ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2018 Vol. 28. No. 9. 999~1006 DOI : https://doi.org/10.5352/JLS.2018.28.9.999

Artemisia scoparia Inhibits Adipogenesis in 3T3-L1 Pre-adipocytes by Downregulating the MAPK Pathway

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Received April 30, 2018 /Revised June 29, 2018 /Accepted July 4, 2018

Obesity is epidemic worldwide and has reportedly been linked to the progression of several metabolic and cardiovascular diseases. The natural products are decreasing the side effects of medicines used for obesity and also have health benefits dut to their numerous bioactive compounds. In this context, *Artemisia scoparia* is a widespread plant that has been suggested as possessing various types of bioactivity. In this study, the crude extract from *A. scoparia* (ASE) was tested for its ability to suppress adipogenesis in mouse 3T3-L1 pre-adipocytes. The molecular pathway by which ASE affects differentiation of 3T3-L1 cells was also investigated. The introduction of ASE to differentiating 3T3-L1 pre-adipocytes resulted in suppressed adipogenesis, as confirmed by decreased intracellular lipid accumulation. The differentiating cells treated with 10 and 100 μg/ml of ASE showed 21.9 and 29.0% less lipid accumulation, respectively, than untreated adipocytes. In addition, the results indicated that ASE treatment lowered the expression of the adipogenesis-related factors PPARγ, C/EBPα, and SREBP-1c. Furthermore, treating with ASE notably decreased levels of phosphorylated p38, ERK, and JNK in 3T3-L1 adipocytes. These results indicate that ASE exhibits significant anti-adipogenesis activity by downregulating the MAPK and PPARγ pathways during the differentiation of 3T3-L1 pre-adipocytes. Therefore, *A. scoparia* may be a potential source of natural products against obesity.

Key words : Adipogenesis, *Artemisia scoparia*, MAPK, PPARγ, 3T3-L1

Introduction

Obesity is characterized as irregular fat metabolism of the body expressed as persistent fat accumulation. Obesity is the main risk factor for most of major diseases such as Type II diabetes [17], heart diseases [15], hypertension [31] and cancer [1]. In metabolic phenotype of obesity, the adipose tissue function is abnormal and affected by several genetic and environmental factors [18, 23]. Triacylglycerols are highly efficient sources of energy in the body, and mammals have developed intricate mechanisms to store triacylglycerols as fats in adipocytes to minimize the loss of energy [30]. Adipose tissue is formed by specialized cell types called adipocytes, which are capable of storing excessive energy as fat and secreting adipose tissue-specific hormones that affect almost all metabolic pathways of the body [5]. During the onset of obesity, the number of adipocytes rises redundantly as the adipose tissue expanses. The role of adipocytes, therefore, is gaining increasing interest towards the efforts to prevent and treat obesity along other metabolic diseases linked with deteriorated adipocyte function [8]. Adipocyte cells are developed from pre-adipocytes through adipogenesis involving the conversion of mesenchymal stem cells (MSCs) to the pre-adipocytes, and differentiation of pre-adipocytes into mature adipocytes.

Differentiation of the pre-adipocytes is strictly regulated by transcription factors and enzymes, mainly peroxisome proliferator-activated receptor γ (PPARγ) and mitogen activated protein kinase (MAPK) pathways [6, 24]. PPARγ is a member of the nuclear-receptor superfamily and has been considered as the master regulator in adipogenesis along CCAAT-enhancer-binding protein α (C/EBPα). Their sequential activation induces the expression of important pro-

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teins and enzymes in order to attain and maintain adipocyte characteristics. Several on the market obesity drugs, acting as PPARγ ligands, target and inhibit this pathway of PPARγ through preventing the upregulation [4]. Recently, considerable attention has been directed to development of active ingredients from natural sources with minor side effects and high biocompatibility for preventing and alleviating obesity.

