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Inhibitory Effect of the Ethyl Acetate Fraction from Tulip Tree Leaf (*Liriodendron tulipifera L.*) on Adipogenesis in 3T3-L1 Cells

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Abstract – The inhibitory effects of adipogenesis on ethyl acetate (EtOAc) fraction from leaves of the Tulip tree (TT) were evaluated. Exposure to TT EtOAc fraction (25~200 μg/mL) for a 72 hr incubation period did not significantly change cell viability. TT EtOAc fraction, with concentrations of 100 and 200 μg/mL, inhibited lipid accumulation in 3T3-L1 adipocytes in a dose dependent manner in adipogenesis. The expression of PPARγ and C/EBPα, essential adipogenic markers, was significantly decreased when TT EtOAc fraction was added to cells for 8 days as compared with the untreated control group. These results suggest that TT EtOAc fraction might be a potential therapeutic agent as an effective, natural alternative material for obesity treatment.
Key words – Anti-obesity, Tulip tree, 3T3-L1 cells, Adipogenesis, PPARγ, C/EBPα

Introduction

Obesity has significantly increased since the 1950s. Now, more than 30 percent adults are obese (Lankford *et al*., 2013). A strong correlation between obesity and several life-threatening diseases, such as cardiovascular disease, type II diabetes mellitus, hyperlipidemia, and osteoarthritis, *etc*. has been reported (Visscher and Seidell, 2001). Currently, cardiovascular disease is the most common cause of death in the western world (Kim *et al*., 2012). Obesity results from energy imbalance caused by overeating and lack of exercise (Reaven *et al*., 2004). Thus healthy dietary and physical activities are very important for management of obesity. Several medications were used for obesity treatment (Kolanowski, 1999). However, currently Orlistat is the only approved drug for long-term use in the treatment of obesity without severe side effects (Zhang and Huang, 2012). Therefore new therapeutic attempts are required for treatment of obesity.

Adipose tissue plays a primary place for energy supply via the hydrolysis of stored triglyceride (TG) by releasing free fatty acid and acting as the storage site for dietary lipid (Stapleton *et al*., 2013). The TG level in adipose tissue plays an important part in the balance between lipogenesis and lipolysis, which are strongly associated with fat cell mass (Marques *et al*., 1998). The expansion of adipose tissue occurs during the development of obesity and obesity is initially characterized by fat cell hypertrophy (Sung *et al*., 2011).

Adipogenesis includes an extremely controlled and organized cascade of transcription factors, such as members of the peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα) (Tang *et al*., 2003). The amount of adipose tissue can be reduced by inhibiting adipogenesis, which may be regulated via these transcriptional key markers (Kong *et al.,* 2010).

Synthetic drugs for the treatment of obesity have high costs and serious side effects (Kolanowski, 1999). So medicinal plants and natural products have been studied to overcome these problems (Vasudeva *et al.,* 2012).

Liriodendron tulipifera L. is native to North America and is known as the tulip tree (TT) or yellow poplar (Graziose *et al.,* 2011). Its bark was traditionally used by the Native Americans as a tonic and treatment for malaria (Graziose *et al.,* 2011). The bark has been reported to have several medicinal benefits such as anti-malaria (Graziose *et al.,* 2011), anti-oxidant (Xu *et al.,* 2011), and anti-cancer effects (Xu *et al.,* 2011). The leaves have been also reported to have a few medicinal benefits such as anti-bacteria (Bae *et al.,* 1990), anti-tumor (Moon *et al.,* 2007), and anti-malaria effects (Graziose *et al*., 2011).

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However, there are little studies on the anti-obesity effect of the TT.

In this study, the anti-obesity effect of ethyl acetate (EtOAc) fraction of TT leaves on adipocyte differentiation and expression of adipogenic markers in 3T3-L1 cells were examined.

Experimental

Materials – 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco modified Eagle medium (DMEM), β-actin antibody, insulin, 3-isobutyl-1-methylxanthine (IBMX), Oil Red O solution, and dexamethasone were provided by Sigma-Aldrich Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS), newborn calf serum (NCS), 25% Trypsin-EDTA, and Antibiotics-Antimycotic were obtained from Gibco (Rockville, MD, USA). A cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). A Quant-iT protein assay kit was purchased from Invitrogen (Auckland, New Zealand). Antibodies against PPARγ and C/EBPα were provided by Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase–conjugated goat anti-rabbit IgG was obtained from Jackson Immuno Research Laboratories (West Grove, PA, USA).

