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ORIGINAL ARTICLE

Silydianin in Chloroform Soluble Fraction of *Cirsium japonicum* Leaf Inhibited Adipocyte Differentiation by Regulating Adipogenic Transcription Factors and Enzymes

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Abstract *Cirsium japonicum*, Compositae, a wild perennial herb found in Korea, Japan and China, has been used in traditional medicines. Effects of various solvent extracts of *C. japonicum* leaf on adipocyte differentiation in 3T3-L1cells were determined, and its mechanism was elucidated. 3T3-L1 cells were incubated with adipogenic hormone mixture mixed with various solvent fractions (hexane, chloroform, ethyl acetate, butanol, and water) of *C. japonicum* leaf. Adipogenesis was evaluated by triglyceride accumulation and expression of adipogenic genes by reverse-transcription-polymerase chain reaction. All solvent fractions of *C. japonicum* leaf inhibited adipogenesis in adipocytes by decreasing triglycerol concentration in a dose-dependent manner. Among solvent fractions of *C. japonicum*, the chloroform-soluble fraction was found to have the highest inhibitory effect on adipocyte differentiation. Silydianin was identified as a major bioactive component in chloroform-soluble fraction of *C. japonicum.* The extract suppressed the expression of genes such as PPARγ, C/EBPα, adiponectin, lipoprotein lipase, and fatty acid synthetase involved in adipogenesis, indicating that chloroform-soluble fraction of *C. japonicum* inhibited lipid accumulation in adipocyte by suppression genes involving adipogenesis. Thus, *C. japonicum* leaf extract containing silydianin could be a good natural candidate for the management of obesity.

Keywords adipocyte differentiation · adipogenic transcription · *Cirsium japonicum* · Silydianin · 3T3-L1 cells

Introduction

Cirsium japonicum (*C. japonicum*) belonging to the *Compositae* family is a wild perennial herb found in Korea, Japan, and China. It has long been used as traditional medicines for hypertension, a uretic, and for the treatment of hepatitis and tumors associated with liver, uterine cancer, and leukemia (Lui et al., 2006). It is also a known source of natural antioxidants and potential anticancer agents (Yin et al., 2008). The main compounds of *C. japonicum* include triterpenoids, steroids, essential oil, and flavones (Zhi et al., 2001). Most of the flavonoids that exist widely in plants are known to inhibit the proliferation of cells and have antiadipogenic effects (Andersen et al., 2010). Liao et al. (2012) reported that *C. japonicum* flavones, pectolinarin and 5,7-dihydroxy-6,4-dimethoxy flavone, enhanced adipocyte differentiation and glucose uptake in 3T3-L1 adipocytes (Liao et al., 2012). Among the flavonoids, silymarin is a mixture of polyphenolic flavonoids composed of silibinin (silybins A and B), isosilyins A and B, silychristins A and B, silydianin, and other phenol compounds.

Human obesity is a significant risk factor of a number of diseases, such as type II diabetes, hypertension, and coronary heart disease (Pi-sunyer, 2006). Adipocyte differentiation has often been a target of anti-obesity strategies, because obesity is caused by not only hypertrophy of adipocytes but also adipocyte hyperplasia (Caro et al., 1989). The 3T3-L1 cell line is commonly used as an adipocyte differentiation model system for the investigation of molecular mechanisms that regulate adipogenesis (Kim and Chen 2004). In 3T3-L1 preadipocytes, the addition of a

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hormone mixture initiates a transcriptional regulatory cascade that results in a gene expression profile specific for adipocyte functions (Cornelius et al., 1994). Several critical transcription factors, including CCAAT/enhancer-binding proteins C/EBPα and the peroxisome proliferator-activated receptor γ (PPARγ), as well as cross-talk among these factors have been well studied. PPARγ acts as a direct regulator of many fat-specific genes that can trigger adipogenesis (Choi et al., 2006). These transcriptional factors are involved in the sequential expression of adipocyte-specific proteins during adipocyte differentiation (Gregorie et al., 1998). Due to the recent increased interest in natural products for anti-obesity, a variety of studies are being conducted to find the flavonoids that play a role in this adipocyte differentiation mechanism. Therefore, in the present study, the anti-adipogenic effects of various solvent fractions of *C. japonicum* leaf in 3T3-L1 adipocytes via regulation of anti-adipogenic transcriptional genes were determined.

