

## Inhibition of Adipocyte Differentiation by MeOH Extract from *Carduus crispus* through ERK and p38 MAPK Pathways

Eun Jeong Lee, Eun Ji Joo, Yoo Na Hong, and Yeong Shik Kim\*

Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

**Abstract** – In this study, the effects of a methanol (MeOH) extract of *Carduus crispus* L. (Asteraceae) on adipogenesis was investigated in 3T3-L1 cells. To differentiate preadipocytes to adipocytes, confluent 3T3-L1 preadipocytes were treated with a hormone mixture, which included isobutylmethylxanthine, dexamethasone, and insulin (MDI). The methanol extract of *C. crispus* significantly decreased fat accumulation by inhibiting adipogenic signal transcriptional factors in MDI-induced 3T3-L1 cells in a dose-dependent manner. In MTT assays and on PI-staining, methanol extract of *C. crispus* inhibited the proliferation of 3T3-L1 cells during mitotic clonal expansion (MCE). The anti-adipogenic effect of the *Carduus* extract seemed to be associated with the up-regulation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways within the first 2 days after MDI treatment. These results suggest that methanol extract of *C. crispus* might be beneficial for the treatment of obesity.

**Keywords** – *Carduus crispus*, adipogenesis, adipocyte, peroxisome proliferator-activated receptor2 (PPAR $\gamma$ 2), obesity

### Introduction

Obesity is rising because of an imbalance in individuals between energy intake and the expenditure of energy (Flier *et al.*, 2004). Adipocytes are the first place in which the triacylglycerols resulting from an excess of energy accumulate. Both an increased number of adipocytes (hyperplasia) due to enhanced differentiation of preadipocytes to adipocytes and an increased size of adipocytes (hypertrophy) due to lipid accumulation have been shown to participate in the expansion of adipose tissue (Spalding *et al.*, 2008).

Adipocyte differentiation is an important process in determining the number and size of adipocytes in the development of obesity. During differentiation, 3T3-L1 preadipocytes undergo not only morphological alterations but also characteristic changes such as adiposity, which means they can accumulate visible lipid in their cytoplasm (Rosen *et al.*, 1999). Preadipocytes express specific genes, such as PPAR $\gamma$ 2 and c/EBP $\alpha$ , related to adipogenesis. PPAR $\gamma$ 2 and c/EBP $\alpha$  are key transcription factors of adipogenesis and lipogenesis that are activated during the early stages of adipocyte differentiation to stimulate the expression of several metabolic genes (Srujana *et al.*,

2007). In the final stage of differentiation, the differentiated cells express markers that are characteristic of the adipocyte phenotype, such as adipocyte selective fatty acid binding protein (aP2), lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), and fatty acid binding protein (FABP) (MacDougald *et al.*, 1995).

In these cells, ERK, p38, c-Jun-NH2-terminal kinase (JNK), and MAPKs are important for their proliferation and differentiation (Pearson *et al.*, 2001). Previously, it was reported that inhibiting MAP kinase could block lipogenesis in the early stages of 3T3-L1 differentiation (Bost *et al.*, 2005). Recently, rosiglitazone and pioglitazone, thiazolidinedione (TZD) containing PPAR $\gamma$ 2 agonists, have been widely used as anti-diabetic agents (Kelly *et al.*, 2002). These can decrease hyperglycemia by direct reduction of insulin resistance leading to increased glucose uptake into skeletal muscle and adipose tissue. However, TZDs can also promote adipocyte differentiation, which may generate negative effects such as increasing subcutaneous adipose tissue (Lebovits *et al.*, 2001; Miyazaki *et al.*, 2002). Consequently, TZDs may result in modest weight gain. Due to the side effects of these drugs, researchers are looking for new types of drugs to be used in the treatment of metabolic disorders. Thus, it would be worthwhile to investigate promising natural products.