Preparation of *Liriodendron tulipifera L.* fraction – The leaves of tulip tree (TT) was provided by the Gangwon Institute of Forest Research (GIFR). A voucher specimen (G-11-15) was deposited in the GIFR herbarium. The TT leaves (1.57 kg) was powdered and extracted twice with 10 L of 70% acetone at room temperature (RT) for 3 days. The total yield was 196.15 g. We got fractions of hexane (2.78 g), EtOAc (8.02 g), and water (122.69 g) from the crude extract. We used the EtOAc fraction in this study.

Cell differentiation and treatment – 3T3-L1 preadipocytes were maintained in DMEM containing 10% NCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 ºC until confluence. To induce adipogenesis, the cells were cultured till two days after confluence (day 0) and then induced by a differentiation mixture containing 5 μg/mL insulin, 0.5 μM dexamethasone, and 0.5 μM IBMX in DMEM with 10% FBS. Thereafter, the medium was changed with 10% FBS/DMEM containing 5 μg/mL insulin every two days. The test compounds were treated with 0, 25, 50, 100, and 200 μg/mL of TTE fraction at the initiation of differentiation and with every medium change for 8 days (day 0~day 8).

Oil Red O staining – Oil Red O staining was performed using the procedures of Kim *et al*. (2010) with minor modifications. In brief, differentiated adipocytes were washed with phosphate-buffered saline (PBS), and then fixed with 10% formalin in PBS for 60 min at room temperature (RT). The fixed cells were then washed 3 times with PBS, and stained with filtered Oil Red O solution for 30 min at RT and then the cells were rinsed 2 times with distilled water. The morphology of the cells was examined under an inverted microscope (ZEISS, Oberkochen, Germany) and the images were captured using a digital camera (Nikon, Tokyo, Japan). Stained oil droplets were dissolved with isopropanol and quantified using luminescence microplate readers (Spectramax/M2^e, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The values were calculated as percentages of the untreated control and expressed as means ± SD.

Cell viability assay – Cell viability was determined using CCK-8, according to the manufacturer's instructions. Briefly, 3T3-L1 cells stimulated by the adipogenic cocktail in the absence or presence of different concentrations of TTE fraction (0, 25, 50, 100, and 200 μg/mL) were employed in this experiment in 96-well plates (5000 cells/well) for 72 hrs. After each indicated time period, DMEM with test compounds were replaced by DMEM with 20 μL of CCK-8 solution and incubated in the dark for 2 hrs. The amount of dehydrogenase activity in viable cells was detected using luminescence microplate readers (Spectramax/M2^e, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Each assay was carried out in triplicate.

Western blot analysis – Cells were seeded in 100 mm dish plates. Adipocyte differentiation and TTE fraction treatment were carried out as described above. The cell extracts were prepared by adding protein extraction solution (PRO-PREP, Intron Biotechnology, Sungnam, Korea). The protein content in cell lysates was determined according to the method described by Bradford (1976). Protein (20 μg) were separated by 10% SDS-PAGE and blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20. Blots were incubated at 4 ºC overnight with primary antibodies, including PPARγ (1:200) and C/EBPα (1 : 5000), and goat anti-mouse IgG-HRP secondary antibody (1 : 5000) in a blocking solution for 2 hrs at RT. The target protein was detected using ECL western blotting detection reagent (GE Healthcare, Seoul, South Korea).

Statistical analysis – Data are presented as the mean ± standard deviation (SD). To determine statistical significance, data were analyzed using student's *t* test. A value of $P < 0.05$ was considered to be statistically significant.