Materials and Methods

Materials. 3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from WellGENE Biopharmaceuticals (Korea). Isobutyl-methyl-xanthine (IBMX), insulin, dexamethasone (DEX), and silydianin were purchased from Sigma (USA). Triglyceride assay reagent was purchased from Asan Pharmaceutical (Korea). Reverse transcription system (ImProm-II^{TM}) was obtained from Promega (USA).

Sample preparation. *C. japonicum* was collected from Pyunggang Botanical Garden, Korea in May 2007. Leaves of *C. japonicum* were crushed in a blender and extracted with 70% ethanol by steam distillation at room temperature followed by filtering through filter papers. The ethanolic extract was then evaporated under vacuum at 50°C. Subsequently, the ethanolic extract was fractionated successively with *n*-hexane, chloroform, ethylacetate, *n*-butanol, and water.

HPLC analysis. Reverse-phase high performance liquid chromatography (HPLC) was conducted by using a Waters SPD-10A series system (USA) consisting of 515 pump, a 717 autosampler, and a UV 2487 detector. Chromatographic separation was accomplished using an Atlantis dC18 reverse phase column (Waters, 4.6×150 mm, 5 μm) and the elution was monitored at 290 nm. For separation, solvents A (20:80 methanol:water, v:v) and B (80:20, methanol:water, v:v) were used. The linear gradient program was initiated with 85:15 solvent A: solvent B for 5 min, followed by 45:55 solvent A:solvent B for 15 min. The proportions of 45:55 were then held for 20 min, after which they were returned to 85:15 after 10 min. The flow rate was 0.75 mL/min.

Adipogenic differentiation and TG assay. The 3T3-L1 cells were maintained in DMEM supplemented with 10% FBS and 100 units of antibiotic/antimycotic solution at 37°C under 5% CO_2. For the adipogenesis experiment, 3T3-L1 cells were differentiated using a hormone mixture (IBMX, DEX, and insulin) in accordance with the previous methods of Hwang et al. (2007). For TG assay, differentiated 3T3-L1 cells were washed with phosphate-buffered saline (PBS) and added to 10% NP-40 in PBS for lysis. Intracellular TG was then measured based on the enzymatic conversion of accumulated TG into glycerol in differentiated adipocytes. The absorbance was determined at 550 nm (Versa Max Microplate Reader) and normalized based on the protein content. The results are expressed as the total TG per mg of cellular protein (Bradford, 1976).

Measurement of free glycerol for lipolysis assay. The differentiated 3T3-L1 adipocytes were incubated with various extract concentrations for 72 h. The incubation medium was transferred to another set of tubes and heated at 70°C for 10 min to inactivate any enzymes released by the cells. The incubated medium was then assayed for free glycerol using a glycerol reagent, and the absorption was measured at 540 nm. The protein content was measured by the Bradford protein assay (Bradford, 1976).

RNA isolation and RT-PCR. Gene expression was determined by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using a dice thermal cycler (Biorad, Bio Inc. Japan) as described by Mackall et al. (1976). Briefly, total RNA was isolated from 3T3-L1 adipocytes using a Trizol reagent according to the manufacturer's protocols (Invitrogen, USA), and 1 μg of RNA was reverse-transcribed by an ImProm-II^{TM} reverse transcription (RT). PCR was then conducted in a reaction composed of the cDNA product and PCR premix (Bioneer, Korea). PCR was conducted by subjecting the samples to the following conditions: 95°C for 3 min (1 cycle), followed by 25 cycles at 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. Primer sequences are shown in Table 1. Bands were visualized on a UV illuminator and photographed using an EL LoGic 100 Imaging System (Kodak, Japan).