Artemisia scoparia is a widespread plant that belongs to a very large flowering plant family of Asteraceae and growing natively across Eurasia. Leaves and flowers of *A. scoparia* are referred in traditional medicine sources with activities such as diuretic, antiphlogistic and for treatment of hepatitis [25]. In addition, several studies reported antioxidant, insecticidal, phytotoxic and anti-inflammatory properties of *A. scoparia* as well as chemical constituents derived from it such as essential oils, flavonoids and coumarins [3, 7, 22, 28]. In the present study, therefore, *A. scoparia* was analyzed to evaluate its effect on the differentiation of 3T3-L1 adipocytes and possible mechanism of action during adipogenesis.

Materials and Methods

Reagents

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Grand Island, NY). Antibodies for Western blotting were procured from Cell Signaling Technology (Danvers, MA, USA). Primers for reverse transcription polymerase chain reaction (RT-PCR) were obtained from Bio-RAD (Hercules, CA, USA). Remaining reagents were purchased from Sigma-Aldrich (St. Tropez, MI, USA) unless otherwise specified.

Crude extract

The sample (3 kg) of *A. scoparia* was air dried and cut into small pieces prior to maceration. Ground sample was extracted twice with methylene chloride (CH_2Cl_2) for 24 hr at room temperature. The extraction solution was dried *in vacuo*. The remains were then subjected to extraction again, twice with methanol (MeOH), using the same procedure as above. Lastly, both extracts were combined and used for further experiments.

Cell culture and adipocyte differentiation

Murine 3T3-L1 pre-adipocytes (ATCC® CL-173™) were cultured in DMEM supplemented with 10% FBS at 37℃ in a humidified atmosphere of 5% CO_2. Differentiation of the pre-adipocytes was induced with a differentiation mixture containing methylisobutylxanthine (0.5 mM), dexamethasone (0.25 μM) and insulin (5 μg/ml) in culture medium after 2 days following the confluence as described earlier [11]. This was replaced with DMEM containing 10% FBS supplemented with insulin only (5 μg/ml) after 2 days of incubation. Initial culture medium was introduced after 2 days incubation and replaced with fresh one every 2 days until the maturation of adipocytes. Successful adipocyte differentiation was confirmed by intracellular lipid droplets observed under a light microscope (Nikon Instruments, Tokyo, Japan). Sample was administered to the cell culture medium starting with introduction of differentiation medium and included in all medium changes. Cytotoxicity level of sample in 3T3-L1 cells was evaluated by MTT assay as previously described [11].

Oil-Red O staining

Accumulation of triglycerides as lipid droplets in 3T3-L1 adipocytes were confirmed by Oil-Red O staining of the triglycerides with common staining procedures reported in our previous study [11]. Following the confirmation of adipogenesis in cultured cells (6-well plate), culture medium was removed, and cells were washed with PBS. Cells were then fixed on wells with 3.7% fresh formaldehyde in PBS at room temperature for 1 hr. Oil-Red O (0.5% w/v) solution (60% isopropanol and 40% water) was filtered and 1 ml of solution was transferred to the wells. After staining incubation of 1 hr, the Oil-Red O solution was aspired from the plates and the plates were washed with distilled water prior to observation. Images of lipid droplets in 3T3-L1 adipocytes were collected by a Nikon Instruments microscope (Tokyo, Japan). Finally, Oil-Red O stain retained in the cells was eluted with 100% isopropanol and quantified by optical absorbance value at 500 nm using a microplate reader (Tecan Austria GmbH, Austria).

RT-PCR

Analysis of mRNA levels was carried out by RT-PCR following the method from previous reports [14]. Briefly, Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent (Invitrogen Co., CA, USA) following the manufacturer's instructions. For synthesis of cDNA from total RNA isolates, RNA (1 μg) was added to RNase-free water and oligo (dT), denaturated at 70°C for 5 min and cooled immediately. RNA was reverse transcribed in a master mix

