Thesis for the Degree of Master of Science

Effect of *Sargassum fulvellum* extract on the lipogenesis of 3T3-L1 cells

by

Jung Min Park

Department of Food and Life Science

The Graduate School

Pukyong National University

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참모자반이 3T3-L1 지방세포의 lipogenesis 에 미치는 영향

Advisor: Prof. Hyeung-Rak Kim

By

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Approved by:

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(Chairman)

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(Member) (Member)

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박 정 민

부 경 대 학 교 대 학 원 식 품 생 명 과 학 과

요 약

참모자반 (*Sargassum Fulvellum*)은 갈조류의 일종으로 한국과 일본에서 식용으로 널리 사용되고 있다. 참모자반으로부터 다양한 생리활성 물질들이 분리되어, 이들의 항알레르기 또는 항산화 활성에 관한 연구가 많이 있지만 비만에 관련된 연구는 거의 없다. 참모자반 에탄올 추출물(*S. fulvellum* ethanol extract, SEE)과 헥산 획분(*S. fulvellum* hexane fraction, SHF)을 전구지방세포 3T3-L1 에 처리한 결과 에탄올 추출물은 400 μg/ml, 헥산 획분은 100 μg/ml 농도까지 세포독성을 나타내지 않았다. 3T3-L1 cell 이 confluent 된 이틀 후, 5 일 동안 3-isobutyl-1- methlylxanthine, dexamethasone 및 insulin (MDI)을 처리하여 세포 분화를 유도한 뒤 8 일 동안 SEE 와 SHF 를 MDI 와 함께 처리하여 Oil Red O 염색을 한 결과 지방 방울의 크기와 축적의 정도가 감소했음을 확인했다. mRNA 수준에서 SEE 또는 SHF 처리에 의하여 지방세포의 분화에 영향을 미치는 전사인자인 CCAAT/enhancer-binding protein α (C/EBPα), peroxisome proliferators activated receptor γ (PPARγ)의 급격한 발현억제가 나타났다. Sterol regulatory element binding protein-1c (SREBP-1c) 또한 mRNA 수준

SEE 와 SHF 에 의해 발현이 억제되었으며, SREBP-1C 의 downstream 에 존재하는 fatty acid synthase (FAS), acetyl-CoA carboxylaseα (ACCα) 및 stearoyl-CoA desaturase-1 (SCD-1)의 발현 또한 억제되었다. 단백질 발현 수준에서도 지방세포 분화와 지방합성에 관련된 전사인자인 PPARγ, C/EBPα, SREBP-1C 의 합성이 억제되었으며, 이들 전사인자들의 조절을 받는 FAS, ACCα 발현억제가 나타났다. 따라서 SEE 와 SHF 의 비만억제 효과는 지방세포분화와 지방합성에 관련된 PPARγ, C/EBPα 및 SREBP1C 를 통한 전사인자의 발현억제와 이들 전사인자의 downstream 에 존재하는 FAS, ACCα 및 SCD-1 의 발현억제로 인한 것으로 판단된다. 본 결과는 SEE 와 SHF 가 MDI 로 분화된 3T3-L1 세포의 지방 합성 과정을 억제할 수 있는 기능성 식품재료로 이용될 수 있음을 시사한다.

1.Introduction

Obesity is increasing rapidly in the developed as well as developing countries, not only in adults but also in children(Smith *et al*., 2010). The World Health Organization has registered obesity as one of the top ten global health problems; some consider it the most dangerous disease in the world today(Evans *et al*., 2004). The health risk of obesity is largely a consequence of the morbidities of diabetes, insulin resistance, hypertension, heart disease, atherosclerosis and cancer(Friedman & Friedman, 1998). Obesity is the result of imbalance between energy intake and expenditure, and is characterized at the cell level by enhanced intracellular lipid accumulation often accompanied by increased number of adipocytes(Gupta & Abu-Ghannam, 2011).

Many mammals have also white adipose tissue (WAT) and brown adiposetissue (BAT). Those are functionally different types of adipose tissue: WAT is the major energy reserve in higher eukaryotes, and storing triacylglycerol in period of energy excess and its mobilization during energy deprivation is its primary purposes(Gregoire *et al*., 1998; Hagen, 1990). WAT development takes place after birth and its mass considerably increases during post-natal life, particularly after the animal has absorbed, to reach a great percentage of body mass in animals or humans(Moulin *et a*l., 2001). WAT is characterized by one large lipid inclusion and very few mitochondria(Néchad, 1986). BAT can be distin-

guished from WAT by multilocular lipid inclusions, numerous and developed mitochodria(Nedergaard *et al*., 1986). BAT develops during fetal life and possesses all the features of mature tissues at birth when the requirements for non-shivering thermogenesis are great than at any other time of life(Nedergaard *et al*., 1986). BAT mitochondria are equipped with a unique protein, called uncoupling protein (UCP) which is a proton translocator in the inner mitochondrial membrane and function as an uncouple of the mitochondrial respiratory chain(Nicholls *et al*., 1986; Ricquier *et a*l., 1991; Klaus *et a*l., 1991). In WAT, preadipocytes exist in close proximity to adipocytes and res-pond to positive energy balance by proliferating and differentiating into adipocytes(Kirkland *et al*.,1994; Wasserman, 1965). In addition to storing and mobilizing neutral lipids in response to various hormones, adipocytes are endowed with intracrine, autocrine/paracrine and endocrine properties(Trayhurn *et al*., 1999). Adipocytes secrete factors that play a central role in the regulation of energy balance, insulin sensitivity, immunological responses, and vascular diseases(Kim & Moustaid-Moussa, 2000; Morrison *et al*., 2000).

These transcription factors are induced early stage of adipocyte differentiation. Member of the C/EBP family are involved in C-terminal basic region-leucine zipper domain that consists of a basic region and C/EBP bZIP region binds to numerous adipose-specific genes when adipogenesis is in initiated(Christy *et al*., 1989; Wedel and Loms Ziegler-Heitbrock, 1995). C/EBP β and C/EBPδ, expressed early stage of differentiation. Regulated expression is

seen for several C/EBP family members during adipogenesis. C/EBPα, C/EBPβ and C/EBP are induced at different time in the differentiation. On the other hand, C/EBPζ is suppressed during the induction of diffeentiation(Darlington *et al*., 1998). Production of the appropriate ligand of PPARγ2 by the differentiating preadipocytes to adipocytes is likely a limiting step in this transcription cascade. C/EBPα and PPARγ appear to serve as pleiotropic transcriptional activators that coordinately induce expression of large group of adipocyte genes including the SCD1, fatty acid synthase (FAS), in addition to its role in the differentiation of adipocytes(Yeh *et al*., 1995; Cristy *et al*., 1989). C/EBPs, PPARγ2, ADD/SREBP- 1c, and several other transcription factors(Reusch *et al*., 2000; Tong *et al*., 2000).