\*Author for correspondence

Tel: +82-2-880-2479; E-mail: kims@snu.ac.kr

*Carduus crispus* L. (Asteraceae) is a biennial growing to 0.9 m, and is mainly found in Asia and China and grows on waste or uncared soil. *C. crispus* has been used in traditional medicine for the treatment of colds, stomach aches, and rheumatism for a long time. Pharmacological screening of its extracts revealed a cytotoxic activity against some human cancer cell lines. The search for the antiproliferative principle of the plant led to the discovery of five novel isoquinoline alkaloids, crispine A-E (Zhang *et al.*, 2002). Also, *Carduus crispus* have been exhibited significant antioxidant activity by hispidulin-7-neohesperidoside and luteolin-7-glucoside in both DPPH assay and peroxyxynitrite (Jeong *et al.*, 2008). However, the effects of methanol extract of *C. crispus* on 3T3-L1 cells has not been reported until now. Therefore, the effects of *Carduus* extracts on adipogenesis in the 3T3-L1 cell line were investigated.

### Methods and materials

**Plant materials** – The whole plant of *C. crispus* L. was collected at Medicinal Plant Garden of Natural Products Research Institute, Seoul National University. It was identified by Prof. Je Hyun Lee, Dong Kuk University. A voucher specimen was deposited at the Herbarium of the Natural Products Research Institute (NPRI-100).

**Plant extracts** – The aerial parts were dried and cut into small pieces. Twenty five grams of *C. crispus* were boiled three times for 2 hr with 250 ml of 100% methanol. All the extracts were combined and concentrated. The solvent was removed by rotary evaporation (N-1000s, Eyela, Japan).

**Cell lines, chemicals, and biochemicals** – 3T3-L1 preadipocyte cells were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (D-PBS), penicillin, streptomycin, trypsin-EDTA, trypan blue, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), Oil Red O, LEPAL CA-630 (NP-40), recombinant human insulin, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), sodium bicarbonate and naphthylethylenediamine dihydrochloride were purchased from Sigma Chemical (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Bioshop (Burlington, Canada). Bovine calf serum (BCS) was obtained from Abclone (Hurstbridge, Australia). Polyvinylidenedifluoride (PVDF) membrane was purchased from Millipore (Bedford, USA). Fetal bovine serum (FBS) was purchased from South Pacific (New Zealand). The Cell

Counting Kit-8 was purchased from Dojindo Laboratories (Tokyo, Japan). Protein assay reagent was from Bio-Rad (Vancouver, Canada). Anti-mouse monoclonal antibodies against PPAR $\gamma$ 2 and c/EBP $\alpha$  were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL plus detection kit was purchased from Amersham (Buckinghamshire, UK).

**Adipocyte differentiation and treatment** – Fibroblasts were grown in DMEM with 10% bovine calf serum (BCS) containing 25 mM HEPES, 25 mM NaHCO $_3$ , 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (growth medium) at 37 °C in 5% CO $_2$ . 3T3-L1 cells were seeded at a density of  $5 \times 10^4$  cells/well in a 6-well plate containing 2 ml of growth medium in each well. The cells were then incubated at 37 °C in 5% CO $_2$  until two days after they became confluent. At this stage, differentiation was induced by exchanging the media with fresh media which included mediated differentiation inducer (MDI), 10% FBS, 0.5 mM IBMX, 1  $\mu$ M DEX, and 10  $\mu$ g/ml insulin for two days (from day 0 to day 2). After two days, the media was replaced with DMEM containing 10% FBS and 10  $\mu$ g/ml insulin, and the cells were incubated for an additional two days (from day 2 to day 4). This media was changed every two days (until day 6).

**Oil Red O staining** – Cells were stained with Oil Red O at day 5 after the induction of differentiation. The adipocytes were washed twice with PBS and fixed with 10% formaldehyde for 1 hr. The cells were then washed twice with distilled water followed by staining with 0.5% Oil Red O solution (in 60% isopropanol) for 2 hr at room temperature. Cells were washed three times with 60% isopropanol to remove unbound dye and were observed under a Leica fluorescence microscope at 100  $\times$  magnification. Stained Oil Red O was eluted with 4% NP-40 in isopropanol (v/v) and was quantified by measuring the optical absorbance at 490 nm.

