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STEM OF *Elsholtzia splendens* INHIBITS ADIPOCYTE DIFFERENTIATION BY REGULATING PPAR γ and C/EBP α GENE EXPRESSION IN 3T3-L1 CELLS

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ABSTRACT

Elsholtzia splendens (*E. splendens*), is a wild herb found in Korea that has been used in traditional medicine. In this study, the effects of *E. splendens* ethanolic extraction lipid accumulation in 3T3-L1 preadipocytes were investigated. Morphological changes and the degree of lipid accumulation were measured by Oil Red O staining and an intracellular triglyceride (TG) assay. Four parts of *E. splendens* (stem, leaf, flower, roots) were decreased lipid accumulation in adipocytes in a concentration-dependent manner. Results of the study demonstrated that 100 μ g/mL of *E. splendens* stem extract (ESS) inhibit intracellular lipid accumulation by 56.4%. In addition, treatment with ESS decreased intracellular TG contents. The levels of peroxisome proliferators-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding proteins (C/EBP α) mRNA were decreased by the *E. splendens* extract. These findings suggest that *E. splendens* inhibits intracellular lipid accumulation by regulating the transcription of PPAR γ and C/EBP α .

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1 Introduction

Many species of the genus *Elsholtzia* are distributed throughout East Asia. Some members of this genus are used as traditional medicine. *Elsholtzia splendens* (*E. splendens*) belongs to family Labiatae, is a wild herb grown in Korea. *E. splendens* has been used as a traditional medicine to treat coughing, pain, and inflammation. Many recent studies have demonstrated that some species of *Elsholtzia* exert positive physiological effects *in vitro* (Kim et al., 2003; Ling et al., 2005; Ling and Lou, 2005). Many Researchers has *in-vitro* identified beneficial biological activities of *E. splendens* such as antioxidant, anti-inflammatory and anti-toxicity actions (Choi et al., 2007; Shim et al., 2008). These findings suggest that *E. splendens* might be used as a functional food and can be potentially used to treat and prevent diseases.

Obesity is condition in which adipocytes accumulate a large amount of fat and become enlarged. It is characterized at the cellular level by an increase in the number and size of adipocyte differentiated from fibroblastic preadipocytes in adipose tissue (Furuyashiki et al., 2004). 3T3-L1 cells are commonly used as a model of adipocyte differentiation for examining the molecular mechanisms responsible for regulating adipogenesis (Choi et al., 2006). The addition of a hormone mixture initiates a transcriptional regulatory cascade in 3T3-L1 pre-adipocytes that results in a gene expression profile specific for an adipocyte phenotype (Cornelius et al., 1994). The roles of several critical transcription factors, including peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding proteins (C/EBP α), as well as cross-talk among these factors in adipocyte differentiation have been extensively studied. In particular, PPAR γ acts as a direct regulator of many fat-specific genes that can trigger adipogenesis (Choi, 2006).

Due to the recently increased interest in natural products with anti-obesity properties, many studies are being conducted to identify flavonoids that influence adipocyte differentiation. *E. splendens* possessed medicinal properties mainly owing to flavonoid contents of the plant and interest in developing food products with health benefits is increasing among both consumers and food industry. Until recently, there has been insufficient information about the effect of *E. splendens* extracts on adipocyte differentiation and the potential of this plant to serve as a functional food with anti-obesity activities. Therefore, the objective of the present study was to evaluate the anti-adipogenic effects of *E. splendens* extracts and investigated the possible underlying mechanisms by measuring the expression of PPAR γ and C/EBP α .

2. Materials and Methods

2.1 Chemicals and reagents

3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Well GENE Biopharmaceuticals (Daegu, Republic of South Korea). Isobutylmethyl-xanthine (IBMX), insulin, and dexamethasone (DEX) were purchased from Sigma (St. Louis, MO). TG assay reagent (Cleantech TG-S) was purchased from Asan Pharmaceutical (Seoul, Republic of South Korea). A reverse transcription system (ImPromTM) was obtained from Promega (Madison, WI).