Peroxisome proliferators-activated receptors (PPARs) are type II nuclear hormone receptor family. PPARs regulate transcription through binding of PPAR retinoid X receptor (RXR) heterodimers to a response element consisting of a direct repeat of the nuclear receptor hexameric DNA recognition motif (PuGGTCA) spaced by one nucleotide(Juge-aubry *et al*., 1995; Tontonoz *et al*., 1994). Three PPAR genes PPARα, PPARδ, and PPARγ encode different members of this family of orphan receptors. The three PPAR family members have distinct functions of tissue distribution, and like typical siblings. PPARα is expressed in metabolically active tissue including liver, kidney, heart, skeletal muscle, and brown fat(Auboeuf *et al*., 1997; Evans *et al*., 2004; Braissant *et al*., 1995). PPARα ligands induced fatty acid β-oxidaiton, and derived from

acachidonic acid via either the lipoxygenaase or cyclooxygenase. Also PPARα activity is enhanced by protein kinase A dependent phosphorylation. PPARβ/δ is abundantly expressed in brain, adipose tissue, and skin. Role for PPARβ/δ has been proposed for embryo implantation, skin proliferation and differentiation, pre-adipocyte proliferation, and modulator of PPARα and PPARγ activity(Hansen *et al*., 2001; Jow & Mukherjee, 1995; Lim *et al*., 1999; Michalik *et al*., 2001; Peters *et al*., 2000; Shi *et al*., 2002; Tan *et al*., 2001). Three different PPARγ mRNA have been characterized. PPARγ1 and PPARγ2 are predominantly present in WAT, PPARγ3 has been reported in the intestine and macrophages(Fransis *et al*., 2003). PPARγ is a master regulator in the formation of fat cells. PPARγ is induced during aidpocyte differentiation, and forced expression of PPARγ in nonadipogenic cells effectively converts them into mature aidpocytes(Tontonoz *et al*., 1994). PPARγ is the ultimate effector of lipogenesis in transcriptional cascade, such as aP2 (fatty acid binding protein), CD36 (receptor for lipoproteins), lipoprotein lipase, FATP-1 (fatty acid transporter), glycerol kinase, SREBP-1c, and SCD-1. PPARγ also induces the expression of genes involved in insulin signaling in both adipose tissue and muscle. Activation of PPARγ attenuates TNF-α(tumor-necrosis factor-α), resistin and adiponectin, which by virtue of serum transport have far-reaching metabolic effect. PPARγ agonists inhibit the expression of TNF-and resistin, which both promote insulin resistance(Combs *et al*., 2001; Berg *et al*., 2001; Yamauchi *et al*., 2001).

Sterol regulatory element-binding proteins (SREBPs) of fat cell-specific genes are helix-loop helix transcription factors which are consist of SREBP-1a, 1c and 2, and regulate genes involved in production and absorption of cholesterol, fatty acid, TG, phospholipid and LDL receptor(Brown *et al.*, 1997; Spiegelman *et al.*, 1998). SREBPs are made as inactive precursors bound to the endoplasmic reticulum (ER) membranes. When it is activated, the precursors have cleavage process to enter the NH_2 -terminal active domain in the nucleus(Shimomura *et al.,* 1998). SREBP-1 activates the fatty acid, triglyceride, and phospholipids metabolism, while SREBP-2 is the isoform supporting cholesterol synthesis(Horton and Simomura, 1998). SREBP-1a is the predominant transcript in growing cells, SREBP-1c transcript predominates in most organs including liver and adipose tissue(Shimomura *et al.,* 1997). The rat SREBP-1 mRNA was induced during adipocyte differentiation in cell culture, so it was named the adipocyte determination and differentiation dependent factor 1 (ADD1). These observations suggested that ADD1/ SREBP-1c is a regulator of genes that are important for lipid accumulation in the adipocyte(Bennett *et al.,* 1995). SREBP-1c, a helix-loop-helix type transcription factor, activates genes of fatty acid synthesis including fatty acid synthase (FAS), stearoyl CoA desaturase-1 (SCD-1), and acetyl CoA carboxylase (ACC)(Lopez *et al.,* 1996; Schultz *et al.,* 2000).

Acetyl-CoA carboxylase (ACC) catalyses the carboxylation of acetyl-CoA to form malonyl-CoA and plays a important role in the regulation of fatty

acid metabolism and a key regulatory molecule in muscle, brain and other tisuues. ACC in dividied into two isoform, ACCα and ACCβ, which play distinct roles in the control of fatty acid synthsis and oxidation(Munday, 2002; Brownsey *et al.,* 2006). The activity of ACC is regulated at transcriptional level and by allosteric activation of citrate, the phosphorylation of multiple serine residues and interactions with other proteins(Brownsey et al., 2006; Tong, 2005).

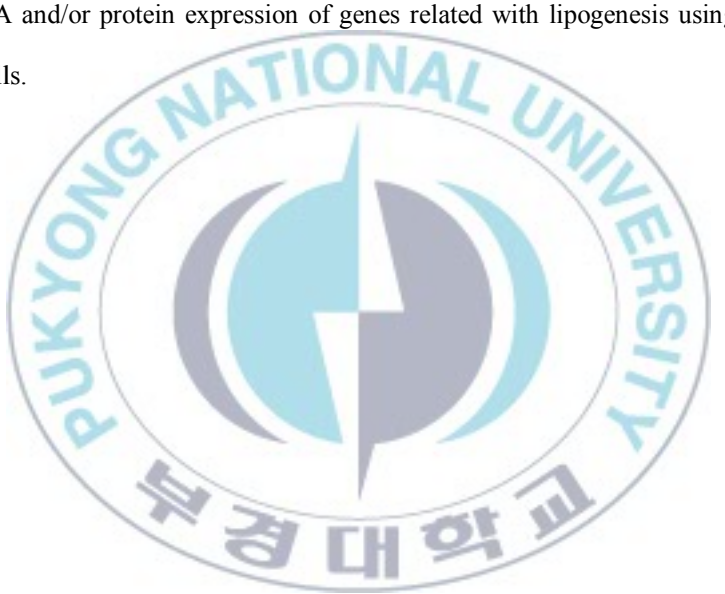
Stearoyl-CoA desaturase (SCD) catalyzeds the biosynthesis of monounsaturated fatty acid, oleate and palmitoleate, are the major fatty cids existed in triglycerides, cholesteryl esters, and phospholipids. A high carbohydrate diet inducing lipogenesis causes increase of gene exression involved in lipogenesis by sterol regulatory element binding protein-1 (SREBP-1C)-med -iated gene transcription, which induces an increase in the synthesis of triglycerides.