containing 1X RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reserve transcriptase and 40 U RNase inhibitor at 42°C for 60 min and at 72°C for 5 min using an automatic T100 Thermo Cycler (Bio-Rad, UK). The target gene in synthesized cDNA was amplified using specific following sense and antisense primers as following: forward 5′-TTT-TCA-AGG-GTG-CCA-GTT-TC-3′ and reverse 5′-AAT-CCT-TGG-CCC-TCT-GAG-AT-3′ for PPARγ; forward 5′-TGT-TGG-CAT-CCT-GCT-ATC-TG-3′ and reverse 5′-AGG-GAA-AGC-TTT-GGG-GTC-TA-3′ for SREBP-1c; forward 5′-TTA-CAA-CAG-GCC-AGG-TTT-CC-3′ and reverse 5′-GGC-TGG-CGA-CAT-ACA-GTA-CA-3′ for C/EBPα; forward 5′-CCA-CAG-CTG-AGA-GGG-AAA-TC-3′ and reverse 5′-AA G-GAA-GGC-TGG-AAA-AGA-GC-3′ for β-actin. The amplification cycles were set as 95℃ for 45 sec, 60℃ for 1 min and 72℃ for 45 sec. After the completion of 30 cycles, the amplified products were separated on 1.5% agarose gel with electrophoresis for 30 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and the bands were visualized by UV light using Davinch-Chemi imager™ (CAS-400SM, Seoul, Korea).

Western blotting

Immunoblotting was performed according to standard procedures [11]. Briefly, cells were lysed in RIPA lysis buffer (Sigma–Aldrich Corp., St. Louis, USA) at 4℃ for 30 min. Cell lysates (25 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis followed by transfer onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., England, UK). The membrane was then blocked with 5% skim milk and hybridized with primary antibodies (diluted 1:1,000) for 1 hr. After incubation with horseradish-peroxidase-conjugated secondary antibody at room temperature for 1 hr, protein bands were visualized using a chemiluminescence ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. Membrane images were captured using a Davinch-Chemi imager™ (CAS-400SM, Seoul, Korea).

Statistical Analysis

The data were presented as mean ± SD. Differences between the means of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 (SAS Institute, Cary, NC, USA) with Duncan's multiple range tests. The significance of differences was defined at the $p<0.05$ level.

Results and Discussion

Crude extract of *A. scoparia* (ASE) was used for evaluation of the anti-adipogenesis effect. Prior to *in vitro* experiments using mouse pre-adipocyte 3T3-L1 cells, any cytotoxic properties of ASE were analyzed with MTT formazan assay. The cells were treated with or without ASE in various concentrations (10, 50, 100, 200 μg/ml). For tested concentrations, ASE did not exhibit any significant cytotoxicity as expected (Fig. 1). Results from MTT assay revealed that ASE is safe to be used *in vitro* experiments up to 100 μg/ml, which was decided as the upper limit of treatment concentration.

3T3-L1 pre-adipocytes were differentiated with differentiation medium treatment in the absence or presence of ASE. Differentiation medium was changed with feeding medium containing insulin only at day 2 and medium was replaced with fresh one every 2 days. Under these conditions, it was assumed that adipocytes were fully matured by day 6. In order to confirm that the cells were fully differentiated and ready for further experiments, intracellular lipid accumulation, an indicator for successful adipogenesis was controlled under a light microscope. At day 6, 70 to 90% of the ASE-untreated control cells were showed adipocyte characteristics by accumulating lipid droplets and accepted as successfully differentiated into mature adipocytes. There-

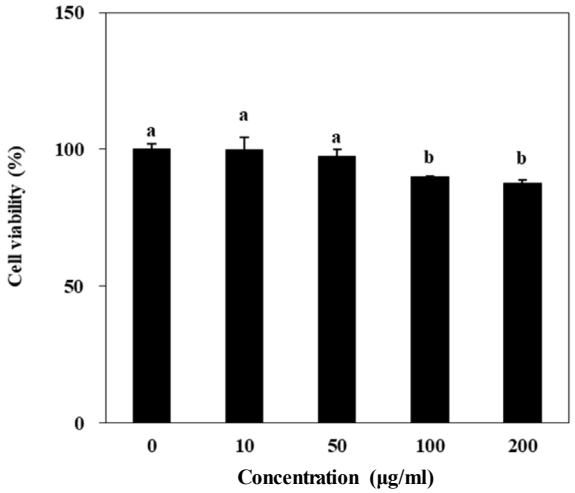

Fig. 1. Effect of *A. scoparia* crude extract on the viability of 3T3-L1 cells. Cytotoxicity of sample in 3T3-L1 cells was evaluated by MTT assay. Cell viability following sample treatment was evaluated by ability to form MTT-formazan crystals and measured by the absorbance values at 540 nm. Viability of the cells was quantified as a percentage of untreated control. Values are means ± SD (n=3). $^{a-b}$Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