2.2 Preparation of *E. splendens* extracts

E. splendens was harvested from Hwacheon (Jeollanam-do, South Korea) during efflorescence (from September to October) in 2006. After washed with water, *E. splendens* were freezing-dried with freeze-dryer (Bondiro, Ilshin Lab Co., Ltd.) for 72 h and crushed in a blender. Total of 10 g of freezing-dried powder from each *E. splendens* plant part underwent three rounds of extraction with 80% ethanol under reflux for 2 h at room temperature. The filtered supernatants were evaporated under vacuum at 50°C yields of each extract from the flower, leaf, root, and stem were 28.3, 18.9, 4.0 and 8.8% of dry weight. Each extract was suspended in DMSO (1% final concentration) and stored at 4°C until further use.

2.3 Cell culture and adipocyte differentiation

3T3-L1 pre-adipocytes were differentiated in the presence or absence of the extracts. Two post-confluence (DAY 0), the cells were treated with the induction hormone mixture (MDI; DMEM containing 10% FBS, 10 μ g/mL insulin, 0.5 μ M DEX, and 0.5 mM IBMX). After the induction, the medium was replaced with DMEM supplemented with 10% FBS and 10 μ g/mL insulin and replenished every 2 days. Extracts from each plant part (10–100 μ g/mL) were added to the medium on during 3T3-L1 cell differentiation. The cells were then incubated until a mature adipocyte state was reached on DAY 8.

2.4 Oil Red O staining and TG assay

Intracellular lipid accumulation and TG contents were analyzed on DAY 8 of differentiation. Lipid accumulation in the 3T3-L1 cells was visualized by Oil Red O staining. Differentiation media were removed and the cells were washed twice with PBS and then fixed with 3.7% paraformaldehyde for 10 min. The fixed cells were then washed twice with Phosphate buffered saline (PBS) and stained with Oil Red O solution (3:2 mixture of 0.2% Oil Red O-isopropanol solution and water) for 1 h at room temperature. For removing Oil red O solution, the cells were washed twice with PBS. To quantify oil red O staining the stained – cells were extracted with

isopropanol and supernatant were measured absorbance at 510 nm using a microplate reader (M2, Molecular Device). To analyze TG contents, the cells were washed twice with PBS and then scraped into 100 μ L of lysis buffer (1 mM EDTA in 20 mM Tris). The lysate was reacted with TG assay reagent (Cleantech TG-S) to measure triglyceride (TG) content according to the manufacturer's instructions. The results are expressed as μ g TG per μ g cellular protein. The protein content was measured by Bradford protein assay (Bradford, 1974).

2.5 RT-PCR to measure PPAR γ and C/EBP α expression

Analysis of PPAR γ and C/EBP α mRNA expression in 3T3-L1 cells was performed by RT-PCR using a MJ Mini Gradient Thermal Cycler

(Bio-Rad, Hercules, CA). Briefly, total RNA was isolated from 3T3-L1 adipocytes with Trizol reagent according to the manufacturer's protocol and 1 μ g of RNA was reverse-transcribed using reverse transcription system (ImPromTM). Next, PCR was conducted in a reaction containing 1 μ L of the cDNA product and PCR premix (Bioneer, Republic of South Korea).

PCR was conducted under the following conditions: 95°C for 3 min (one cycle) followed by 25 cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min with a final extension at 72°C for 5 min. The primer sequences were as follows; PPAR γ forward 5'-ACC ACT CGC ATT CCT TTG AC-3', reverse 5'-TCA GCG GGA AGG ACT TTA TG-3', C/EBP α forward 5'-ATG GAG TCG GCC GAC TTC TAC-3', reverse 5'-CAG GAA CTC GTC GTT GAA GGC-3', GAPDH forward 5'-AAC TTT GGC ATT GTG GAA GGG C-3', reverse 5'-GAC ACA TTG GGG GTA GGA ACA C-3'. Bands were visualized with a UV illuminator and photographed using an EL LoGic 100 Imaging System (Kodak, Rochester, NY).

