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RESEARCH ARTICLE

Eleutheroside E, an Active Compound from *Eleutherococcus senticosus,* Regulates Adipogenesis in 3T3-L1 Cells

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Abstract In this study, we investigated the anti-adipogenic effects of *Eleutherococcus senticosus* and its active compounds *in vitro* to examine new functions. We first analyzed the active compounds in *E. senticosus* growing in Korea using HPLC and found that the concentration of eleutheroside B and E was higher in stems and roots than in other plant parts. There were no significant ($p<0.05$) differences in eleutheroside concentration between plant ages. Anti-adipogenic effects of *E. senticosus* on lipid accumulation in 3T3-L1 cells were examined. Extracts of stems and roots more effectively inhibited lipid accumulation in 3T3-L1 cells than extracts of other plant parts. Eleutheroside E was responsible for the pharmacological anti-adipogenic effects via regulation of the mTOR pathway. This is the first report of an anti-adipogenic effect of *E. senticosus* and the active compound eleutheroside E.

Keywords: *Eleutherococcus senticosus,* eleutheroside E, anti-adipogenic effect, mTOR

Introduction

Eleutherococcus is a genus of thorny shrubs and trees in the family Araliaceae that is widespread in Northeastern Asia. The best-known *Eleutherococcus* species in the West is *Eleutherococcus senticosus* (Rupr. et Maxim.) Harms (sometimes referred to as *Acanthopanax senticosus*), which is also called Siberian ginseng in English-speaking countries, Ciwujia in China, and Gasiogapi in Korea. The stem of this plant used as an adaptogen for treatment of various ailments (1,2), contains pharmacologically active compounds, such as eleutherosides, vitamins, flavonoids, and complex polysaccharides (3). These compounds exhibit anti-oxidant, anti-diabetes, anti-microbial, anti-inflammatory, anti-stress, and anti-tumor activities (3-9). Because of a therapeutic efficacy, many people have become interested in *E. senticosus* and cultivation studies are increasing.

In order to use *E. senticosus* for development of dietary supplements in promotion of health, it is important to establish a practical biological marker of raw material quality for monitoring of the production process. Plant parts and plant age should be considered when creating quality standards for raw materials (10). A large number of compounds have been isolated from *E. senticosus*, including eleutheroside B, eleutheroside E, isofraxidin, and chlorogenic acid (Fig 1A). Among these compounds, eleutheroside B and eleutheroside E may be appropriate indicators of the pharmacological quality of raw *E. senticosus*, as their activities are well known (11).

Although, various therapeutic functions of *E. senticosus* are well known, anti-obesity effects have not been reported. In this study, the eleutheroside contents of different parts of the *E. senticosus* plant and in plants of different ages were evaluated. The anti-adipogenic effects of *E. senticosus* in 3T3-L1 cells were investigated to explore new pharmacological functions of this plant.

Materials and Methods

Materials HPLC grade acetonitrile, water, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphoric acid, eleutheroside B (#90974), eleutheroside E (#08198), chlorogenic acid (C3878),

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isobutylmethylxanthine (IBMX, I7018), dexamethasone (D4902), insulin (#16634), and Oil Red O (O0625) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against PPARγ (sc-7273) and TSC2 (sc-893) and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against FAS (#3180), β-actin (#4967), C/EBPα (#2295s), p-S6K1 (#9205), S6K1 (#9202), p-Akt (#9271), Akt (#9272), and p-TSC2 (#3615s) were purchased from Cell Signaling Technology (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine calf serum (CS), sodium pyruvate, and penicillin-streptomycin were obtained from Gibco BRL (Grand Island, NY, USA).

Extract preparation *E. senticosus* was obtained from the Hambakjae Farming Corporation (Gunsan, South Korea) and identified by Professor Seong-Gyu Ko, College of Oriental Medicine, Kyunghee University (Seoul, South Korea). Voucher specimens were deposited in the herbarium of the Korea Food Research Institute, South Korea (number E0112). Freshly picked plants were air-dried at room temperature for 1 week. Dried plants were ground using a mill and passed through a 50-mesh sieve. The ground powder was subjected to extraction twice using repeat sonication for 30 min at room temperature with 70% ethanol. The extract was filtered using Whatman No. 2 paper filter, and the active compounds eleutheroside B, eleutheroside E, and chlorogenic acid were analyzed using HPLC. Extracts were dried using a vacuum evaporator to investigate anti-adipogenic effects. Dried extracts were dissolved in DMSO for cell experiments.