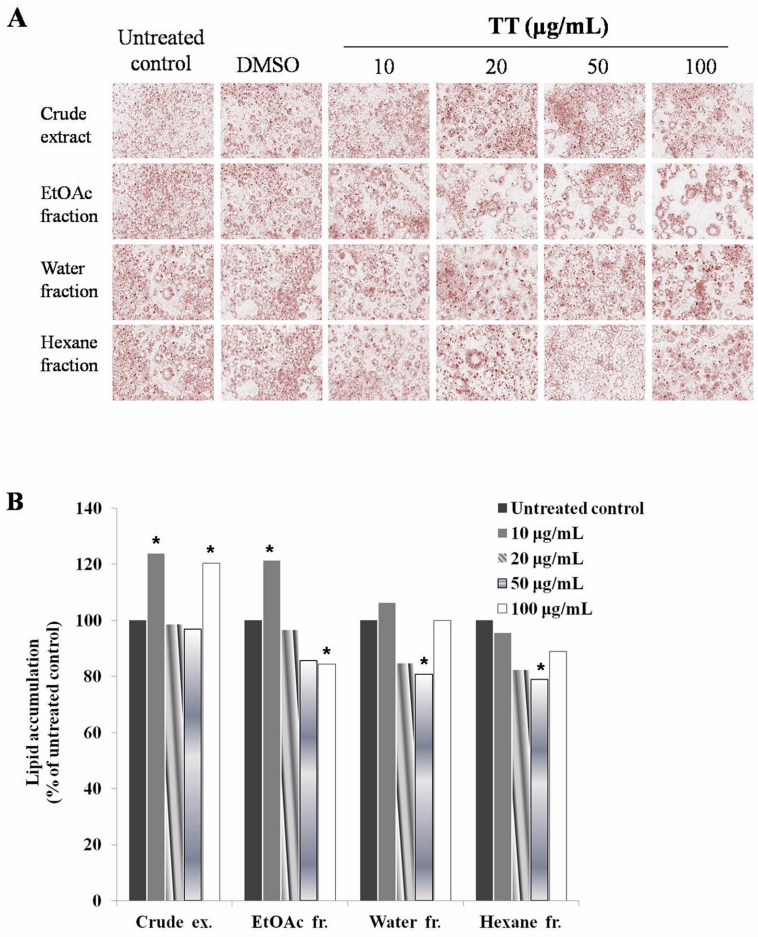

Fig. 1. The effect of TT acetone extract and its fractions (EtOAc, water, and hexane) on lipid accumulation in adipocyte differentiation. 3T3-L1 preadipocytes were treated with samples for the entire differentiation process (days 0 - 8). Representative microscopic morphological images of the adipocytes were stained with Oil Red O after 8 days of treatment with 0, 10, 25, 50, and 100 μg/mL. (A). The OD values were measured from the isopropanol elution of the Oil Red O stained cells (B). Values are expressed as means ± SD of 3 separate experiments. *$P < 0.05$ compared with an untreated control.

Results and Discussion

Effect of TT EtOAc on anti-adipogenesis during adipocyte differentiation – For the screening of the anti-obesity effect, TT acetone extract and its fractions (EtOAc, water, and hexane) were treated to 3T3-L1 preadipocytes at a concentration of 10~100 μg/mL during the whole differentiation period. At a 50 μg/mL concentration of TT water and hexane fractions showed significant inhibitory activity on adipogenesis, and the effect was not dose-dependent (Fig. 1A and B). Among those, EtOAc was the only fraction found to exhibit significant inhibitory activity on adipogenesis in a dose-dependent manner (Fig. 1A and B). Thus, we measured the concentration effects of EtOAc fraction. The inhibitory effect of the TT EtOAc fraction on adipogenesis was observed after Oil Red O staining. As shown in Fig. 2, the EtOAc fraction of TT showed significant inhibitory activity as compared with the untreated control cells. As shown in morphological observations, lipid accumulation in the TT EtOAc fraction treated cells was lower than in untreated control cells (Fig. 2A). In the OD values of TT EtOAc fraction treated cells, a significant decrease of lipid accumulation was detected as compared with the untreated control cells (Fig. 2B). The results showed that 100 and 200 μg/mL concentrations of TT EtOAc fraction were effective in inhibiting the lipid content to up to 23 and 49% of the untreated control cells, respectively. These results suggest that TT EtOAc fraction might have an anti-adipogenic effect.