2.6 Statistical analysis

Data are presented as the mean \pm standard deviation (SD) of at least three independent experiments performed in triplicate. Statistical differences among the groups were evaluated with a one-way analysis of variance. For all analyses, p -values < 0.05 were considered statistically significant. All analyses were conducted using SPSS 10.0 (SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1. Effect of *E. splendens* on intracellular lipid accumulation in 3T3-L1 cells

The inhibitory effects of various extracts (flower, stem, leaf, and root) of the *E. splendens* plant on intracellular lipid accumulation are presented in Table 1. Overall, all parts of *E. splendens* were associated with decreased lipid accumulation in adipocytes in a concentration-dependent manner. Amongst various tested extracts, the extract obtained from the stems and leaves had highest inhibitory effects. These two extracts suppressed 56.4 and 53.0 %, lipid accumulation respectively, at a concentration of 100 μ g/mL as compared to the group treated with a hormone mixture (IBMX, Dexamethasone, Insulin; MDI) alone. However, root extract at 50 and 100 μ g/mL concentration induced cytotoxicity during adipocyte differentiation. Shim et al. (2008) reported that *E. splendens* extracts induced the chromosomal abnormality in Chinese hamster lung (CHL) cells. For characterize of the *E. splendens* stem extract (ESS) effects on the intracellular lipid accumulation, fully-differentiated 3T3-L1 cells were stained with Oil red O and cellular TG contents were directly measured using a TG assay. ESS reduced the number and size of cytosolic lipid droplets (Figure 1A). As shown in Figure 1B, ESS significantly decreased intracellular TG contents by 57.6 μ g/mL compared to cells treated with MDI alone (82.7 μ g/mL). Previous phytochemical investigations have shown that flavonoids are major components in *Elsholtzia*. Phenylpropanoids, terpenoids, phytosterols, and cyanogenic glycosides are also main chemical constituents in members of this genus (Guo et al., 2012). Shen et al. (1994) isolated flavonoids such as 5, 6-dihydroxy-7, 8-dimethoxyflavone from *E. splendens*. Previous studies have reported that the major active component of antioxidant is apigenin in *E. Splendens* ethanolic extracts (data not shown). Hsu and Yen (2007) also investigated the ability of naturally occurring flavonoids to inhibit adipogenesis in 3T3-L1 cells. Ono and Fujimori (2011) report that apigenin suppressed adipocyte differentiation of mouse adipocytic 3T3-L1 cells and reduced the accumulation of intracellular lipids. Thus, inhibitory effects of *E. splendens* on adipocyte are in confirmation of these previous finding and this differentiation might be attributed to flavonoids produced by the plant.

Table 1. Effects of extracts from different *E. Splendens* plant parts on 3T3-L1 adipocyte differentiation.

	Intracellular Lipid Accumulation (% of MDI-treated cells ^a)			
	Stem	Leaf	Flower	Root
10 (μ g/mL)	105.4 \pm 3.0	87.8 \pm 4.1	96.1 \pm 4.8	93.3 \pm 3.9
50 (μ g/mL)	96.0 \pm 1.8	79.2 \pm 1.6*	99.1 \pm 1.3	Toxic ^b
100 (μ g/mL)	43.6 \pm 1.7*	47.0 \pm 13.0*	97.4 \pm 4.1	Toxic ^b

^aMDI is the hormone mixture used to induce adipocyte differentiation (DMEM containing 10% FBS, 10 μ g/mL insulin, 0.5 μ M DEX, and 0.5mM IBMX).

^bThe root extract had cytotoxic effects during adipocyte differentiation.

* p < 0.05 compared to the cells treated with MDI alone (n = 3).