Table 1 Primers for RT-PCR and Real time-PCR

GENE		Sequences
PPARγ	Sense	5'-ACC ACT CGC ATT CCT TTG AC-3'
	Anti-sense	5'-TCA GCG GGA AGG ACT TTA TG-3'
C/EBPα	Sense	5'-ATG GAG TCG GCC GAC TTC TAC-3'
	Anti-sense	5'-CAG GAA CTC GTC GTT GAA GGC-3'
Adiponectin	Sense	5'-AAC CCC TGG CAG GAA AGG-3'
	Anti-sense	5'-TGA ACG CTG AGC GAT ACA CAT-3'
LPL	Sense	5'-CAA GCT GGT GGG AAA TGA TG-3'
	Anti-sense	5'-ACTGGGGGCTTCTGCATACT-3'
FAS	Sense	5'-ATGGACACAATGCTGCAGGT-3'
	Anti-sense	5'-ACCAGCTGTTCTTGCTGCC-3'
GAPDH	Sense	5'-AAC TTT GGC ATT GTG GAA GGG C-3'
	Anti-sense	5'-GAC ACA TTG GGG GTA GGA ACA C-3'

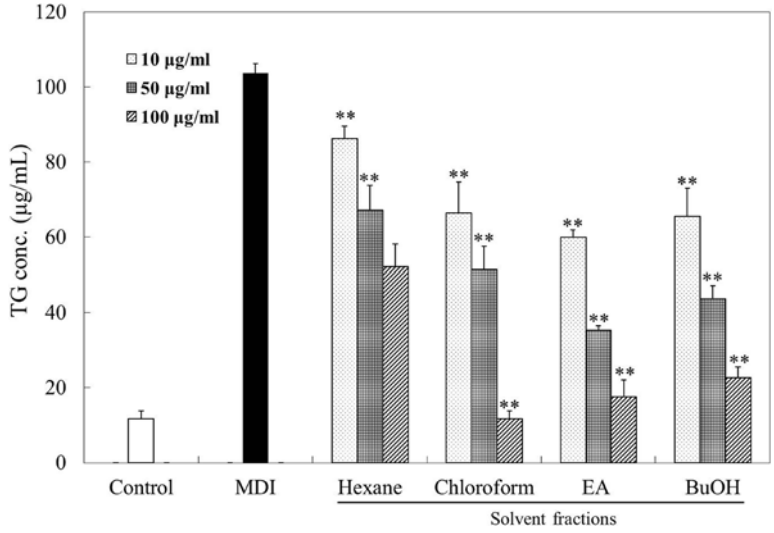

Fig. 1 Inhibitory effects of fractions from *C. japonicum* leaf on intracellular triglyceride concentration. The values were calculated as mg of TG per mg of total protein. Data are expressed as the means ± SEM of triplicate experiments. *Significant at p <0.05, **Significant at p <0.01.

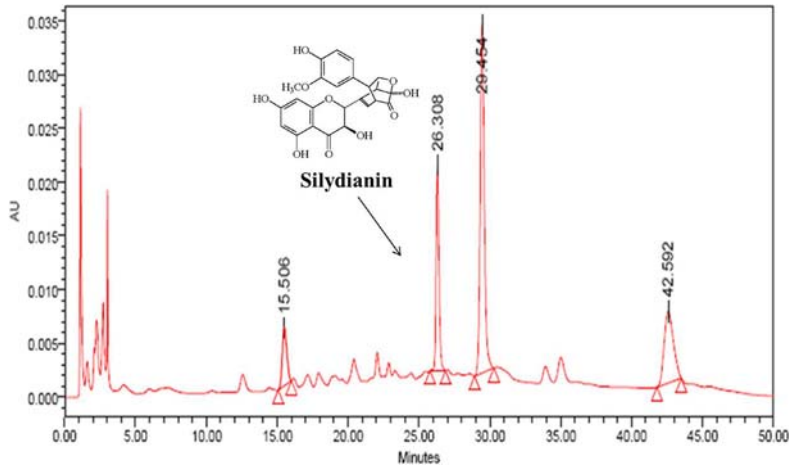

Fig. 2 Chromatogram of the chloroform-soluble fraction of the leaf of *C. japonicum*