High-performance liquid chromatography analysis The active compounds in ethanol extracts of *E. senticosus* were analyzed using a Jasco HPLC system consisting of a Jasco PU-2089 plus quaternary gradient pump, a Jasco AS-2057 plus intelligent sample injector, a Jasco UV-2075 intelligent UV/VIS detector, and Borwin chromatography software version 1.5. Separation was performed using an XTerra RP18 column (5.0 μm, 4.6×250 mm i.d.; Waters, Milford, MA, USA) and a guard column. Analyses were performed using a mobile phase consisting of the 2 solvents of water containing 1% phosphoric acid (solvent A) and acetonitrile (solvent B). The flow rate was 1.0 mL/min. Elution was performed using the following programmed gradient elution: 0-40 min, elution with gradually increasing concentration of solvent B (5%-40%); 40-50 min, isocratic elution with 40% solvent B; and 50-60 min elution with gradually decreasing concentration of solvent B (40%-5%). Compounds were detected at 216 nm.

Cell culture and differentiation 3T3-L1 pre-adipocyte cells were purchased from ATCC (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% calf serum (CS) and 1% penicillin-streptomycin at 37ºC in a 5% CO_2 incubator. The cells were seeded into a 6-well plate at a density of 4×10^5 cells, and cell differentiation was induced. In brief, beginning at 2 days post-confluence (day 0), cells were incubated for 2 days using a differentiation media of 0.5 mM 3-IBMX, 1 μM dexamethasone, and 1 μg/mL of insulin (MDI) in DMEM with 10% FBS. After induction, the medium was replaced with DMEM containing 10% FBS and 1 μg/mL of insulin, and the cells were incubated for 2 more days. Cells were then maintained in DMEM containing 10% FBS until maturity. From days 0 to 2, cells were exposed to one of several test eleutheroside extracts.

Oil Red O staining Oil Red O staining was performed on day 8. Prior to quantification, cells were fixed using 10% neutral formalin for 1 h at room temperature, washed with phosphate-buffered saline (PBS), then stained for 1 h with 0.5% Oil Red O in 60% isopropanol. After stained cells were washed with distilled water, they were observed under a fluorescence microscope. The stained Oil Red O was extracted from cells using 100% isopropanol. Optical density was measured at a wavelength of 490 nm.

Western blotting 3T3-L1 adipocytes were scraped into lysis buffer containing 40 mM Hepes (pH 7.4), 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, and 1% Triton X-100 supplemented with EDTA-free phosphatase and a protease inhibitor cocktail (#78441; Thermo Scientific, Waltham, MA, USA), then sonicated (on ice) twice for 15 s for complete lysis. After centrifugation at 10,000×*g* for 20 min at 4ºC, supernatants were boiled in SDS-loading buffer, loaded onto Tris-glycine gels, transferred to polyvinylidene fluoride (PVDF) membranes and visualized using a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ, USA).

Statistical analysis Differences between groups were evaluated using a one-way analysis of variance (ANOVA) with Prism5 software (GraphPad, San Diego, CA, USA). The Bonferroni post-hoc test was used when significant differences were identified using ANOVA ($p<0.05$). Data are expressed as mean±standard deviation (SD).