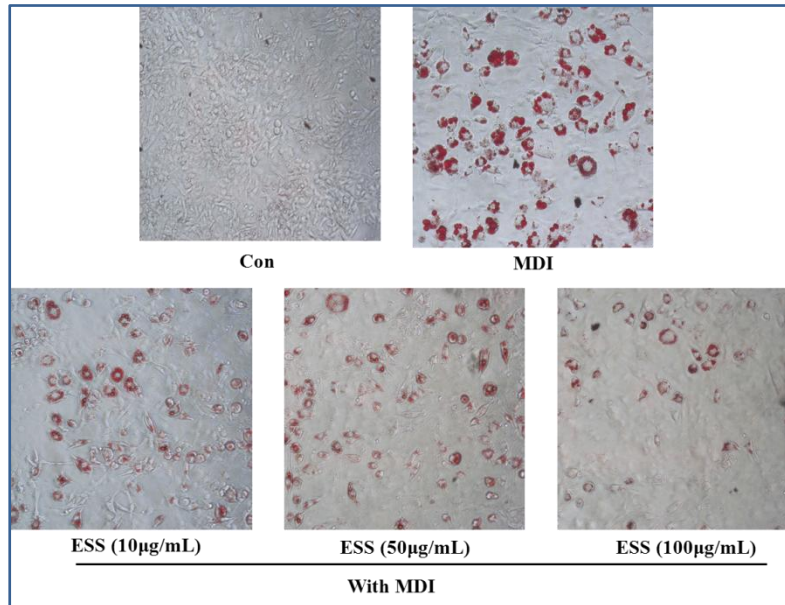


Figure 1. Inhibitory effects of extract from *E. Splendenstem* (ESS) on intracellular TG accumulation 3T3-L1 cells underwent differentiation into adipocytes for 8 d with vehicle (media) or ESS extract at the indicated concentration (10-100 µg/mL). (A) Image of cells stained with Oil Red O. Magnification $\times 200$.

(B) Intracellular TG contents were measured with a TG kit (Cleantech TG-S). Results are expressed as µg TG contents per µg total cell protein. Data are expressed as the mean \pm SD of triplicate experiments. Means without a common letter differ significantly ($p < 0.05$).

3.2. Effect of ESS on the mRNA expression of adipogenic transcription factors

Adipocyte differentiation is regulated by several transcription factors including PPARs and C/EBPs (Gregorie, 2001). To investigate the ability the ESS to inhibit the differentiation of 3T3-L1 cells, expression levels of adipogenic genes including PPAR γ and C/EBP α were measured by RT-PCR. As shown in Figure 2, mRNA expression of PPAR γ and C/EBP α in cells treated with the ESS were decreased in a dose dependent manner compared to that of cells treated with MDI alone. Guo et al. (2011) reported that pharmacological activities of *Elsholtzia* species were regarded on antioxidant, antiviral and

antibacterial activities. Yoon et al. (2011) reported that *E. ciliate* extract inhibits lipid accumulation in 3T3-L1 cells and ameliorates high-fat diet-induced obesity by down-regulating PPAR γ expression in the fat tissue of mice. During hormone-stimulated adipogenesis, C/EBP β expression is rapidly and transiently induced. The expression of PPAR γ and C/EBP α is subsequently increased. PPAR γ and C/EBP α then act as factors that promote the development of adipogenic characteristics including lipid accumulation and insulin sensitivity (Takashi, 2004). Results from the current study indicated that ESS inhibits intracellular lipid accumulation by decreasing the expression of transcription factors such as PPAR γ and C/EBP α in 3T3-L1 cells.

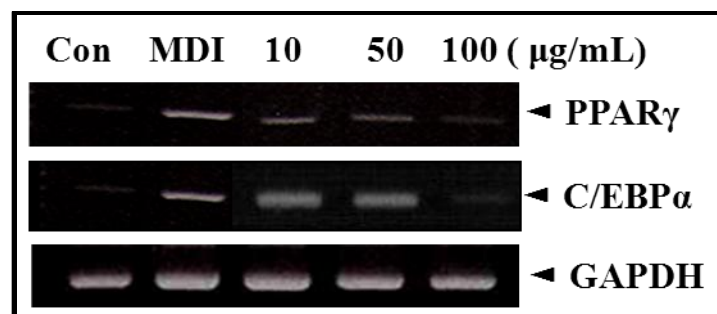


Figure 2. Inhibition of PPAR γ and C/EBP α expression by treatment with ESS extract 3T3-L1 cells underwent differentiation into adipocytes for 8 d in the presence of vehicle (media) or ESS extract at the indicated concentration (10-100 µg/ml). RT-PCR analysis of adipogenic transcription factors is shown

4. Conclusions

In conclusion, we found that extract of *E. splendens* stems exerted anti-adipogenic effects on 3T3-L1 adipocytes. This was accomplished through the regulation of PPAR γ and C/EBP α . These findings suggest that *E. splendens* extract may be a good candidate for the management of obesity.

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