fore, it was accepted to be sufficient to carry out further experiments. Progression of obesity occurs with redundant accumulation of lipid by adipose tissue. During the formation of new adipocytes through adipogenesis, ability to accumulate lipid droplets is the main factor that marks the successful adipogenic differentiation [10]. Accordingly, one of the main approaches to prevent and treat obesity is inhibition of fat storage in adipocytes [19]. In this respect, lipid accumulation of differentiated cells under ASE treatment was assessed by staining the lipid droplets with Oil Red O staining assay. As seen in Fig. 2, the amount of lipid droplets in ASE treated cells was decreased gradually in a dose-dependent manner. Staining of lipid droplets was quantified by elution of the Oil Red O stain that was bind to the lipid droplets. As expected from cell images, amount of stain bound by intracellular lipid was relatively low in ASE-treated cells compared to untreated control. Ability of ASE to decrease the storage of triglycerides in differentiating pre-

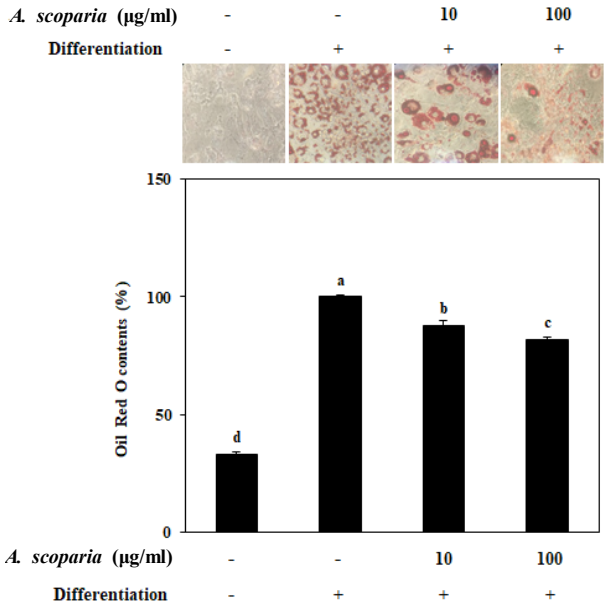

Fig. 2. Effect of *A. scoparia* crude extract on the intracellular lipid accumulation of the differentiated 3T3-L1 cells. Cells were induced to adipogenesis with a differentiation mixture with different concentrations (10 and 100 μg/ml). Following the successful differentiation of the cells, intracellular lipid droplets were stained by Oil-Red O staining and images were taken. Lipid accumulation was measured by the absorbance values of the eluted stain from cells at 500 nm. Accumulated lipid droplets were quantified as a percentage of the fully differentiated untreated control cells. Values are means ± SD (n=3). $^{a-e}$Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

adipocytes was regarded as a marker for intervening the either adipogenesis or lipid accumulation pathways of 3T3-L1 cells. Additionally, inhibiting the possible fat storage of adipocytes suggested that ASE might contain substances that were able to act against obesity-related characteristics of the adipose tissue.

The adipocyte differentiation is comprehensively studied and reported to be activated and regulated by a complicated signaling cascades of transcription factors and enzymes. PPARs, especially PPARγ, are ligand-activated transcription factors and reported to play pivotal roles in energy metabolism and, in this context, in differentiation of adipocytes [6]. Along PPARs, C/EBPs are also key proteins in the pathway that activate PPARγ resulting in the progression and maturation of differentiating pre-adipocytes. Following the introduction of adipogenic stimuli, activated C/EBPα induces the further activation and expression of PPARγ, which sequentially progress differentiation of adipocytes and regulate insulin sensitivity in mature adipocytes. During final steps of adipogenesis another transcription factor, SREBP-1c, is closely linked with fatty acid metabolism where the storage of fat in differentiated adipocytes occur [26]. The effect of ASE on the PPARγ pathway regulation of adipogenesis, was analyzed by reverse transcription polymerase chain reaction and immunoblotting of PPARγ, C/EBPα and SREBP-1c for gene and protein expression, respectively.