Statistical analysis. Data were presented as the means ± SEM of at least three independent experiments performed in triplicate. Statistical differences among groups were evaluated by one-way analysis of variance. For all analyses, p <0.05 was considered to indicate significance. Statistical analyses were conducted using SPSS 10.0 (SPSS Inc., USA).

Results and Discussion

Effect of various solvent fractions of *C. japonicum* on adipocyte differentiation. Inhibition of adipocyte differentiation on MDI-induced cell adipogenesis by various solvent fractions of *C. japonicum* leaf was investigated (Fig. 1). Overall, all of the solvent fractions exhibited a decreasing pattern of triglycerol concentration in adipocyte in a concentration-dependent manner. Among them, chloroform-soluble fraction was found to have the greatest inhibitory effect, indicating that it suppressed lipid accumulation by 66.5, 51.5, and 11.7% at the concentrations of 10, 50, and 100 mg/mL, respectively.

Identification of bioactive component in chloroform fraction of *C. japonicum*. HPLC was carried out for identification and quantification of bioactive component in chloroform-soluble fraction of *C. japonicum* (Fig. 2). As a major bioactive component, silydianin was identified according to relative retention time as of the standard. Chloroform-soluble fraction was detected at 194.2 μg/mL for silydianin.

Lipolysis effects of various solvent fractions from *C. japonicum* leaf on the level of glycerol secretion in differentiated 3T3-L1 cells. Lipolysis, the process by which fat cells degrade and release their content of triglycerides, is a potential therapeutic target for obesity. Lipid accumulation is

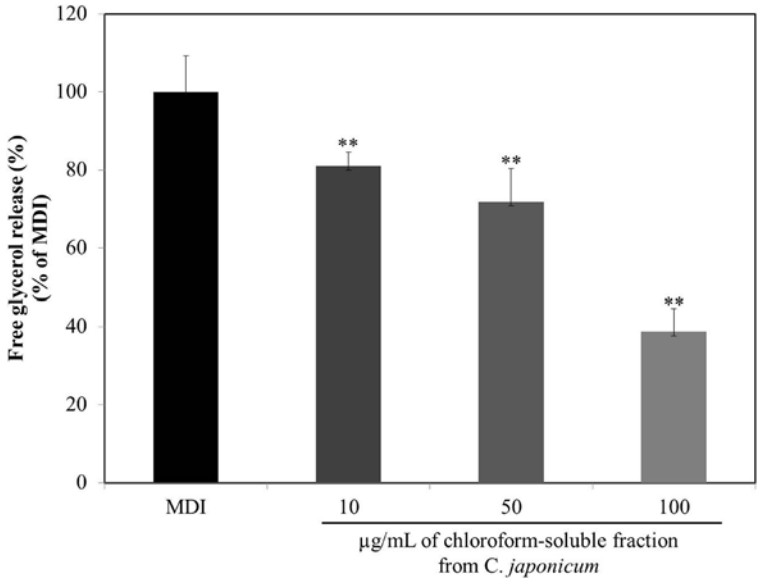

Fig. 3 Values indicate free glycerol content of the differentiated cells. Data are mean ± SD values, each performed in triplicate. *Significant at $p < 0.05$, **Significant at $p < 0.01$.

controlled by a balance between lipogenesis and lipolysis (Rayalam et al., 2008). Triglyceride hydrolysis proportionally released glycerol and free fatty acid from adipocytes, causing lipolysis (Moussalli et al., 1986). To determine whether chloroform-soluble fraction exerts lipolytic effects in 3T3-L1 adipocytes, matured adipocytes were treated with various concentrations of extracts. The level of glycerol secretion into the medium showed higher decrease by treatment with chloroform-soluble fraction compared to the MDI group (Fig. 3).