**Western blot analysis** – 3T3-L1 cells and animal tissues were extracted in ice-cold lysis buffer containing 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM Na $_2$ EDTA, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM Na $_3$ VO $_4$ , 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protein inhibitor cocktail (Sigma MO) for 1 hr. Proteins (25  $\mu$ g/lane) were loaded onto a 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), chromatographed, and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS-T, 50 mM Tris-HCl, pH 7.6 NaCl, 0.05% Tween 20). It was then incubated with primary antibodies (PPAR $\gamma$ 2, c/EBP $\alpha$ ) for 3 hr and

washed three times in TBS-T. The membrane was incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Proteins were visualized using a chemiluminescent substrate kit AB frontier, Seoul, Korea).

**Proliferation assay** – Within one or two days after confluence, 3T3-L1 cells were treated with MDI containing methanol extract of *C. crispus*. At the indicated time points, 5 mg/ml of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added and incubated for 4 hr. The formazan crystals were dissolved by addition of 200  $\mu$ l of DMSO. After 30 min, the amount of colored formazan derivative was determined by measuring the absorbance using an ELISA microplate reader at 570 nm.

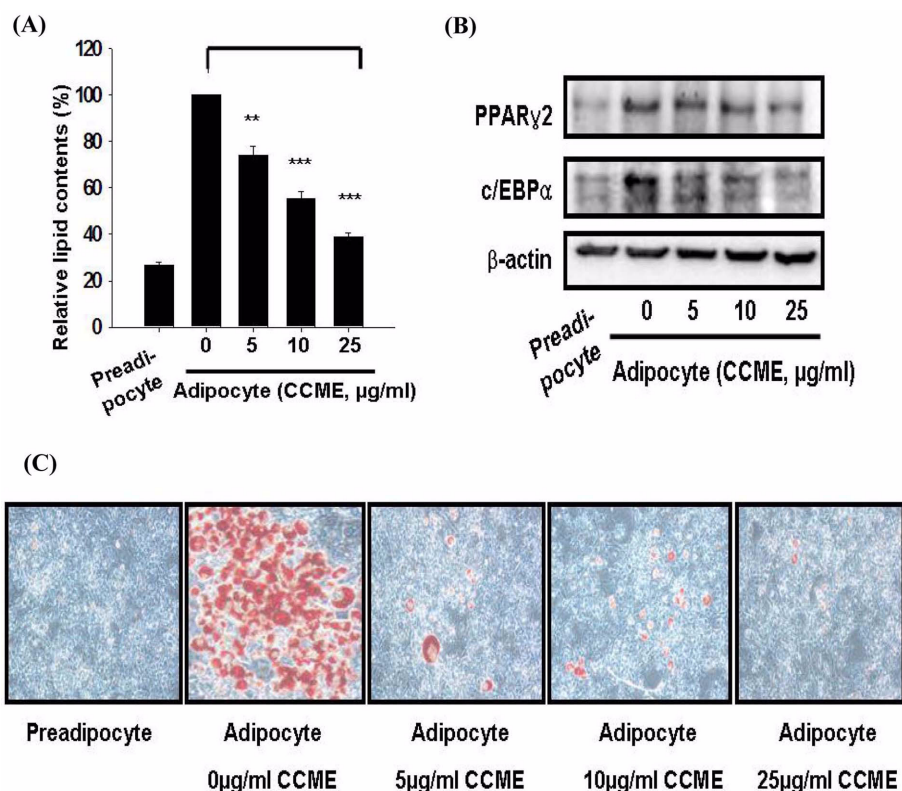
**Annexin V-FITC and Propidium iodide (PI) staining** – Within one or two days after confluence, cells were washed carefully by ice-cold DPBS twice and then 1 X Annexin V binding buffer once. The cells were stained with Annexin V-FITC and PI, and then diluted 1 : 10 in 1

X binding buffer for 20 min at room temperature in a dark chamber. After washing once with 1 X binding buffer, the stained cells were observed under fluorescence microscopy (Olympus CKX41, Japan).

**Statistics** – All results are expressed as mean  $\pm$  S.D. The statistical significance between two groups was analyzed by Student's *t* test. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was also applied to assess the statistical significance of the differences between the study groups (SPSS version 10.0, Chicago, IL).