At the beginning of the differentiation 3T3-L1 cells were treated with different concentrations of ASE (10, 50 and 100 μg/mL) during the differentiation period and its effect was observed after the maturation into adipocytes. The mRNA expression levels of PPARγ, C/EBPα and SREBP-1c were all increased in the mature adipocytes compared to non-differentiated pre-adipocytes (Fig. 3A). Treatment with ASE dose-dependently suppressed the mRNA levels. At the concentration of 100 μg/ml, ASE lowered the mRNA expression levels of PPARγ, C/EBPα and SREBP-1c to 71.9, 71.9 and 54.4% of the untreated control cells, respectively. These results suggested that ASE was able to hinder the activation of PPARγ pathway which resulted in the suppressed adipogenesis in 3T3-L1 cells. In order to strengthen this suggestion, protein levels of transcription factors were evaluated by immunoblotting. Similarly, presence of ASE ameliorated the overexpressed levels of adipogenesis markers. Following ASE treatment of 100 μg/ml, PPARγ, C/EBPα and SREBP-1c protein levels were lowered by 68.7, 55.5 and 68.2% compared to untreated control cells (Fig. 3B). Combines results

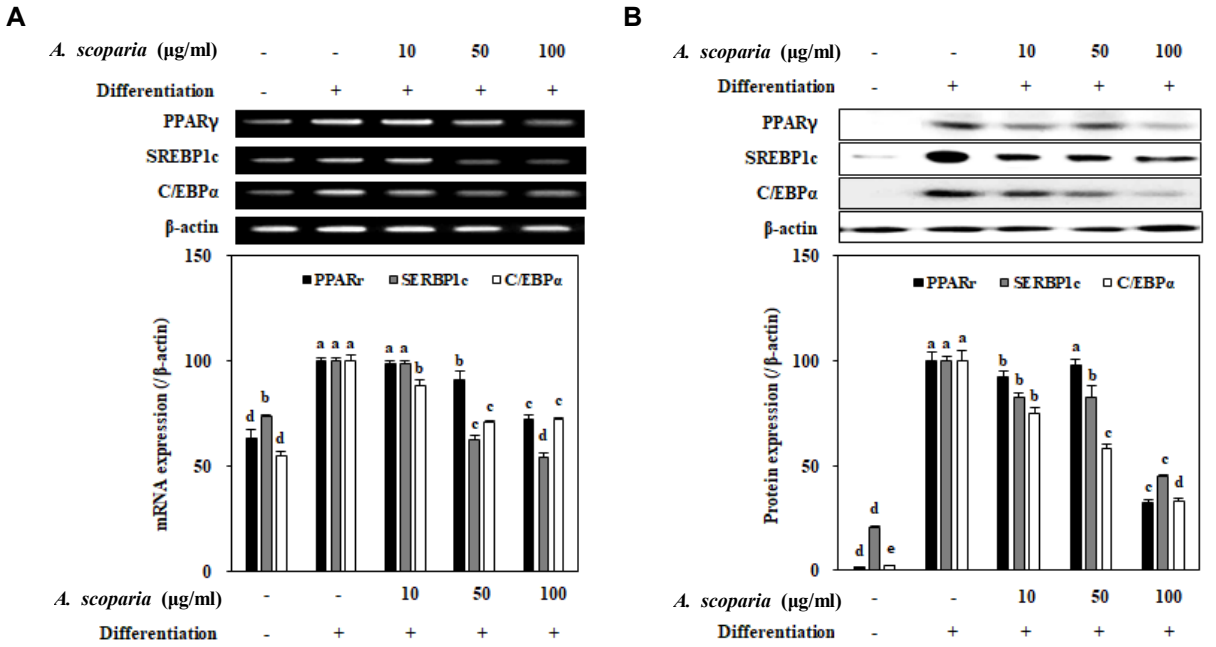

Fig. 3. Effect of *A. scoparia* crude extract on the expression of key adipogenesis transcription factors; PPARγ, SREBP-1c and C/EBPα: (A) Effect of the sample on the mRNA levels of the transcription factors was analyzed by RT-PCR. Regulation of the mRNA levels was quantified by the density of the bands and normalized against the housekeeping gene β-actin. Effect of the sample on mRNA levels was given as the percentage of the fully differentiated untreated control cells; (B) Effect of the sample on the protein levels of the transcription factors was analyzed by Western blotting. The protein levels was quantified by the density of the bands and normalized against the housekeeping protein β-actin. Effect of sample on protein levels was given as the percentage of the fully differentiated untreated control cells. Values are means ± SD (n=3). $^{a-e}$Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

were confirmed that ASE possessed ability to intervene with the PPARγ pathways and lowered the expression of associated crucial proteins.