Fatty acid synthase (FAS), a single, homodimeric, multifunctional protein, is liopogenic enzyme that catalyzes the entire pathway of palmitate synthesis from malonyl-CoA in mammal(Smith et al., 2003). The activity of FAS is controlled by transcription level in response to diet, glucose, thyroxine and SREBBP-1C(Kim et al., 1998). The concentration of FAS play important role in deciding the maximal capacity of a tissue to synthesize fatty acids. Most tissues including adipose, liver, lung, brain, kidney, and small intestine have transcription process of FAS, mRNA. In addition, the amount of FAS mRNA in a tissue determines the rate of FAS protein synthesis, and ultimately the tissue content of FAS protein(Clarke, 1993).

Many of the advances in studies on adipogenesis are based on studies in murine 3T3-L1 cells(Green and Meuth, 1974). This was clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19-day mouse embryos(Green and Kehinde, 1976; Green and Meuth, 1974). 3T3-L1 cells resemble fibroblast. When induced by dexamethasone, 3-isobutyl-1-methyl- xanthin, insulin (MDI) a significant proportion of preadipocytes were differentiated without mitotic clonal expansion(Lee, 2006). After stimulation with MDI, transcription factors involved in adipogenesis and lipogenesis are regulated by several transcription factors, including CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferators -activated receptors (PPAR) and sterol regulatory element binding proteins (SREBPs) (Rosen *et al*., 2000; Wu *et al*., 1999).

Brown seaweeds have been used as important culinary items for centuries in Far Eastern countries. Due to richness of polysaccharides, minerals, and polyunsaturated fatty acids, these are known to be a good source of healthy food. *Sargassum fulvellum* is used as a favorite culinary item in Korea. A variety of biological compounds such as phlorotannins and fucoxanthin were isolated from *Sargassum sp.* and have shown anti-allgic and antioxidant activities(Kim *et al.*, 2009; Jung *et al.*, 2009). Kang et al. reported that solvent extracts from *S. fulvellum* has antipyretic, analgesic, and anti-inflammatory activities in mice(Kang et al., 2008). As part of our ongoing research to detect biological activities from marine brown algae, we found anti-lipogenic activity in the ethanol fraction of *S. fulvellum* in 3T3-L1 adipocyte. Our objection is to investigate the anti-obesity

effect of *S. fulvellum* and possible mechanisms by analyzing the expression the expression the expression level of proteins and genes related on obesity. Hence, substances which inhibit the productions of these obesity transcription factors are considered as potential anti-lipogenesis agents. For the development of functional foods for the prevention and treatment of obesity, this study performed the inhibitory effect of SEE and SHF on the accumulation of triglyceride and mRNA and/or protein expression of genes related with lipogenesis using 3T3-L1 cells.



2. Materials and methods

2-1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin mixture, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and fetal bovine serum (FBS) and bovine serum albumin(BSA) were purchased from WelGENE (Daegu-si, Korea), TRIzol reagent was puchsed Ambion (Life Technlogies, CA, USA). CellTiter$^{96®}$ AQ_{ueous} One Solution Cell Proliferation assay kit was purchased from Promega (Madison, WI), dimethyl sulfoxide (DMSO), dexamethasone, 3-isobutyl-1-methylxanthine and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemiluminescence (ECL) detection kit was purchased GE Healthcare Bio-Science (Piscataway , NJ, USA). Culture dish, 6-well plate, 96 well plate, 50 ml tube, 15 ml tube and cell scrapper were purchased SPL, Western blot primary antibody and secondary antibody were purchased Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology Inc. (Danvers, MA, USA).

2-2. Preparation of *S. fulvellum*

S. fulvellum was collected from the coast of Busan, South Korea, in April 2010. Taxonomic identification was confirmed by an agal taxonomist (C.K Choi), at the Department of Ecological Engineering, Pukyong National University, Korea. The seaweed was rinsed in tap water to remove salt, and air-dried under sunshine

for 3 days. Dried powder (1.5 kg) of *S. fulvellum* was extracted three times with 96% (v/v) ethanol (EtOH) for 3 h at 70℃. The combined extracts were concentrated under reduced pressure to obtain the EtOH extract (215.6 g). For further fractionation of the EtOH extract, the extract was resuspended in water:EtOH (9:1, v/v) and partitioned successively with n-hexane, dichloromethane, ethyl acetate, and n-butanol (Fig.1).

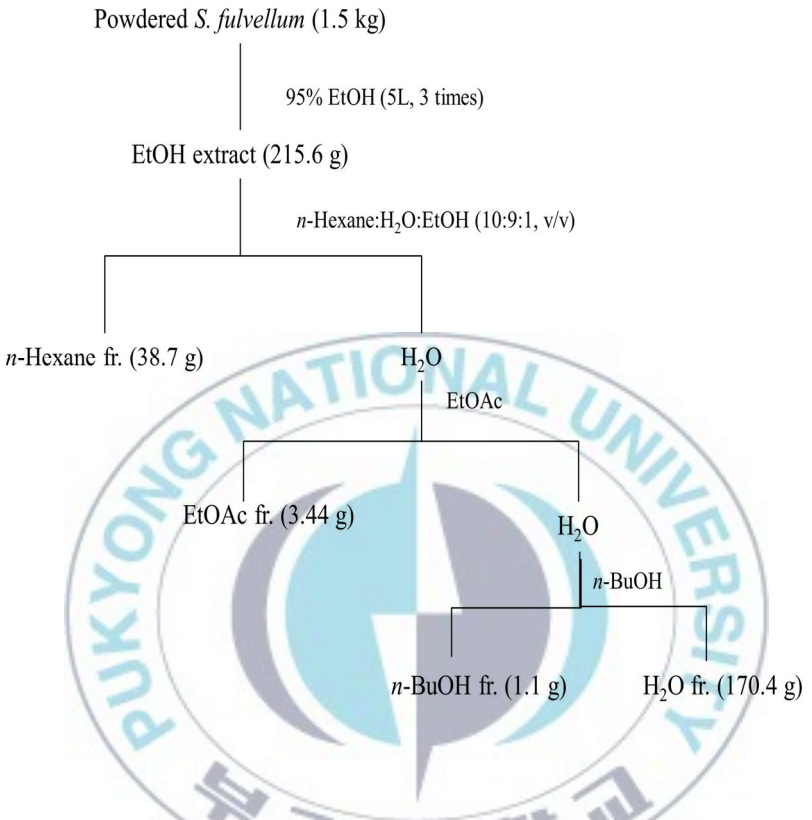


Fig. 1. Extraction and fractionation of *Sargassum fulvellum*

2-3. Methods

2-3-1. Cell culture and treatment

Murine 3T3-L1 preadipocytes (ATCC, Rockville, MD) were cultured at 37℃ in DMEM supplemented with 10% BSA, penicillin (100 units/ml), and streptomycin sulfate (100 ug/ml). After 2-day postconfluence, 3T3-L1 preadipocytes were induced to differentiate by the addition of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 10 μg/ml insulin (MDI). On day 3, the MDI medium was replaced with DMEM containing 10% FBS and 10 μg/ml insulin, which was changed at every 2 days until analysis. On day 5, cells were cultured in the absence or in the presence of the ethanol fraction of *S. fulvellum* (SEE) and the hexane fraction of *S. fulvellum* (SHF) concentrations. During the period of SEE and SHF exposure, the medium was changed every 2 day for control and treated groups. On 13 day, cells were harvested. SEE and SHF were reconstituted as 100 mg/ml stock solutions in DMSO and stored at –20℃.