## Results and Discussion

Obesity is characterized by a pathological growth of adipocytes, which is intrinsically a consequence of adipocyte hypertrophy and differentiation (Hwang *et al.*, 2005). Although adipocyte differentiation is an important process for cellular function under normal conditions, pathological adipocyte differentiation confers abnormal expression of adipokines, which are implicated in



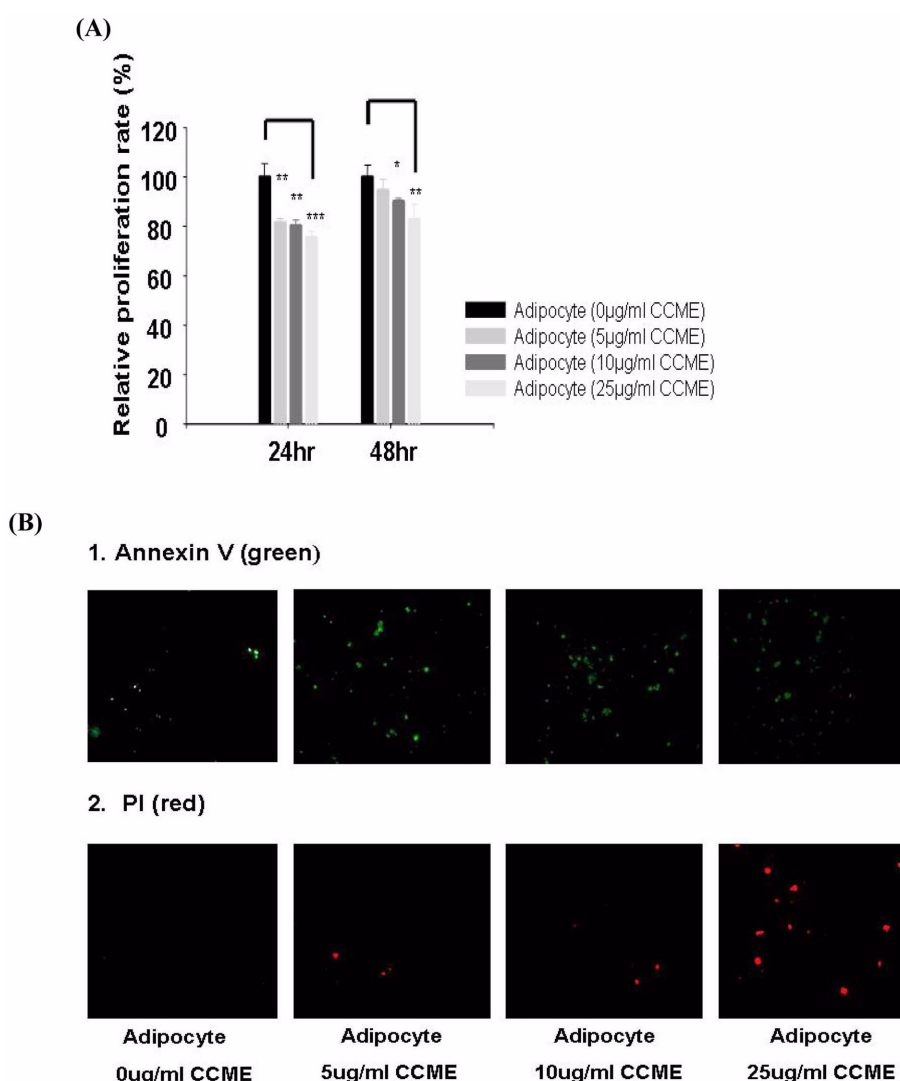
**Fig. 1.** Methanol extract of *C. crispus* (CCME) inhibited lipid accumulation in 3T3-L1 cells. 3T3-L1 cells were differentiated with MDI in the absence or presence of methanol extract of *C. crispus* (0, 5, 10 and 25  $\mu$ g/ml) for 9 days and then the lipid contents were measured by Oil Red O staining (A). The stained Oil Red O was eluted with 4% NP-40 solution and the stained lipid content was quantified by measuring the absorbance at 490nm (B). Changes in protein expression of PPAR $\gamma$ 2 and c/EBP $\alpha$ , the major transcriptional factors related to lipogenesis in 3T3-L1 cells. Western blots showed that PPAR $\gamma$ 2 and c/EBP $\alpha$  proteins were reduced in 3T3-L1 cells treated with methanol extract of *C. crispus* on the sixth day of differentiation (C). These signals were normalized to  $\beta$ -actin. The error bars represent the standard deviation of the mean of three independent experiments (\*\**p* < 0.05, \*\*\**p* < 0.001 compared with differentiated control).

metabolic disorders (Halberg *et al.*, 2008). Therefore, inhibition of adipocyte differentiation was suggested to play a role in the pathogenic development of obesity.