Various transcription factors like PPARγ and C/EBP family are involved in MAPK pathways during adipocyte differentiation [2]. In order to provide insights towards the action mechanism during inhibitory effect of AES, activation levels of other crucial MAPK cascade proteins, namely p38, ERK and JNK, were observed. Effect of AES treatment (1, 5 and 10 μg/mL) on the phosphorylated (p-) p38, ERK and JNK levels were evaluated by protein levels through Western blotting. In fully differentiated mature adipocytes these protein levels were increased significantly while AES treatment suppressed the levels substantially in a dose-dependent manner (Fig. 4). Adipogenesis of 3T3-L1 cells resulted in elevated levels of activated p38, ERK and JNK compared to non-differentiated blank cells. Treatment with AES lowered the levels of phosphorylated MAPK proteins to 46.4, 36.3 and 65.6% of the untreated fully differentiated control cells for p38, ERK and JNK, respectively.

Natural products, especially the plants from traditional medicine references are gaining much interest in alternative and complementary medicine studies due to their various benefits such as few side effects and high biocompatibility. In this context, *A. scoparia* is a widely distributed plant that has been reported to possess several bioactivities including but not limited to antimicrobial, anti-inflammatory, antioxidant and antihepatotoxic [7, 22, 28]. Current results indicated that *A. scoparia* suggestively disrupts the differentiation of pre-adipocytes into adipocytes. For prevention and treatment of obesity, hindering the expansion ability of the adipose tissue by means of stopping formation of new adipocytes is a viable target. Several PPARγ ligands that act as adipogenesis suppressors are on the market for their beneficial effects against obesity [21]. Effect of *A. scoparia* on adipogenesis in 3T3-L1 adipocytes indicated a promising chemical composition that may yield substances that can be used natural products against obesity. Previous phytochemical investigations of *A. scoparia* resulted in the isolation of numerous chemical compounds such as coumarins, essential oils and flavonoids where all of them were previously reported adipogenesis inhibitory substances from different sources [9,

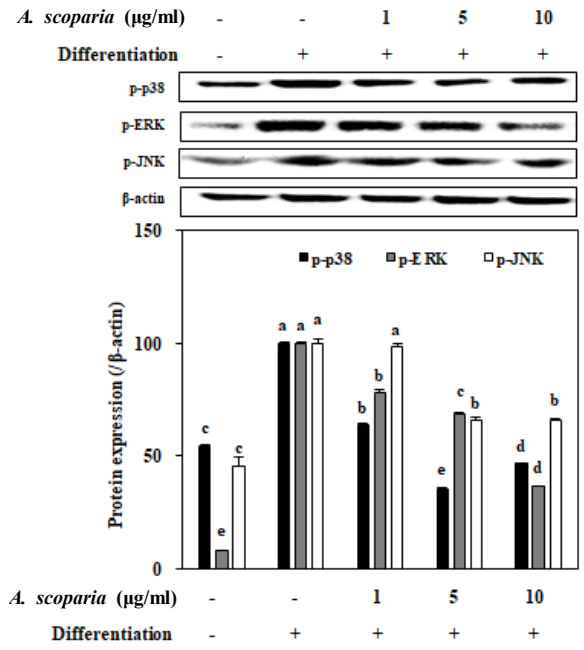

Fig. 4. Effect of *A. scoparia* crude extract on the protein levels of the phosphorylated (p-) MAPK pathway proteins p38, ERK and JNK analyzed by Western blotting. The protein levels was quantified by the density of the bands and normalized against the housekeeping protein β-actin. Effect of sample on protein levels was given as the percentage of the fully differentiated untreated control cells. Values are means ± SD (n=3). [a-e]Means with the different letters are significantly different (p<0.05) by Duncan's multiple range test.