2-3-2. Cytotoxicity assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy- pheny-l)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using a CellTiter96® AQueous One Solution Cell Proliferation assay kit according to the manufacturer's instruction. Briefly, preconfluent 3T3-L1 preadipocytes were inoculated at a density of 1.5×10^3 cells/well into 96-well plate in DMEM

with 10% FBS and cultured at 37℃ for 24 h. The culture medium was replaced with 200 μl of serial dilutions of SEE and SHF, and the cells incubated for 24 h. The medium was replaced with 95 μl fresh medium and 5 μl MTS solution. After 1 h, the absorbance at 490 nm was measured using a microplate reader (Glomax Multi Detection System, Promega, USA).

2-3-3. Oil red O staining

The lipids accumulated in the adipocytes were quantified after staining the 3T3-L1 cells with oil red O staining. Briefly, after differentiation the cells were fixed with 10% formalin for 1 hour and washed with 60% isopropanol. After air drying the plates were stained with oil red O working solution (6 parts of 0.35% stock Oil red O and 4 parts of distilled water) for 10 min and rinsed with water. Images were obtained using an Olympus CX40 microscope (Center Valley, PA, USA) at 40 × magnification. To quantify the amount lipid contents, the oil red O stain was eluted by adding 100% iso-propanol for 10 min, and the absorbance was measured using microplate reader (Glomax Multi Detection System, Promega, USA) at 500 nm.

2-3-4. RNA isolation

3T3-L1 cells were washed with cold PBS and the harvested cells were lysed with TRIzol reagent in microcentrifuge tubes. Tubes were added add 0.2 ml of chloroform per 1 ml of trizol reagent and then were shaken vigorously for 15s.

Mixture was centrifuged at no more than 12,000 × g for 20 min at 2 to 8℃. The aqueous phase was transferred 4℃ to a fresh tube and the RNA was precipitated by centrifugation at no more than 12,000 × g for 10 min at 2 to 8℃ with isopropyl alcohol. 0.5 ml of isopropyl alcohol was used to 1 ml TRIzol reagent. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by shaking. After centrifugation at 7,500 × g for 5 min at 2 to 8℃, the RNA pellet was briefly dried and dissolved into 0.1% DEPC treated water. The concentration of RNA was determined by measuring the UV absorbance at 260 and 280 nm, and the RNA was stored at -70℃ until RT-PCR analysis.

2-3-5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Three micrograms of RNA were reverse-transcribed using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA), oligodT-15 primer, deoxynucleo tide triphosphate (10 Mm) in a final volume of 25 μl. RT reactions were performed at 42℃ for 50 min, and 95℃ for 5 min in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems). cDNA was amplified by 5x PCR master Mix (Elpisbiotech, Korea) 4 μl., 10 pmole forward primer and reverse primer each other 1 μl., autoclaved D.W. in a final volume of 20 μl, performing 27 cycle of the following protocol: 30 s denaturation at 94°C, 30 s annealing at 59°C, followed by 45 s extension at 72° C. CDNA was amplified by PCR with following primers:

PPARγ forward, 5'-CTG ATG CAC TGC CTA TGA GCA C-3'

PPARγ reverse, 5'-GCC TGA TGC TTT ATC CCC ACA G-3'

SREBP-1c forward, 5'-GCG GGA CAG CTT AGC CTC TA-3'

SREBP-1c reverse, 5'-GTA GTG CCT CCT TTG CCA CTG-3'

C/EBPα forward, 5'-TGA TCA AAC AAG AGC CCC GC-3'

C/EBPα reverse, 5'-TAC CCG GTA CTC GTT GCT GT-3'

FAS forward, 5'-CAC ACA CAA TGG ACC CCC AG-3'

FAS reverse, 5'-CAG AGG TGT TCG GCT TCA GG-3'

SCD-1 forward, 5'-CCT TCT TGC GAT ACA CTC TGG TG-3'

SCD-1 reverse, 5'-TCA GCT ACT CTT GTG ACT CCC G-3'

ACCα forward, 5'-AGC ATG TCT GGC TTG CAC C-3'

ACCα reverse, 5'-CCC ACA CTG CTT GTA CAG GTA TC-3'

GAPDH forward, 5'-TGG CAC AGT CAA GGC TGA GA-3'

GAPDH reverse, 5'-CTT CTG AGT GGC AGT GAT GG-3'

2-3-6. Western immunoblot analysis

3T3-L1 cells were washed twice with ice-cold PBS, scraped with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet-40, 1% Tween-20, 0.1% SDS, 1 mM Na3VO4, 10 μg/ml leupeptin, 50 mM NaF, 1 mM PMSF) and stood on ice for 30 min for lysis. After centrifuge at 14240 g for 20 min, supernatant was collected and used as cellular protein×. Aliquots of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris-HCl, pH

8.0, 150 Mm NaCl, 0.06% Tween-20) for 1 hr and incubated for overnight with primary antibody in TBST buffer. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer for 1hr, and immune complex was detected using ECL detection kit.

2-3-8. Statistical analysis

Data are expressed as the mean ± SDs. Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair of Student's t-test for multiple comparisons. Differences were considered significant if $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL).

3. Results

3.1 Cytotoxicity of SEE and SHF on 3T3-L1 cells

To examine cytotoxicity of SEE and SHF on 3T3-L1 preadipocyte, 3T3-L1 cells were cultured to 70-80% in 96 well, and idifferent concentrations of concentrations SEE and SHF were added and then further cultured for 24 hr. Cell viability was determined by MTS assay. Fig. 2 showed that 3T3-L1 preadipocytes were not affected from 10 to 400 μg/ml SEE concentration. Also, SHF was not affected from 10 to 100 μg/ml. These results suggest that SEE and SHF are non-cytotoxic effect on 3T3-L1 preadipocytes (Fig. 3).