Effects of chloroform-soluble fraction of the *C. japonicum* leaf on mRNA expression of adipogenic transcription factors. In order to determine mechanism on the anti-adipogenic effects of chloroform-soluble fractions of the *C. japonicum* leaf, a reverse-transcription RT-PCR was carried out. Adipocyte differentiation includes a series of programmed changes in specific gene expressions. PPARγ and C/EBPα are the two central transcriptional regulators that are induced prior to the transcriptional activation of most adipocyte-specific genes. Over-expression of these transcription factors can accelerate adipocyte differentiation (Gregorie et al., 1998). To determine if the decrease in adipogenesis is caused by the inhibition of adipogenic transcription factor, reverse-transcription PCR was conducted to evaluate the expression levels of the mRNA of transcription factors PPARγ and C/EBPα in 3T3-L1 cells. The mRNA level of the PPARγ and C/EBPα decreased in a dose-dependent manner (Fig. 4). These results indicate that lipid accumulation was inhibited by transcription factors such as PPARγ and C/EBPα. A previous study found that PPARγ and C/EBPa synergistically activate downstream adipocyte-specific gene promoters such as FAS and LPL (Gregorie et al., 1998). LPL in adipose tissue involves the accumulation of the TG droplet in fat cells, and the high regulation of LPL activity in adipocytes is

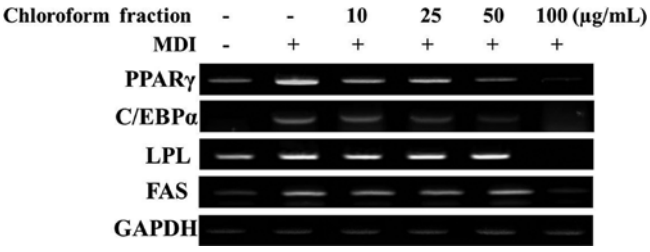

Fig. 4 Inhibitory effects of chloroform-soluble fraction from *C. japonicum* leaves on the expression of transcription factors in adipocyte differentiation. The 3T3-L1 preadipocytes were treated as a MDI or with chloroform-soluble fraction (10, 50, 100 μg/mL) for 72 h. The mRNA expressions of PPARγ, C/EBPα, adiponectin, LPL, and FAS were determined by RT-PCR.

closely linked with obesity (Bulló et al., 2002). Furthermore, activated PPARγ is involved in a metabolic cascade that leads to the expression of FAS and is clearly able to cross-activate the FAS promoters (Palmer et al., 2002). Therefore, in the present study, the effect of chloroform-soluble fraction of *C. japonicum* leaf extract were investigated on the regulation of the adipogenic target genes FAS and LPL using reverse-transcription PCR. The mRNA levels of FAS and LPL decreased in response to treatment with the chloroform-soluble fraction of *C. japonicum* leaf extract at a concentration of 100 mg/mL. Suppression of LPL and FAS expressions indicated a reduction of PPARγ and C/EBPα expression, suggesting that the chloroform-soluble fraction of *C. japonicum* leaf extract suppresses adipocytes differentiation by acting with adipogenic enzyme such as FAS and LPL. To the best of our knowledge, the present study is the first to report the characterization of silydianin and the potential mechanism of the

chloroform-soluble fraction of *C. japonicum* on anti-obesity.

In conclusion, we found that the chloroform-soluble fraction of *C. japonicum* leaves showed anti-adipogenic effect in adipocytes via regulation of adipogenic enzymes and transcription factors. These findings suggest that *C. japonicum* extract may be a good candidate for the management of obesity.

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