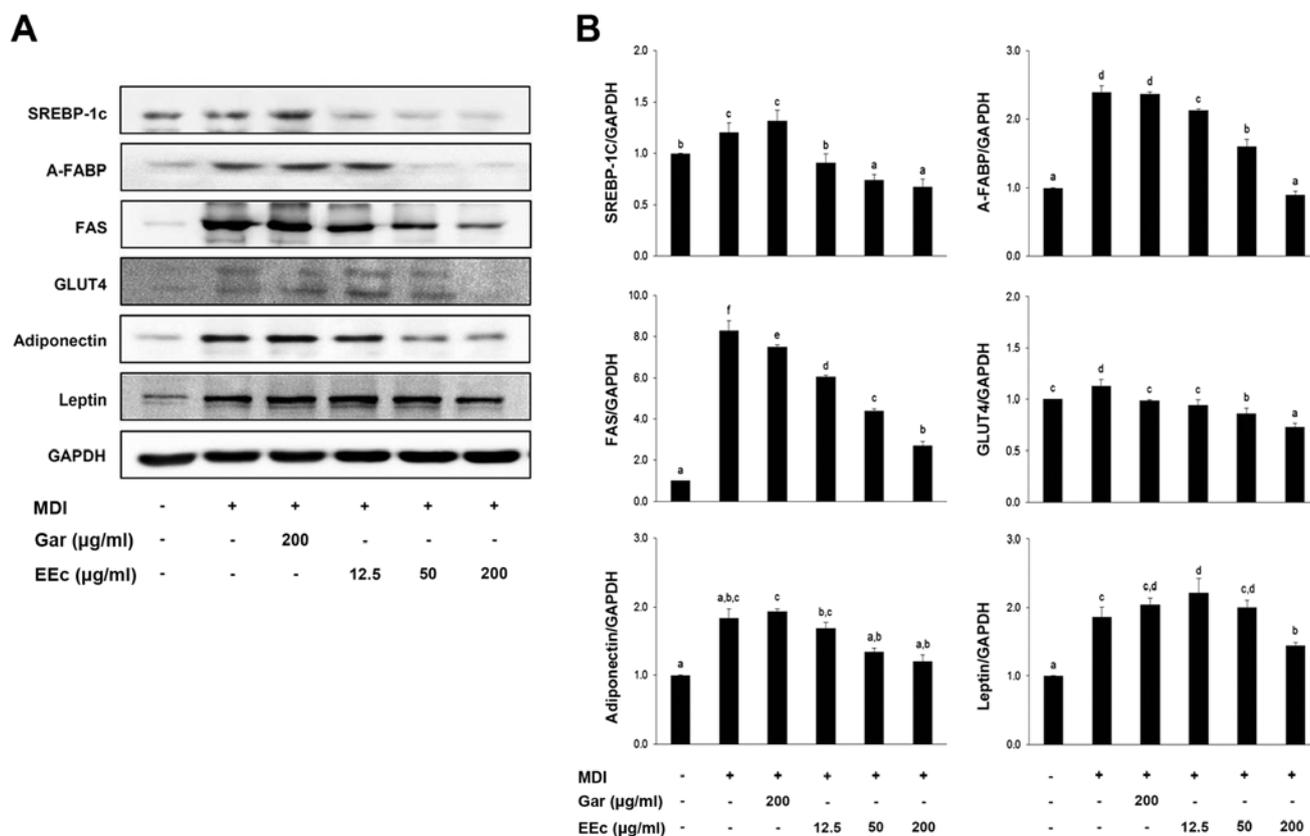
Figure 5. Treatment with the EEc extract inhibits expression of adipogenesis-related proteins. (A) 3T3-L1 adipocytes were incubated with or without various concentrations of the EEc extract for 24 h. Protein levels of SREBP-1c, A-FABP, FAS, GLUT4, adiponectin and leptin were examined by western blotting as described in Materials and methods. (B) The bands were normalized to an internal control (GAPDH), and the relative ratio is graphed. Data are presented as the means ± standard deviation (P<0.05) from three independent experiments. Gar, *Garcinia cambogia* extract; EEc, enzyme-treated *Ecklonia cava* extract; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; SREBP-1c, sterol regulatory element binding protein-1c; A-FABP, adipocyte fatty acid binding protein; FAS, fatty acid synthase. The different letters at all concentrations represent significant differences (P<0.05) as determined by Duncan's multiple range test.

of 3T3-L1 cell differentiation by *E. cava* extracts has been previously studied (69,70). The diethyl ether fraction (69), methanolic extract and its solvent-partitioned fraction (70) of *E. cava* extract were examined for their antiadipogenic effect on 3T3-L1 adipocytes. Dieckol from the diethyl ether fraction was found to inhibit adipogenesis (69), and eckstolonol from the n-BuOH fraction inhibited lipid accumulation (70).

In the present study, an *E. cava* extract was prepared using an enzyme-treatment high-yield extraction method with a high concentration of polyphenols, and then the effects of this extract on adipogenesis were determined.