3.2 Effect of SEE on lipid accumulation during differentiation.

Mature adipocytes accuculate TG from either circulating lipids or de novo lipogenesis(Darimont et al., 2006). To examine dose-dependent effect of SEE and SHF on lipid accumulation during differentiation of 3T3-L1 cells, the lipid contents in the cells were determined. To quantify the amount lipid contents, the oil red O stain was eluted by adding 100% isopropanol , the absorbance was measured using microplate reader at 500 nm. Fig. 4 and fig. 5 show dose-dependent inhibitory effect of SEE on lipid accumulation of 3T3-L1 cells. Also, fig. 6 and fig. 7 show dose-dependent inhibitory effect of SHF on lipid accumulation of 3T3-L1 cells. Kong et al. reported 1-(3′,5′-dihydroxyphenoxy)-7-

(2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin from *Ecklonia cava* was significantly reduced in the presence of compound in a concentration-dependent manner Oil-Red O staining. Lipid accumulation in cells was concentration dependently inhibited in the presence of compound(Kong et al., 2010). Also, Okada et al. reported neoxanthin during the adipocyte differentiation caused significant reduction of intracellular lipid accumulation(Okada et al., 2008). And Park et al. reported fucoidan showed anti-adipogenic properties as evidenced by decreased lipid and triglyceride accumulation ins 3T3-L1 cells(Jeong et al., 2010). Also, our results showed and inhibited the differentiation of adipocytes and cotributed to decrease lipid accumulation in adipocytes. Together, these observations suggest that SEE and SHF inhibit lipogenesis in 3T3-L1 adiocytes.

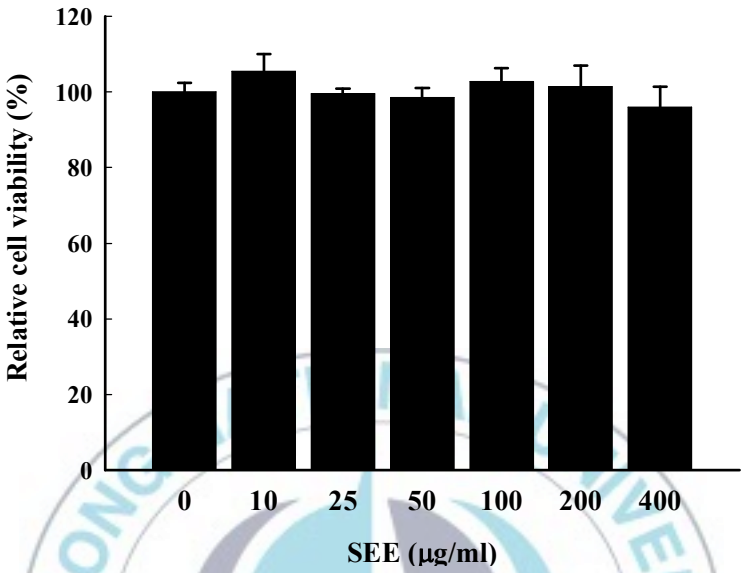


Fig. 2. Cytotoxicity of EtOH extracts of *S. fulvellum* (SEE) in 3T3-L1 preadipocytes. 3T3-L1 cells were treated with 10, 25, 50, 100, 200 and 400 μg/ml of SEE for 24 hrs. Cell viability was determined by MTS assay. Values are the means ± SDs of three independent experiments.

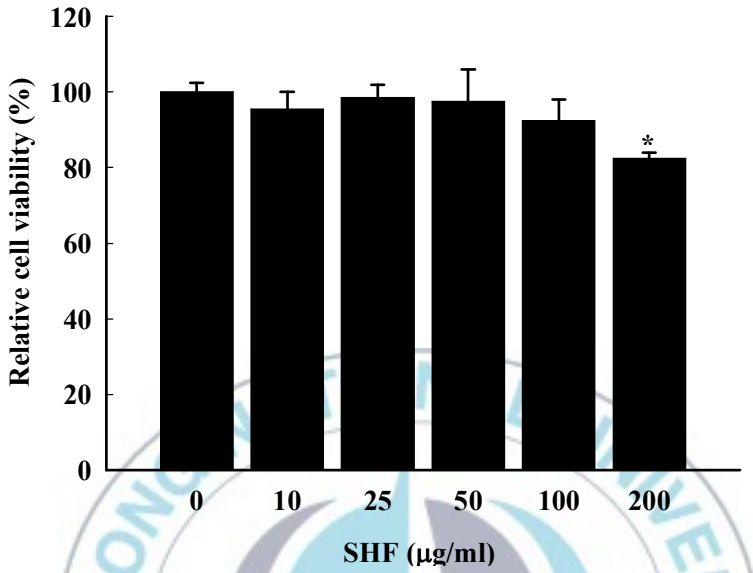


Fig. 3. Cytotoxicity of Hexane extracts of *S. fulvellum* (SHF) in 3T3-L1 preadipocytes. 3T3-L1 cells were treated with 10, 25, 50, 100 and 200 μg/ml of SHF for 24 hrs. Cell viability was determined by MTS assay. Values are the means ± SDs of three independent experiments. *$p<0.05$ indicate significant differences compared to SHF-untreated group.

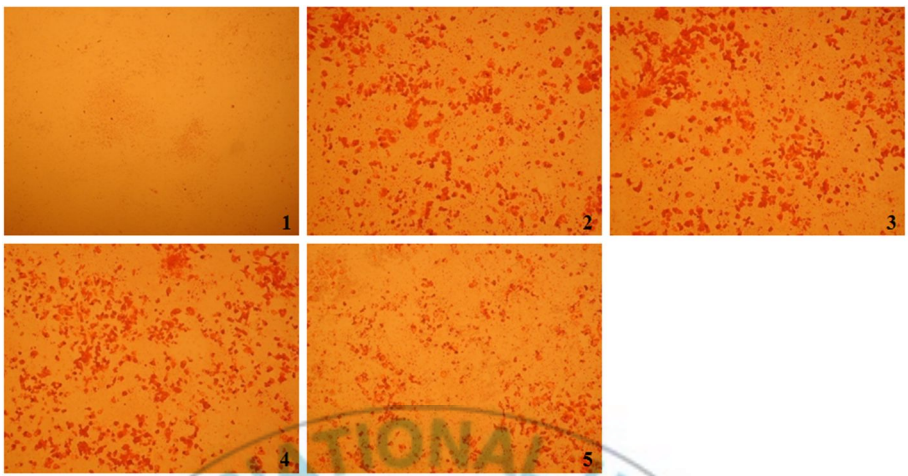


Fig. 4. Microscopic observation of differentiated 3T3-L1 adipocytes stained with Oil Red O. 1, Control (no treatment); 2, MDI (IBMX, dexametathone, insulin); ; 3, MDI + SEE 100 μg/ml; 4, MDI + SEE 200 μg/ml; 5, MDI + SEE 400 μg/ml. ×40