Results and Discussion

Previous studies have reported several kinds of chemical

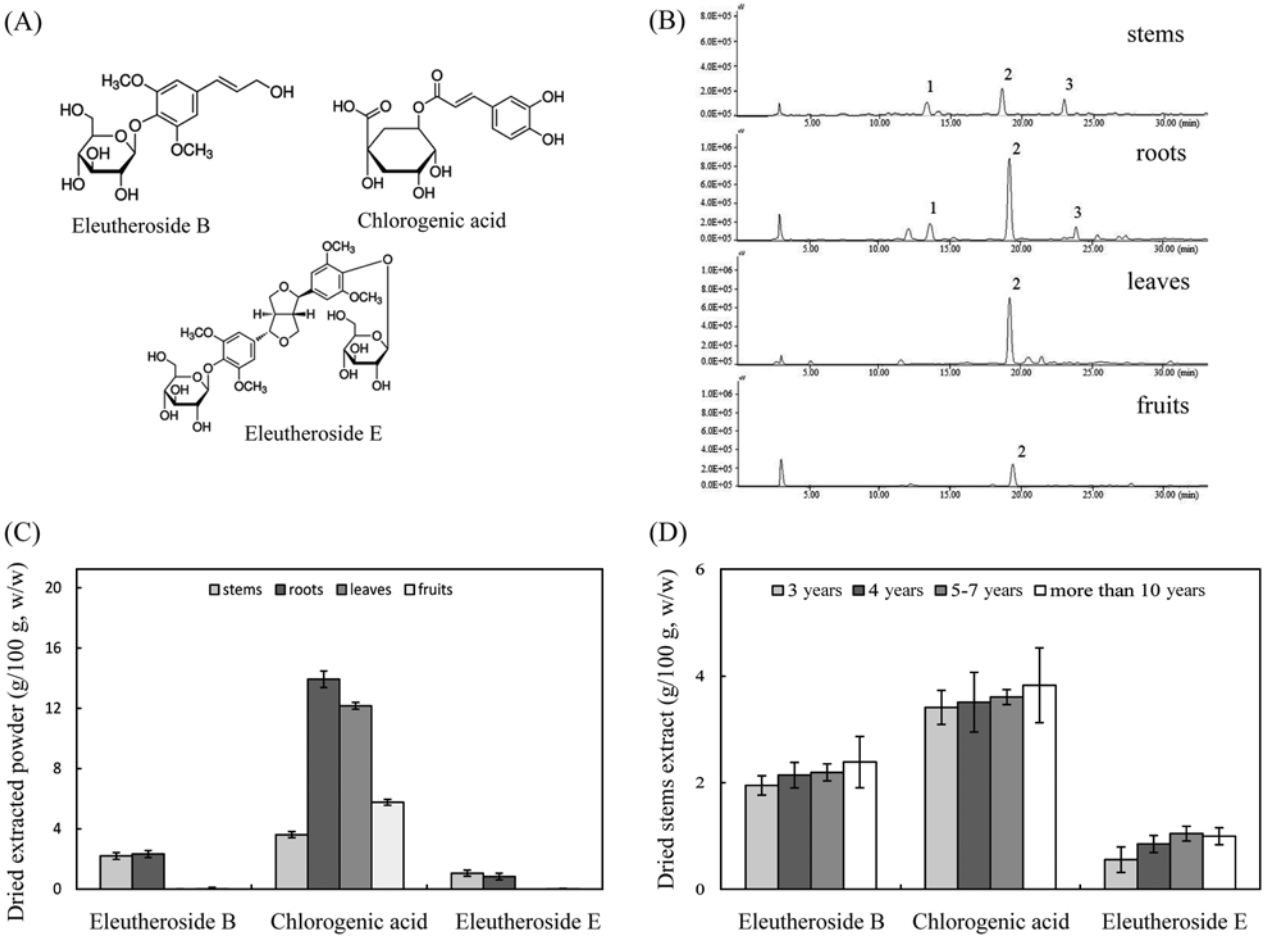

Fig. 1. Biologically active compounds in different *Eleutherococcus senticosus* plant parts and from plants of different ages. (A) Chemical structures of the active compounds in *E. senticosus*. (B) HPLC chromatograms of eleutheroside B, chlorogenic acid, and eleutheroside E from different plant parts. Peaks 1, 2, and 3 represent eleutheroside B, chlorogenic acid and eleutheroside E, respectively. (C) The concentrations of the 3 active compounds in different plant parts. (D) The concentrations of the 3 active compounds in stems of *E. senticosus* harvested at different plant ages. Values represent means±SD (n=3).

compounds in *E. senticosus*, including eleutherosides, phenolics, saponins, and lignans (3). Eleutherosides, such as eleutherosides B and E are considered to be the most pharmacologically active compounds in *E. senticosus* (12). The concentration of pharmacologically active compounds can vary according to plant parts and plant age. Here, active compounds from different parts of the *E. senticosus* plant growing in Korea were analyzed using HPLC (Fig. 1B). Higher concentration of eleutheroside E and eleutheroside B were identified in the stems and roots than in extracts of fruit and leaves (Fig. 1C). The extraction yields for dried stems, roots, leaves, and fruits powders of *E. senticosus* were 4.1, 4.2, 42.9, and 34.7%, respectively (w/w, data not shown).

For *Panax* ginseng, plant age is an important consideration when determining the optimal harvest time for maximizing active compound concentrations. To determine the concentration of active compounds in stems of *E. senticosus* at different ages, samples from plants grown in the same cultivation area that were harvested at different times were obtained. The eleutheroside B and eleutheroside E concentrations in these plants were then determined. No significant differences (p>0.05) in bioactive component concentrations were identified among *E. senticosus* plants of different ages (Fig. 1D), suggesting that harvest time does not affect the eleutheroside content of *E. senticosus.* Consistent with previous reports that chlorogenic acid as one of the major phenolic compounds in *E. senticosus* (12,13), chlorogenic acid was identified as one of the main phenolic acids in *E. senticosus* in this study, with the highest concentration in the extracts of leaves.