Effect of TT EtOAc fraction on cell viability – To investigate the effect of TT EtOAc fraction on the viability of 3T3-L1 cells in the early stage of adipocyte differentiation, the differentiating 3T3-L1 cells were

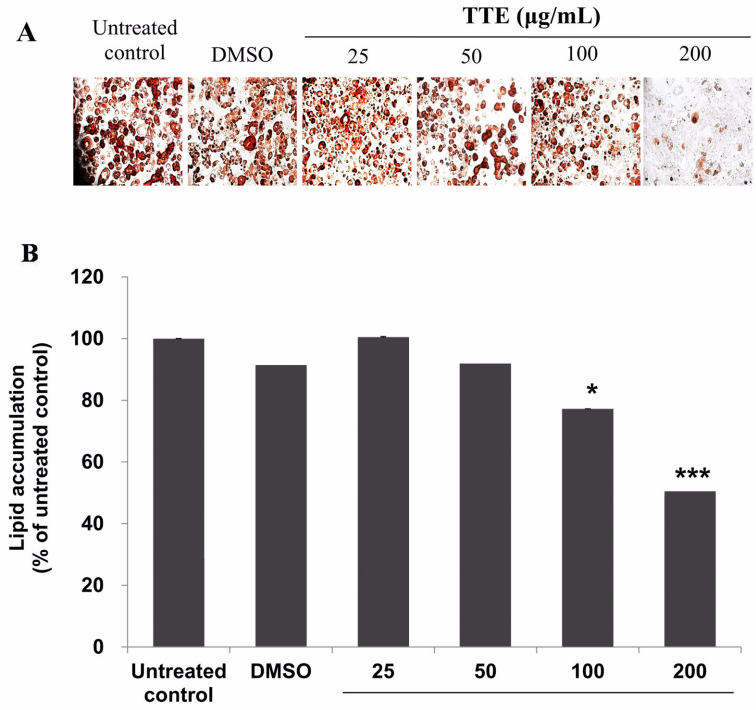

Fig. 2. The effect of TT EtOAc fraction on lipid accumulation in adipocyte differentiation. 3T3-L1 preadipocytes were treated with samples for the entire differentiation process (days 0 - 8). Representative microscopic morphological images of the adipocytes were stained with Oil Red O after 8 days of treatment with 0, 25, 50, 100, and 200 μg/mL. TT EtOAc fraction and microscopic pictures were taken at 200 × magnification (A). The OD values were measured from the isopropanol elution of the Oil Red O stained cells (B). Values are expressed as means ± SD of 3 separate experiments. *P < 0.05, ***P < 0.005 compared with an untreated control.

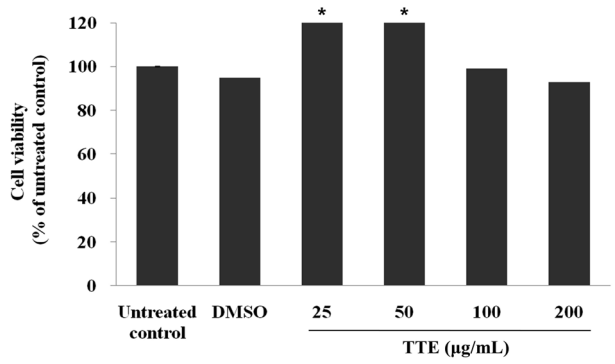

Fig. 3. The effect of TT EtOAc fraction on the viability of differentiating adipocytes. 2 days post confluent 3T3-L1 cells were incubated for 72 hrs with an adipogenic cocktail and various concentrations of TT EtOAc fraction (0, 25, 50, 100, and 200 μg/mL). The CCK-8 assay for testing cell viability was performed after 72 hrs of treatment. Values are expressed as means ± SD of three separate experiments.

treated with various concentrations (0, 25, 50, 100, and 200 μg/mL) of TT EtOAc fraction for 72 hrs. The results showed that 25 and 50 μg/mL concentrations of TT EtOAc fraction were effective in cell viability up to 20% of the untreated control cells (Fig. 3). The activation of PPARγ is essential to significantly promote adipocyte differentiation and increase the number of differentiated cells in human bone marrow mesenchymal stem cells (Noh and Lee, 2010). Wu *et al*. (2012) reported that troglitazone, synthetic anti-diabetic compounds, shown to anti-diabetic effect, but can leads to side effects specially lipid increase due to PPARγ agonist action. Low-dose TTE, thus, may have PPARγ agonist action. Further study is required to explain the relative mechanisms of this cell over-proliferating effect. Also treating cells with TT EtOAc fraction did not showed any cytotoxic effect when compared with untreated control cells (Fig. 3).