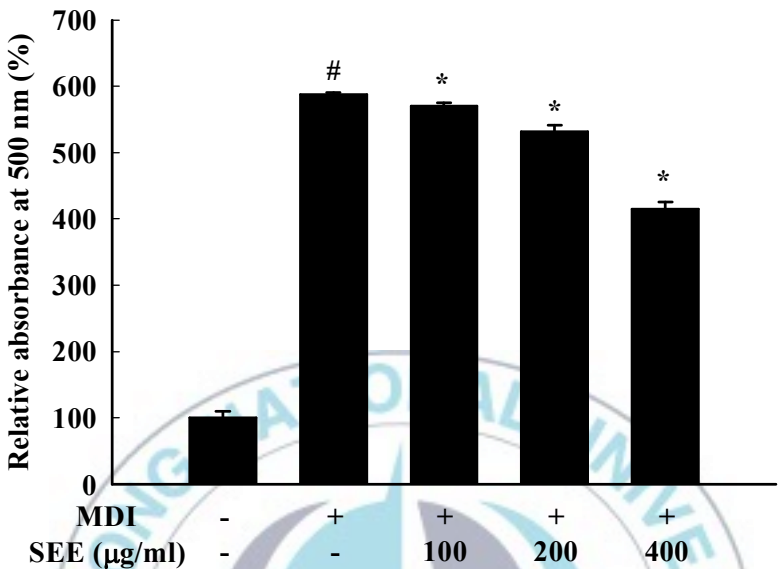


Fig. 5. Effect of SEE on lipid accumulation in 3T3-L1 adipocytes. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SEE. Medium was changed at day 7, 9 and 11, and the cells were stained on day 13. Stained lipid was extracted with 100% isopropanol, and its absorbance was measured at 500 nm. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $^{*}P<0.05$ indicates significant differences compared with MDI-only treated group.

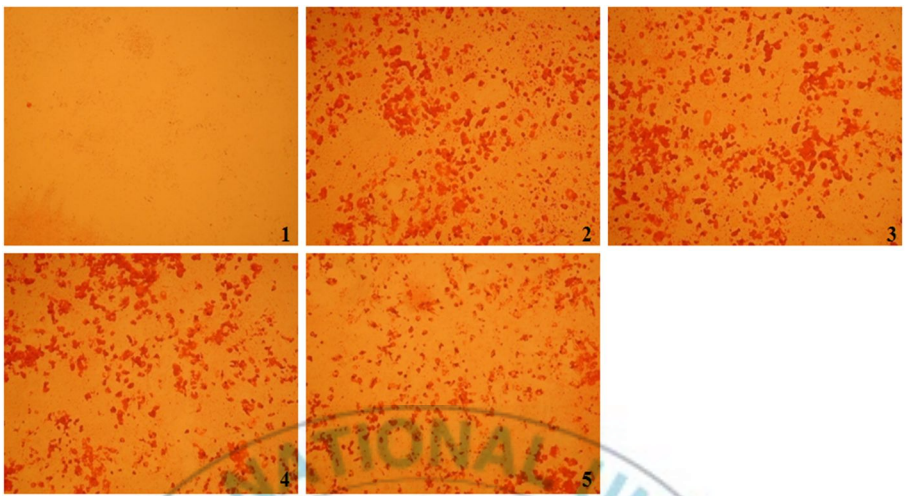


Fig. 6. Microscopic observation of differentiated 3T3-L1 adipocytes stained with Oil Red O. 1, Control (no treatment); 2, MDI (IBMX, dexametathone, insulin); ; 3, MDI + SHF 25 μg/ml; 4, MDI + SHF 50 μg/ml; 5, MDI + SHF 100 μg/ml. ×40

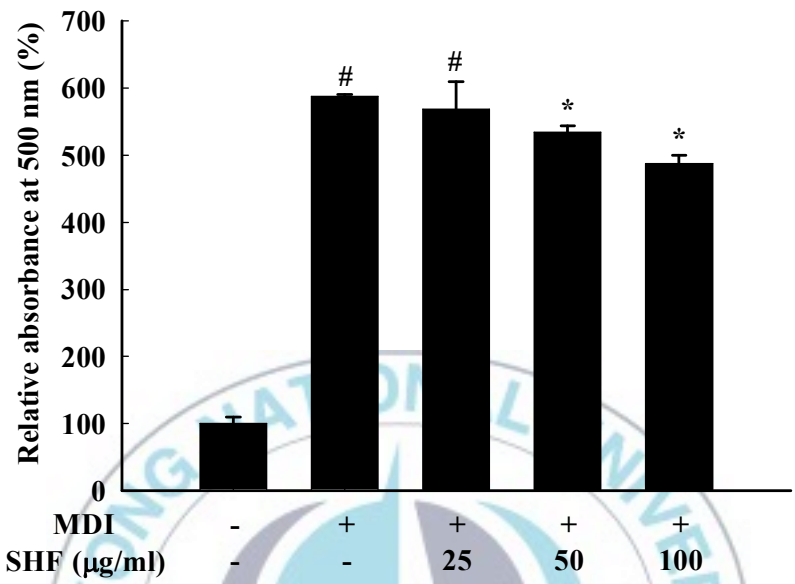


Fig. 7. Effect of SHF on lipid accumulation in 3T3-L1 adipocytes. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SHF. Medium was changed at day 7, 9 and 11, and the cells were stained on day 13. Stained lipid was extracted with 100% isopropanol, and its absorbance was measured at 500 nm. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $*P<0.05$indicates significant differences compared with MDI-only treated group.

3.3 Change of gene expression in 3T3-L1 cells by SEE and SHF.

3-3-1. RT-PCR results.

Fat cells secrete adipocyte specific genes involved in lipogenesis as differentiation is preceded. C/EBPα and PPARγ are known as important regulators of lipogenesis and lipid storage(Sheu et al., 2007). Accordingly, the effect of SEE and SHF on the expression of C/EBPα, PPARγ and down-regulation of the SREBP-1c-dependent lipogenic pathway including SREBP-1C target genes, such as SCD-1, FAS and ACCα, were evaluated by RT-PCR. As a result of Figure 3, differentiated adipocytes had substantially increased levels of the C/EBPα, SREBP-1C, PPARγ, SCD-1, FAS and ACCα compared to preadipocytes. In contrast, figure 3 shows that SEE inhibited C/EBP mRNA expression, which is key transcription factors at an early stage of differentiation, which augments lipid accumulation in fully differentiated adipocytes. Furthermore, SEE(400 μg/ml) effectively inhibited mRNA expression of genes related with SREBP-1c-dependent lipogenic pathway and also inhibited various genes related with lipogenesis.