Although *in vitro* and animal models have shown *E. senticosus* to have a variety of biological effects, potential for prevention of obesity has not yet been explored. In this study, the anti-adipogenic effects of different parts of the *E. senticosus* plant were studied using 3T3-L1 pre-adipocytes. *E. senticosus* stems and root extracts significantly suppressed MDI-induced differentiation of 3T3-L1 preadipocytes,

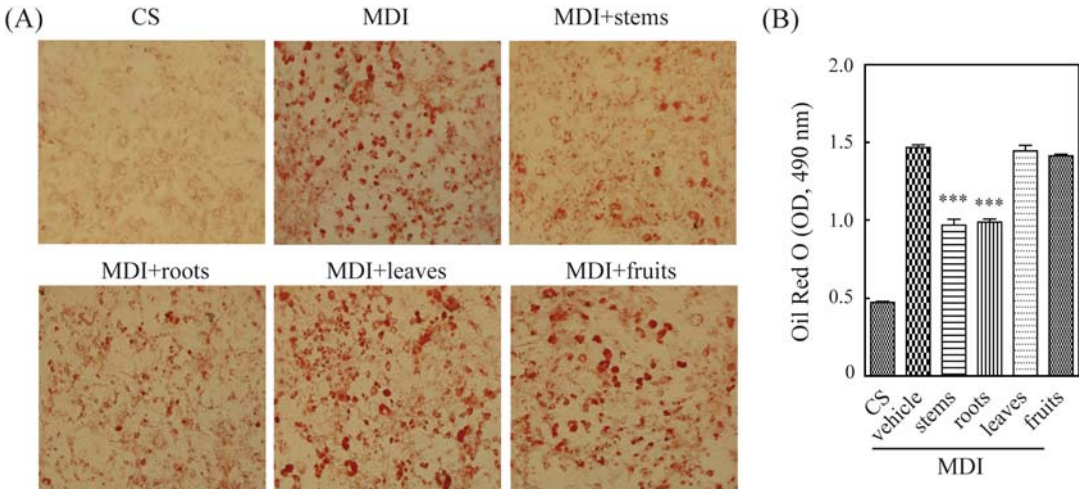

Fig. 2. Anti-adipogenic effects of ethanol extracts from different *Eleutherococcus senticosus* plant parts. (A) Stem and root extracts of *E. senticosus* inhibited cell differentiation. 3T3-L1 cells were treated with 100 μg/mL of each extract. After 8 days of cell differentiation, cells were stained with Oil Red O. (B) Quantitative analysis of 3T3-L1 cell differentiation. The intracellular TG content was quantitated using Oil Red O extracted from cells at an excitation wavelength of 490 nm. Values are displayed as means±SD (n=3, ***p<0.001).