In the present study, the effect of methanol extract of *C. crispus* on the adipogenic differentiation of 3T3-L1 cells was examined. The optimal concentration of methanol extract of *C. crispus* for treating adipocytes without altering cell viability was 25  $\mu\text{g}/\text{ml}$  as determined by MTT assay (data not shown).

To differentiate preadipocytes into adipocytes, 2-day post-confluent 3T3-L1 preadipocytes were stimulated with medium differentiation inducer (MDI) in the

presence or absence of methanol extract of *C. crispus* and diluted with DMEM at concentrations of 0, 5, 10 and 25  $\mu\text{g}/\text{ml}$ . As shown in Fig. 1(A), lipid accumulation in methanol extract of *C. crispus* treated cells was lower than lipid accumulation in control cells. Therefore, the results show that methanol extract of *C. crispus* blocked adipocyte differentiation in a dose-dependent manner. The stained Oil Red O was eluted with 4% NP-40 solution and the absorbance was measured at 490nm by a UV spectrophotometer (Fig. 1B). The results show that 25  $\mu\text{g}/\text{ml}$  of methanol extract of *C. crispus* was effective for reducing the lipid content up to 58.3% of the positive



**Fig. 2.** Methanol extract of *C. crispus* (CCME) inhibited adipocyte differentiation in 3T3-L1 cells within the first 2 days of treatment, indicating that the anti-adipogenic effect of methanol extract of *C. crispus* originated from the anti-proliferation of the cells. Post-confluent 3T3-L1 cells were induced to differentiate with MDI plus methanol extract of *C. crispus* (0, 5, 10 and 25  $\mu\text{g}/\text{ml}$ ). MTT assay was done at 24 and 48 hr for calibration of 3T3-L1 cell proliferation (A). Reconfirmation of the inhibitory effect of methanol extract of *C. crispus* on the associated proliferation within 24 hr. Cells were stained with Annexin-PI solution (B). The error bars represent the standard deviation of the mean of three independent experiments (\* $p < 0.01$ , \*\* $p < 0.05$ , \*\*\* $p < 0.001$  compared with differentiated control).

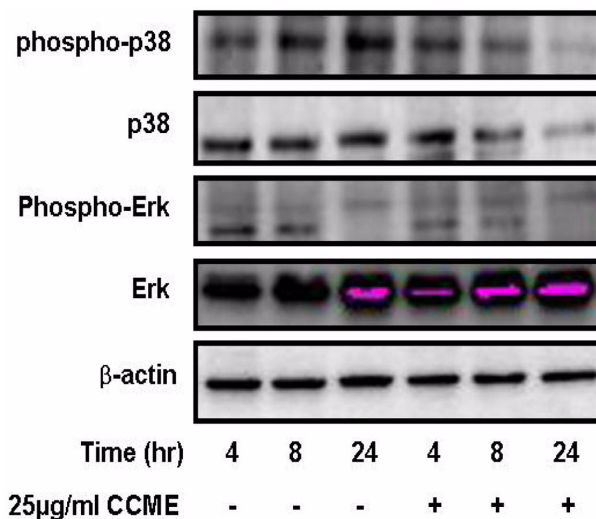
control value in differentiated 3T3-L1 adipocytes. Western blot analysis was done to examine the change in expression of PPAR $\gamma$ 2 and c/EBP $\alpha$ , the major transcriptional factors related to the regulation of lipogenesis. Consistent with the decreased lipid accumulation, methanol extract of *C. crispus* treatment significantly decreased the expression of PPAR $\gamma$ 2 and c/EBP $\alpha$  during differentiation (Fig. 1C).