13].

Effect of *A. scoparia* was suggested to be raised from its content of flavonoids and essential oils, as current results were also exhibited an inhibitory effect on the MAPK pathway during adipogenesis similar to that of flavonoids and essential oils, observed by suppressed levels of PPARγ, SREBP1c and C/EBPα along with activated levels of key MAPK proteins p38, ERK and JNK. A study by Cha et al. [3] reported that *A. scoparia* and *A. capillaris* were rich in essential oils. Study showed that these species dominantly contain camphor (11.0%), 1,8-cineole (21.5%), and β-caryophyllene (6.8%) as the major essential oils Among them, β-caryophyllene [16] and camphor [29] were suggested to inhibit adipogenesis via similar pathway suggested in this study. Another study by Xie et al. [32] investigated the chemical composition of *A. scoparia* and isolated 7-methoxycoumarine, isosabandin, 6,7-dimethylesculetin, 7-methylesculetin, scopoletin, capillarisin, chlorogenic acid butyl ester, isoscopoletin-β-D-glucoside and β-sitosterol. Yang et al. [33] and Shin et al. [27] reported adipogenesis inhibitory effects of similar compounds from different sources in 3T3-L1 cells. Using *A. scoparia*, Nam et al. [20] isolated and characterized 3,5-dicaffeoyl-epi-quinic acid as an active compound against inflammation. Kim et al. [12] showed that same compound isolated from *Ainsliaea acerifolia* exerted a strong anti-obesity effect in 3T3-L1 cells. In this context, the anti-adipogenesis effect of *A. scoparia* extracts in current study may be credited to its chemical composition of bioactive essential oils and derivatives of coumarines and flavonoids. Therefore, further studies to elucidate the possible active constituents of *A. scoparia* are urged. Nevertheless, the results presented here showed that *A. scoparia* was a potential source of natural products that may be used to reduce obesity by preventing the forming of adipocytes and hence, reducing the fat accumulation.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1A2B4009588).

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초록 : 비쑥 추출물이 3T3-L1 지방세포 분화 및 MAPK 신호 전달 경로에 미치는 영향

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비쑥(*Atermisia scoparia*)은 국화과에 속하는 한해살이 풀로서 유라시아 지역 분포하며, 염생습지에 자생하는 염생식물의 일종이다. 비쑥은 민간요법에서 이뇨제, 소염제, 간염치료제로 사용되어 왔으며, 비쑥에서 분리한 플라보노이드, 쿠마린 화합물의 항산화, 항염증 등의 생리활성이 보고되어 있다. 본 연구에서는 비쑥 추출물이 3T3-L1 지방전구세포 모델에서 지방세포 내에서의 중성지방 생성 및 지방세포 분화조절 인자 발현에 미치는 영향을 검토하였다. 지방전구세포 3T3-L1을 지방세포로 분화하여 Oil Red O 염색법으로 지방세포 분화 정도를 측정한 결과, 비쑥 추출물 처리군에서 농도 의존적으로 지방세포 형성이 억제되었다. 또한 지방세포 분화 관련 인자인 PPARγ, C/EBPα, SREBP-1c의 발현을 mRNA 및 단백질 수준에서 확인한 결과, 비쑥 추출물을 처리한 군에서 지방세포 분화 인자 발현이 감소하는 것으로 나타났다. 지방세포 분화 및 증식에 관여하는 것으로 알려져 있는 MAPK 신호전달 경로를 확인한 결과 비쑥 추출물을 처리한 군에서 p38, ERK, JNK의 인산화가 억제되었다. 이를 통해 비쑥 추출물은 MAPK 신호전달 경로를 통한 지방세포 분화 인자 조절을 통해 지방 생성과 합성을 억제하는 것으로 사료된다. 따라서 본 연구 결과로부터 비쑥 추출물의 MAPK 신호전달 경로 억제를 통한 항비만 효과를 확인하였으며, 나아가 건강 기능성 식품 소재로서의 개발 가능성이 기대된다.