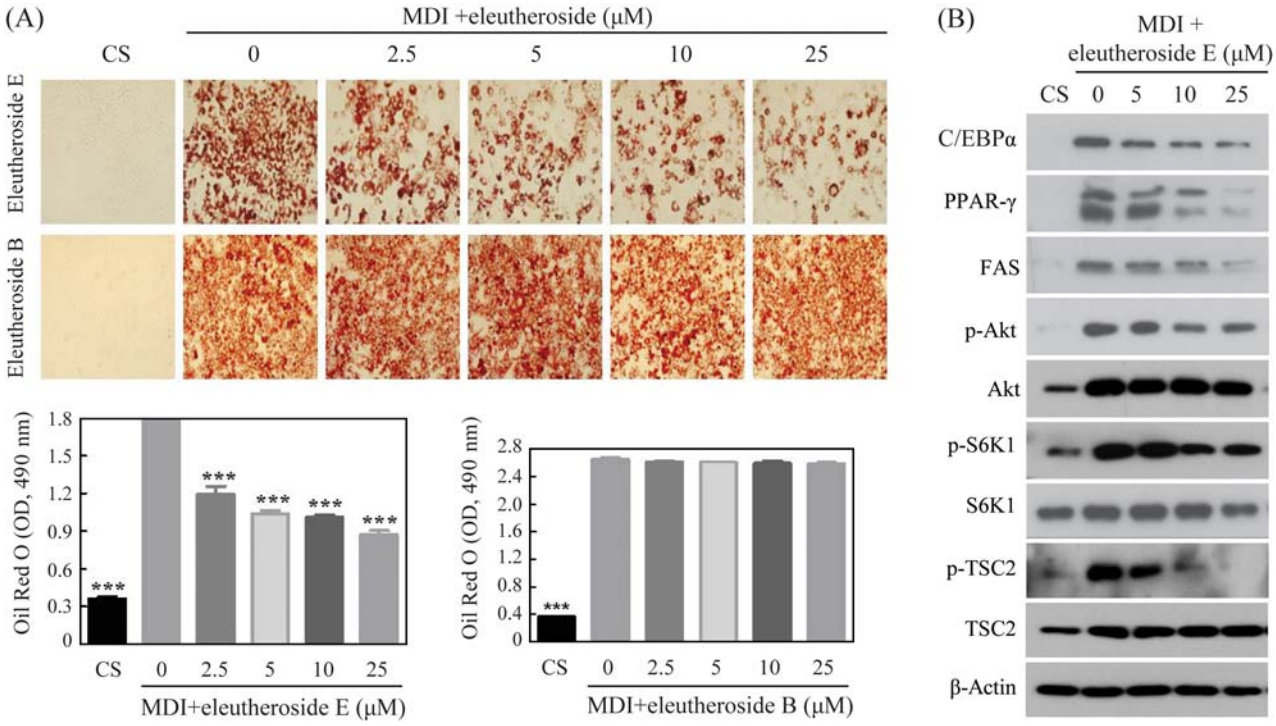

Fig. 3. Anti-adipogeneic effects of eleutheroside E. (A) Eleutheroside E inhibited cell differentiation. Cells were stained with Oil Red O after 8 days of cell differentiation in the presence of different concentrations of eleutherosides B and E. (B) Quantitative analysis of 3T3-L1 cell differentiation. Values represent means±SD (n=3, ***p<0.001). (C) Effects of eleutheroside E on the regulation of the mTOR pathway during adipocyte differentiation. Cells were treated with eleutheroside E at 2 d post confluence (day 0). Cells were scraped into lysis buffer and sonicated (on ice) twice for 15 s for complete lysis and were analyzed by Western blotting.

suggesting that the bioactive components eleutherosides B and E, but not chlorogenic acid, are responsible for the observed anti-adipogenic effect (Fig. 2A, 2B). The viability of 3T3-L1 cells was not affected when exposed to extract concentrations of 100 μg/mL (data not shown). To identify the plant components responsible for the anti-adipogenic effect observed in the *in vitro* model, 3T3-L1 cells were treated with 2.5, 5, 10, and 25 μM of either eleutheroside B or eleutheroside E during differentiation. Amounts of eleutheroside E and B were 2.0 and 5.75 μmol/mL in stem

extracts, respectively. Cytotoxicity of these eleutherosides was not observed up to a 25 μM concentration (data not shown). Thus, a concentration of less than or equal to 25 μM was used for subsequent experiments. Eleutheroside E inhibited cell differentiation of 3T3-L1 pre-adipocytes in a dose-dependent manner, whereas eleutheroside B did not affect lipid accumulation (Fig. 3A, 3B).

Western blotting results indicated that an eleutheroside E treatment suppressed the levels of PPAR-γ, C/EBPα, and FAS that are upregulated during adipogenesis (Fig. 3C). Recent studies have suggested that the mammalian target of rapamycin (mTOR) plays a critical role in adipogenesis in mammalian cells (14,15). Eleutheroside E reduced the levels of S6K1 phosphorylation on Thr389, the mTOR-dependent phosphorylation site. Eleutheroside E also inhibited the phosphorylation of both Akt and TSC2 during cell differentiation. TSC2 is a substrate of Akt and is a negative regulator of mTOR (16). TSC2 activation is inhibited by Akt-mediated phosphorylation, suggesting that eleutheroside E inhibits adipocyte differentiation in 3T3-L1 cells by attenuating the Akt/mTOR pathway. These results suggest that eleutheroside E in *E. senticosus* is responsible for the observed anti-adipogenic effects.

E. senticosus had higher concentrations of the main active compounds eleutheroside E and eleutheroside B in stems and roots than in leaves and fruits. Eleutheroside E markedly inhibited the accumulation of lipid droplets in adipocytes, whereas eleutheroside B had no effect. This is the first study to indicate that eleutheroside E is responsible for the anti-adipogenic effects of *E. senticosus*. Further investigation of the potential anti-obesity effects of *E. senticosus* using animal models is required, along with an examination of the precise mechanism underlying the anti-obesity effects of eleutheroside E.

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