Preadipocytes, which were in a state of pre-confluent proliferation, grew until reaching the confluence state; G0/G1 growth was arrested by contact inhibition, and then the cells were left for another 2 days (Kim *et al.*, 2008). This period is when MCE occurs, resulting in a doubling of the cell numbers (Zhang *et al.*, 2004). Thus, MCE is an event associated with adipocyte differentiation and proliferation (Tang *et al.*, 2003).

To confirm whether inhibition of adipogenesis was due to the inhibition of cell proliferation by methanol extract of *C. crispus*, the MTT assay was used to measure the cell viability of the adipocytes during the MCE period. The results show that 25  $\mu$ g/ml of methanol extract of *C. crispus* was effective in reducing the 3T3-L1 adipocyte proliferation up to 23.2% of control, and methanol extract of *C. crispus* exhibited its inhibitory activity on the proliferation of 3T3-L1 cells within the first 24 hours of the MCE period (Fig. 2A). To confirm the inhibitory effect of methanol extract of *C. crispus* on the associated proliferation that occurred within 24 hours of treatment, cells were stained with annexin V-PI solution. Annexin V-PI double-staining analysis showed that treating the cells with methanol extract of *C. crispus* reduced the number of adipocyte cells in a dose-dependent manner (Fig. 2(B)).

MAPK is expressed constitutively by growth-arrested 3T3-L1 preadipocytes and is rapidly, within 1 hr after induction, and transiently phosphorylated (Boney *et al.*, 2000). To confirm which signaling pathway methanol extract of *C. crispus* affects for the inhibition of adipocyte differentiation, 3T3-L1 cells were differentiated with MDI in the absence or presence of methanol extract of *C. crispus* (25  $\mu$ g/ml) for 4, 8 and 24 hr. The signals for ERK and p38 were then analyzed by western blots. Methanol extract of *C. crispus* significantly decreased the expression of ERK and p38 proteins (Fig. 3).

Therefore, methanol extract of *C. crispus* inhibited adipocytic differentiation of 3T3-L1 cells during the 2 days after confluence (MCE), indicating that the anti-adipogenic effect of *Carduus* originated from an antiproliferation effect on the cells. Similar results have been published: pigment epithelium-derived factor (PEDF) suppressed adipogenesis via inhibition of early activation



**Fig. 3.** Methanol extract of *C. crispus* (CCME) inhibited adipocyte differentiation of 3T3-L1 cells through p38 and Erk pathways within 24 hr of treatment. The cells were treated with 25  $\mu$ g/ml of methanol extract of *C. crispus* and MDI for 4, 8 and 24 hr and the signals were analyzed by western blots. These signals were normalized to  $\beta$ -actin.

of ERK and MCE (Wang *et al.*, 2009). Also, genistein inhibited the mitotic clonal expansion of 2-day post-confluent 3T3-L1 preadipocytes (Harmon *et al.*, 2001). In addition, treatment with natural products such as quercetin (Yang *et al.*, 2008), evodiamine (Wang *et al.*, 2009), vitisin A (Kim *et al.*, 2008), and resveratrol (Yang *et al.*, 2008) suppressed adipogenesis via inhibition of the MAPK/ERK pathway in 3T3-L1 preadipocytes.

In summary, methanol extract of *C. crispus* exhibited its inhibitory activity on the proliferation of 3T3-L1 cells during the 2 days after confluence (MCE) and inhibited the expression of the adipogenic transcription factors, c/EBP $\alpha$  and PPAR $\gamma$ 2 through the ERK and p38 pathways. These findings suggest that methanol extract of *C. crispus* might be used for the amelioration of obesity.

### Acknowledgements

This work was supported by the National Research Foundation of Korea, Basic Research Promotion Fund (2010-0008384) and the MRC grant funded by the Korean government (MEST) (No. 20090083533).

### References

- Boney, C.M., Gruppuso, P.A., Faris, R.A., and Frackelton, A.R., The critical role of Shc in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. *Mol. Endocrinol.* **14**, 805-813 (2000).