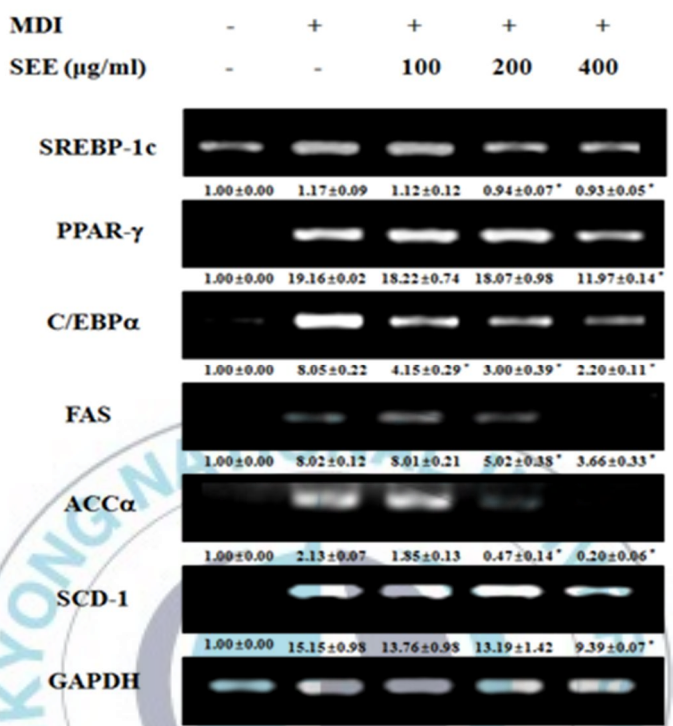


Fig.8 . Effect of SEE on the mRNA expression of PPARγ, SREBP-1c, C/EBPα, FAS, SCD-1 and ACCα in 3T3-L1 cells. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SEE. Medium was changed at day 7, 9 and 11, and the cells were harvested on day 13 for RT-PCR analysis. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $^{*}P<0.05$ indicates significant differences compared with MDI-only treated group.

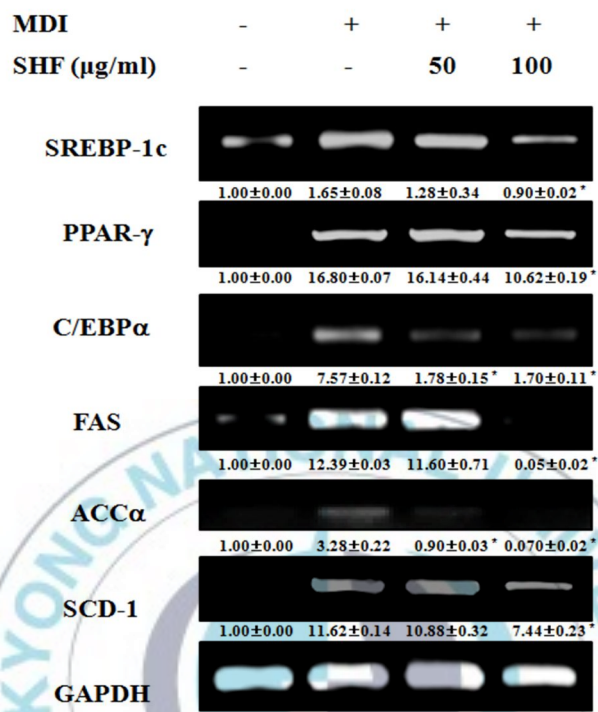


Fig.9 . Effect of SHF on the mRNA expression of PPARγ, SREBP-1c, C/EBPα, FAS, SCD-1 and ACCα in 3T3-L1 cells. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SHF. Medium was changed at day 7, 9 and 11, and the cells were harvested on day 13 for RT-PCR analysis. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $^{*}P<0.05$ indicates significant differences compared with MDI-only treated group.

3-3-2. Western immunoblot results

Since PPARγ and C/EBPα are also key regulators of lipogenesis, we asked whether SEE could also influence the expression of these proteins in differentiated adipocytes. Mature 3T3-L1 adipocytes were treated with SEE and the expression level of PPARγ and C/EBPα was determined by Western blotting. At figure 4, SEE at 400 μg/ml was sufficient to suppress the expression of PPARγ and C/EBPα in these cells. Furthermore, these results demonstrate the inhibitory effect of SEE on the expression of PPARγ and C/EBPα and activation of PPARγ, and suggest SEE inhibits lipogenesis by regulating PPARγ and C/EBPα. Also, the inhibition of SREBP-1c protein expression was accompanied by a reduction in the protein expression of SREBP-1c target genes such as SCD-1, FAS and ACCα. These experimental data demonstrated that SEE markedly suppresses adipocytic differentiation of 3T3-L1 cells by inhibiting SREBP-1 expression and transcriptional factors PPAR-γ and C/EBP-α (Sheu et al., 2007).

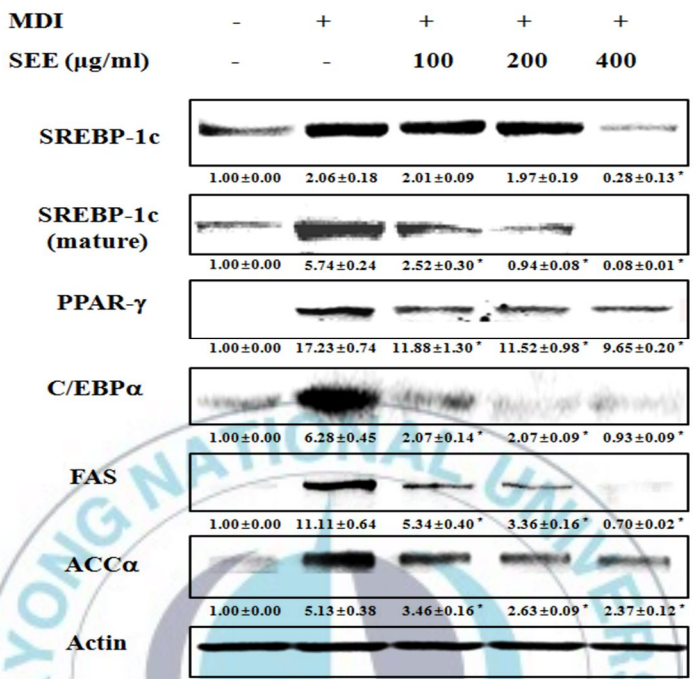


Fig. 10. Effect of SEE on the protein expression of PPARγ, SREBP-1c, C/EBPα, FAS and ACCα in 3T3-L1 cells. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SEE. Medium was changed at day 7, 9 and 11, and the cells were harvested on day 13 for Western blot analysis. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $*P<0.05$ indicates significant differences compared with MDI-only treated group.