We tested various methods to determine the optimal conditions for extraction of the extract of *E. cava*. We evaluated the following extraction methods: hot water treatment (60°C, 90°C), ethanol treatment (60%, 80%), and enzymatic treatment (Protex™ 6L, an endo-type protease; Rapidase® X-Press L, a pectinase-cellulase-hemicellulase enzyme complex; Rohament® CL, a cellulase-β-glucanase-hemicellulase enzyme complex). The resulting yields were as follows: hot water extraction, 19.31-27.75%; ethanol extraction, 3.24-11.42%; and enzyme extraction, 19.08-21.87%. Specifically, the enzyme extraction yields were 21.06% with Protex 6L, 19.08% with Rapidase X-Press L, 18.66% with Rohament CL, and 21.87% with the Rapidase X-Press L-Rohament CL complex. Hot water extraction resulted in a higher yield compared to enzyme extraction; however, we decided that enzyme extrac-

tion was the ideal method. The total polyphenol content was also measured for each extraction method and ranged from 206.74 to 812.17 μg/ml, with the 60% ethanol extraction having the highest content. We decided that this method had low economic efficiency as raw materials of *E. cava* were obtained at a lower yield. Therefore, enzyme extraction using the Rapidase X-Press L-Rohament CL complex was selected based on a consideration of both the total yield and cost effectiveness. High-performance liquid chromatographic analysis of the EEc extract revealed three components (eckol, dieckol, and phlorofucofuroeckol-A), and dieckol was the most abundant at 16 mg/g. The hexamer of phloroglucinol and dieckol exhibits various biological properties, including antioxidant and anti-allergenic activity, and plays a role in immunomodulation (74-76).

We considered two directions when planning the present experiment. In other words, we considered whether to treat the sample during or after completion of differentiation. In many studies, it was known that sample treatment after the completion of differentiation inhibited the adipogenesis-related protein expression levels (64-70). Thus, we examined the inhibitory effects of the EEc extact treatment upon completion of adipocyte differentiation and adipogenesis *in vitro* in the 3T3-L1 adipocytes. First, we determined that treatment of 3T3-L1 pre-adipocytes with 12.5, 50, or 200 μg/ml Gar or EEc extract did not result in cytotoxicity (Fig. 1). Glucose utiliza-

tion and TG accumulation were confirmed following EEc extract treatment in the 3T3-L1 adipocytes. Both the Gar and EEc extract-treated groups showed similar glucose utilization at 200 μg/ml (96.7±6.2 mg/dl vs. 84.4±17.9 mg/dl), as well as similar TG accumulation at 50 μg/ml (92.4±1.3 mg/dl vs. 90.8±3.5 mg/dl) (Fig. 2). Overall, we found that EEc extract treatment decreased glucose utilization and TG accumulation in the 3T3-L1 adipocytes.

Lipid accumulation in adipose tissues occurs at a late stage in adipogenesis. For this reason, Oil Red O staining was performed following EEc extract treatment in the 3T3-L1 adipocytes. We found that the EEc extract-treated group exhibited a marked decrease in the number of lipid droplets and lipid storage organelles compared to the MDI-treated group (Fig. 3). To confirm that the decrease in lipid accumulation shown in Figs. 2 and 3 was due to downregulation of the differentiation-related transcription factors C/EBPα/β/δ and PPARγ, we examined their expression levels by Western blot analysis. Differentiation of pre-adipocytes into adipocytes is tightly controlled by the sequential activation of several transcriptional factors, including C/EBPα, C/EBPβ, C/EBPδ and PPARγ (16,17). Generally, C/EBPβ functions rapidly following the induction of pre-adipocyte differentiation, followed by the expression of C/EBPα and PPARγ (18,19). As shown in Fig. 4, treatment with EEc extract decreased the expression of C/EBPα but did not affect the expression of C/EBPβ, C/EBPδ, or PPARγ.

Adipogenesis is regulated by a complex transcriptional cascade, in which members of the C/EBP family and PPARγ play important roles in the expression of adipogenesis-related genes, such as SREBP-1c, A-FABP, FAS, GLUT4, LPL and SCD-1, which are involved in insulin sensitivity, lipogenesis and lipolysis (16-20). A role for C/EBP in coordinating transcription during pre-adipocyte differentiation was indicated by its ability to transactivate the promoters of several adipogenesis-specific genes (21-24). The activation of C/EBPα promotes differentiation of pre-adipocytes by cooperating with PPARγ, resulting in transactivation of adipogenesis-specific genes such as A-FABP and FAS (26).

SREBP-1 is the first transcription factor involved in adipocyte differentiation, and it increases the expression of several lipogenesis-related genes, including LPL, ACC and FAS (38). To determine the expression of adipogenesis-related genes, SREBP-1c, A-FABP, FAS, GLUT4, adiponectin and leptin were examined by Western blotting. As shown in Fig. 5, EEc extract treatment decreased the expression of SREBP-1c, A-FABP, FAS and adiponectin in a dose-dependent manner compared to levels noted in the MDI-treated group. Our results demonstrated that EEc extract treatment appears to have an inhibitory effect on adipogenesis by reducing the expression of differentiation-related transcription factors and adipogenesis-related proteins in 3T3-L1 adipocytes.

In the present study, most genes examined were early markers of adipogenesis and related transcription factors. Based on the present findings, future research will assess markers at different time points including the early stage of differentiation using pre-adipocytes.

Our results demonstrated that EEc extract treatment inhibited adipogenesis in 3T3-L1 adipocytes, shown by the significant reduction in glucose utilization and TG accumu-

lation without cytotoxicity. The suppressive effects of EEc extract treatment may be mediated by downregulation of the expression of adipogenesis-related genes. Therefore, EEc extract treatment may be a potential therapeutic agent for the prevention of obesity.

Acknowledgements

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