- Bost, F., Aouadi, M., and Caron, L., The role of MAPKs in adipocyte differentiation and obesity. *Biochimie*. **87**, 51-56 (2005).
- Flier, J.S., Obesity wars: Molecular progress confronts an expanding epidemic. *Cell*. **116**, 337-350 (2004).
- Halberg, N., Wernstedt, A.I., and Scherer, P.E., The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am*. **37**, 753-768 (2008).
- Harmon, A. and Harp, J., Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. *Am J Physiol Cell Physiol*. **280**, C807-C813 (2001).
- Hwang, J.T., Park, I.J., Shin, J.I., Lee, Y.K. and Lee, S.K., Genistein, EGCG and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. *Biochem Biophys Res Commun*. **338**, 694-699 (2005).
- Jeong, da. M., Jung, H.A., and Choi, J.S., Comparative antioxidant activity and HPLC profiles of some selected Korean thistles. *Arch Pharm Res*. **31**, 28-33 (2008).
- Kelly, D.E., Mckolanis, T.M., and Kelley, C.A., Comparative effects of rosiglitazone and metformin on fatty liver and visceral adiposity in type 2 diabetes mellitus. *Diabetes*. **51**, (Suppl. 2) 142 (2002).
- Kim, J.K., So, H., Youn, M.J., Kim, H.J., and Kim, Y., Hibiscus sabdariffa L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway. *J Ethnopharmacol*. **114**, 260-267 (2007).
- Kim, S.H., Park, H.S., Lee, M.S, Cho, Y.J., and Kim, Y.S., Vitisin A inhibits adipocyte differentiation through cell cycle arrest in 3T3-L1 cells. *Biochem Biophys Res Commun*. **372**, 108-113 (2008).
- Lebovitz, H.E., Dole, J.F., and Patwardhan, R., Rosiglitazone monotherapy is effective in patients with type 2 diabetes. *J Clin Endocrinol Metab*. **86**, 280-288 (2001).
- MacDougald, O.A. and Lane, M.D., Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem*. **64**, 345-373 (1995).
- Miyazaki, Y., Matsuda, M., and DeFronzo, R.A., Dose response effect of pioglitazone on insulin sensitivity and insulin secretion in type 2 diabetes. *Diabetes care*. **25**, 517-523 (2002).
- Pearson, G., Robinson, F., Gibson, T.B., Xu, M., and Cobb, M.H., Mitogen-activated protein (MAP) kinase pathway : Regulation and physiological functions. *Endocr Rev*. **22**, 153-183 (2001).
- Rosen, E.D. and Spiegelman, B.M., Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol*. **16**, 145-171 (2000).
- Spalding, K.L., Arner, E., Westermark, P.O., Bernard, S., and Buchholz, B.A., Dynamics of fat cell turnover in humans. *Nature*, **453**, 783-787 (2008).
- Srujana, R., Mary Anne, D.F., and Clifton, A.B., Phytochemicals and regulation of the adipocyte life cycle. *J Nutr Biochem*. **19**, 717-726 (2007).
- Tang, Q.Q., Otto, T.C., and Land M.D., Mitotic clonal expansion: A synchronous process required for adipogenesis. *Proc Natl Acad Sci*. **100**, 44-49 (2003).
- Wang, M., Wang, J.J., Li, J., Park, K., Qian, X., Ma, J.X., and Zhang, S.X., Pigment Epithelium-derived factor (PEDF) suppresses adipogenesis via inhibition of the MAPK/ERK pathway in 3T3-L1 preadipocytes. *Am J Physiol Endocrinol Metab*. **297**, E1378-87 (2009).
- Wang, T., Wang, Y., and Yamashita, H., Evodiamine inhibits adipogenesis via the EGFR-PKCalpha-ERK signaling pathway. *FEBS Lett*. **583**, 3655-3659 (2009).
- Yang, J.Y., Della-Fera, M.A., Rayalam, S., Ambati, S., Hartzell, D.L., Park, H.J., and Baile, C.A., Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sci*. **82**, 1032-1039 (2008).
- Zhang, J.W., Tang, Q.Q., Vinson, C., and Lane, M.D., Dominant-negative c/EBP $\alpha$  disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. *Proc Natl Acad Sci*. **101**, 43-47 (2004).
- Zhang, Q., Tu, G., Zhao, Y., and Cheng, T., Novel bioactive isoquinoline alkaloids from *Carduus crispus*. *Tetrahedron*. **58**, 6795-6798 (2002).

Received October 6, 2011

Revised November 11, 2011

Accepted November 15, 2011