Effect of TT EtOAc fraction on protein expression of PPARγ and C/EBPα – PPARγ and C/EBPα are closely related to adipogenesis (Lee *et al.,* 2011). Thus, to find the molecular mechanism underlying the anti-adipogenic effect of TT EtOAc fraction on 3T3-L1 cells, we analyzed the expression of PPARγ and C/EBPα directly involved in adipogenesis. After 3T3-L1 preadipocytes were exposed to TT EtOAc fraction for 8 days, we measured the expression levels of PPARγ and C/EBPα using western blot analysis. TT EtOAc fraction-treated cells significantly suppressed the expression of these proteins compared with untreated control cells (Fig. 4). The expression of PPARγ was significantly decreased

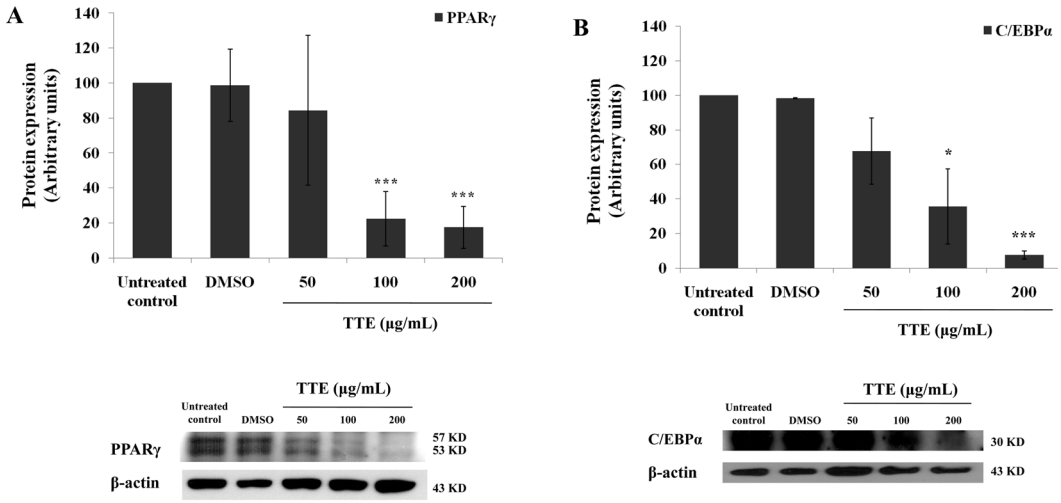

Fig. 4. Effects of TT EtOAc fraction on the expression of adipogenic-related proteins by Western blot analysis. The results showed that the expression levels of PPARγ (A) and C/EBPα (B) were reduced in 3T3-L1 cells treated with TT EtOAc fraction on day 8 of differentiation. These signals were normalized to β-actin. Values are expressed as means ± SD of 3 separate experiments. *$P < 0.05$, ***$P < 0.005$ compared with an untreated control.

when 100 and 200 μg/mL concentrations of TT EtOAc fraction was added to cells for 8 days as compared with the untreated control cells, respectively ($P < 0.005$, $P < 0.005$) (Fig. 4A). The expression of C/EBPα was significantly decreased when 100 and 200 μg/mL concentrations of TT EtOAc fraction was added to cells for 8 days as compared with the untreated control cells, respectively ($P < 0.05$, $P < 0.005$) (Fig. 4B). PPARs have also been reported to be key regulators of inflammatory and immune responses (Ricote and Glass, 2007). Therefore, since obesity is usually accompanied by chronic, low-grade inflammation in adipose tissue (Xu *et al*., 2003), PPARγ has received much interest as a new target in therapeutic drugs for the treatment of chronic inflammatory diseases such as obesity-induced insulin resistance, atherosclerosis, and neurodegenerative diseases (Kostadinova *et al*., 2005).

In conclusion, our results indicate that the EtOAc fraction of TT significantly inhibited adipogenesis as a result of down-regulating PPARγ and C/EBPα without cell toxicity. These results thus suggest that TT EtOAc fraction may be an effective, natural alternative for the prevention and/or treatment of obesity.

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