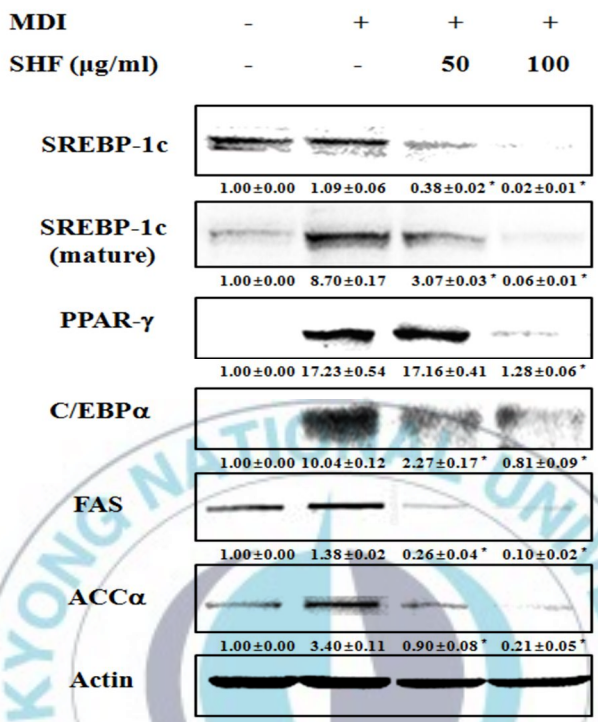


Fig. 11. Effect of SHF on the protein expression of PPARγ, SREBP-1c, C/EBPα, FAS and ACCα in 3T3-L1 cells. After 2-day postconfluence, 3T3-L1 preadipocytes (0 day) were induced by MDI. On day 3, the MDI medium was replaced with DMEM containing 10% FBS and insulin. On day 5day, MDI medium was replaced with DMEM/10% FBS culture medium containing insulin and indicated concentrations of SHF. Medium was changed at day 7, 9 and 11, and the cells were harvested on day 13 for Western blot analysis. $^{\#}P < 0.05$ indicates significant differences compared with non-treated control group. $*P<0.05$ indicates significant differences compared with MDI-only treated group.

4. Discussion

Obese condition increases the risk of fat-related disorders such as hyperglycemia, hyperlipidemia, hypercholesterolemia, and diabetes. There are many reports that brown seaweeds have efficacies in disease-associated metabolic disorder and lipid metabolism(Yin et al., 2008; Samad et al., 2009). The aim of this study was to determine the obesity therapeutic potential of SEE and SHF in 3T3-L1 adipocytes. Adipocyte differentiation is regulated by the coordinated expression of various transcription factors, including C/EBPα and PPARγ. Both C/EBPα and PPARγ play important roles in the regulation of lipid metabolism. We investigated whether the effect of SEE and SHF on differentiation and lipid accumulation in adipocytes is influenced by the adipocyte-related key transcription factors C/EBPα, PPARγ, and their target genes(Kim & Lee, 2012; Quan et al., 2012). On the molecular level, C/EBPα, PPARγ and SREBP-1c are induced during adipocyte differentiation(Gregoire et al., 1998). Specifically, PPARγ and SREBP-1c mRNAs start to be expressed at the very early/early stage (day 1-2 postconfluence) followed by the expression of C/EBPα at the intermediate stage (day 4 post-confluence)(Kim & Spiegelman, 1996). These transcription factors further controls expression of adipocyte-specific genes at the late stage (day 5 post-confluence) leading to the fat droplet formation(Ntambi & Young-Cheul, 2000). Therefore, the inhibition of PPARγ, SREBP-1c and C/EBPα, by SEE and SHF at the early/intermediate stages results in the suppression of expression of

adipocyte specific genes and lipid formation at the late stage of adipocyte differentiation. First, recent study reported dietary sesamin decreased lipogenic enzyme gene expression through the reduction of the SREBP-1 mRNA level and protein content of the precursor and its mature forms(Yamauchi et al., 2001). SEE and SHF decreased SREBP-1c mRNA levels and the expression of the related mature 68 kDa protein. Because of the mature form of SREBP-1c is known to promote lipogenic gene expression, decrease in the levels of the 68 kDa SREBP-1c protein contribute to suppressed lipogenesis in SEE-treatd cells and F SHF-treatd cells in mature cells. In agreement with SEE-induced down-regulation of SREBP-1c at transcriptional and posttranslational level, we observed a reduction in adipocyte lipogenic activity and decrease in the expression in SREBP-1c target genes, such as SCD-1, FAS and ACCα, important enzymes for lipogenesis(Braissant et al., 1995; Osborn & Dooley, 2000). SREBP-1c plays a critical role in the transcriptional regulation of a number of genes in the lipogenic pathway, including FAS and SCD-1(Kim et al., 1998). Decreased expression of SREBP-1c in the SEE-treated 3T3-L1 caused suppressed expression of lipogenic genes, FAS and SCD-1 displayed in figure 3 and figure 4. This result suggests that suppression of lipogenesis by SEE treatment in 3T3-L1 cells was mainly caused by down-expression of lipogenic genes (FAS and SCD-1) through suppression of SREBP-1c. With treatment of SEE during differentiation of 3T3-L1 preadipocytes, expression level of C/EBPα was remarkably reduced, however, that of PPARγ and its target gene was moderately suppressed by expo-

sure to high concentration of SEE. Since C/EBP and PPAR ie key genes in differentiated adipocytes, it is likely that the suppression of C/EBPα and PPAR at 400 μg/ml concentration of SEE partially influenced on the adipocyte differentiation. At 400 μg/ml concentration, SEE remarkably changed adipocyte differentiation. Also, at 100 μg/ml concentration, SHF remarkably changed adipocyte differentiation. And This result can be brought together with the observation that C/EBPα and PPARγ gene and protein expression with decreased level at this 400 μg/ml concentration of SEE and at thish 100 μg/ml concentration of SHF. Supression of PPARγ at low concentration of SEE and SHF may influence on the adipogenesis of 3T3-L1 as shown in fig. 9 and fig. 10. It is generally recognized that whereas the lipogenic activity has a central role in energy storage in cultured preadipose cell lines and in adipose tissue from rodent species, this pathway has been reported to exert an accessory function in human adipose tissue(El Hadri et al., 2004; Diraison et al., 2004). In humans, the liver is the central organ for de novo lipogenesis. As suggested in our experimental results, the anti-lipogenic properties of SEE and SHF in adipocyte may help to ameliorate obesity in addition to liver function. In summary, evidence was provided that SEE and SHF decrease lipid accumulation in differentiating preadipocytes through suppression of lipogenic and adipogenic genes. Our results suggest that consumption of SEE and SHF has the potential to prevent obesity. The present study indicated that possible mechanism for the suppression of lipid accumulation by SEE and SHF is relevant to the down-regulation of SREBP-1c and their

target genes. Further experimental investigations will be helpful to understand which SEE and SHF have key role in the suppression of lipogenesis in 3T3-L1 adipose cells.

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