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Fucoxanthin-containing Ethanolic Extract from *Sargassum hemiphyllum* Inhibits Adipogenesis in 3T3-L1 Cells

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ABSTRACT

Obesity is the main clinical manifestation of the disturbance of energy intake and consumption, and is a significant risk factor for diabetes mellitus, hypertension, dyslipidemia, and cardiovascular disease. In the present study, *Sargassum hemiphyllum* ethanolic extract (SHEE) containing 7.56 mg of fucoxanthin per gram of dry matter was investigated to determine its potential in inhibiting adipogenesis in adipocytes 3T3-L1 cells. SHEE at concentrations of 250, 500, and 750 μg/ml had no cytotoxic effect on 3T3-L1 cells. When cells were treated with 750 μg/ml SHEE, lipid accumulation was significantly reduced to 1.26 ± 0.06 mM compared with control cells (1.62 ± 0.05 mM). In relation to adiponectin activation, secretion and mRNA expression were significantly increased at 500 and 750 μg/ml SHEE. In addition, 3T3-L1 cells treating with 750 μg/ml SHEE significantly decreased mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ) 0.44 ± 0.09-fold compared with the control. These results suggest that fucoxanthin-containing SHEE inhibited adipogenesis in 3T3-L1 cells through downregulation of PPARγ.

Key words: *Sargassum hemiphyllum*, fucoxanthin, 3T3-L1 cells, adiponectin, PPARγ

INTRODUCTION

Adipocytes are specialized connective cells that function as major storage sites for fat in the form of triglycerides. Furthermore, adipocytes have endocrine functions as they secrete adipokines, such as leptin, adiponectin, resistin, tumor necrosis factor (TNF)-α, and IL-6, among others. These adipokines regulate energy balance and glucose and lipid levels and may contribute to diseases associated with obesity (Flier, 2004). In this study, we focused on adiponectin, a protein synthesized and secreted by mature adipocytes (Berg *et al*., 2002). It is an adipose tissue-specific secretory adipokine that has direct insulin sensitizing activity (Nakano *et al*., 1996). The concentration of plasma adiponectin is reduced in obese humans/mice and in insulin resistance (Hu *et al*., 1996; Arita *et al*., 1999), and the synthesis of adiponectin increases in weight loss and improved insulin sensitivity (Milan *et al*., 2002; Yang *et al*., 2001). The role of adiponectin in obesity is currently being clarified. We know that obesity is a medical condition that may lead to diabetes mellitus, hypertension, dyslipidemia, and cardiovascular disease (Fasshauer *et al*., 2004). Antiobesity drugs such as orlistat and sibutramine can be used for some patients to lose weight. Although these drugs are very effective, there are still concerns about their safety, including with increases in blood pressure and pulse rate (Padwa *et*

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al., 2007). Natural ingredients may be more effective and practical treatment for antiobesity.

Fucoxanthin, the characteristic carotenoid found in brown seaweed, is a natural product with proven efficacy and safety in the management of body weight (Lee *et al*., 2011). In animal studies, fucoxanthin has been shown to upregulate the expression of mitochondrial uncoupling protein 1 (UCP 1) in white adipose tissue (WAT), which may contribute to reduce the weight of WAT (Maeda *et al*., 2005). In fucoxanthin-fed mice, adipocyte size is significantly reduced, and lipogenic mRNA expression and fatty acid β-oxidation enzymes are significantly downregulated in a dose-dependent manner. Furthermore, fucoxanthin also elevates the adiponectin concentration in these mice (Woo *et al*., 2009). Fucoxanthin has thus been shown to reduce lipid accumulation as well as to stimulate adiponectin secretion. Flavonoids such as phenolic acid (Hsu and Yen, 2007) and ginseng (*Panax quinquefolius*) extract (Yeo *et al*., 2011) have been reported to reduce lipid accumulation and stimulate adiponectin expression simultaneously. However, there has been no report of brown seaweed extract functioning in both ways simultaneously.

The brown seaweed *Sargassum hemiphyllum* grows around the coasts of Taiwan, Korea, Japan, Hong Kong, and East China and is used in traditional Chinese medicine in Taiwan (Yao, 2003). *S. hemiphyllum* has been found to have valuable antioxidative, immunomodulatory (Hwang *et al*., 2010), and antiinflammatory activity (Hwang *et al*., 2011). However, no data is currently available on its possible antiadipogenesis effects. Thus, the objective of this study was to investigate the effects of *S. hemiphyllum* ethanolic extract (SHEE) on proliferation, lipid accumulation, and adiponectin secretion of 3T3-L1 cells. We also examined the effects of SHEE on gene expression of adiponectin and peroxisome proliferator-activated receptor γ (PPARγ) in 3T3-L1 cells.

MATERIALS AND METHODS

1. Materials and Reagents

Fresh *S. hemiphyllum* was collected from the coast of Penghu county, Taiwan from December 2008 to January 2009. The seaweed was washed and dried in a dryer with current air (RISEN Co., Taiwan) at 40°C for 90 min. The dried sample was ground to a powder with a mini blender (YOUQI, Taiwan) and dried again with the dryer at 50°C for 10 min. The dried seaweed (100 g) was then incubated with shaking (125 rpm) in 2 l of 95% ethanol at 50°C for 6 h. The extract was centrifuged at 10,000×*g* for 20 min, and the supernatant was evaporated under reduced pressure at a temperature below 40°C, yielding dry residues considered to constitute SHEE. Once SHEE was obtained, it was stored at −20°C until use. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. Purification of Fucoxanthin

Analysis of the fucoxanthin content of SHEE was performed by high performance liquid chromatography (HPLC) after purification according to the method described by Sugawara *et al*. (2001) with a minor modification. SHEE was dissolved in 95% ethanol, applied to a cartridge column (Column PD-10, GE) with silica gel (Keisel gel 60, Merck, Darmstadt, Germany) equilibrated with hexane, and eluted by stepwise elution with a hexane:ethyl acetate mixture (10:0-4:6, v/v). Fucoxanthin was recovered from the hexane:ethyl acetate fraction (5:5–4:6, v/v).

3. High Performance Liquid Chromatography Analysis

Reverse-phase HPLC was performed with an Hitachi L-7000 system on a Develosil ODS-UG-5 column (250×4.6 mm i.d., 5.0 μm particle size; Nomura Chemical Co., Japan) fitted with a guard column (10×4.0 mm i.d.) containing the identical stationary phase. A mixture of methanol and acetonitrile (70:30, v/v) at a flow rate of 1.0 ml/min was used as the mobile phase. Fucoxanthin was monitored at 450 nm using a UV–Vis detector (Kotake-Nara *et al*., 2001).

4. Cell Culture and Proliferation Assay

3T3-L1 cells (preadipocytes derived from Swiss mouse embryos) were obtained from the American

Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 110 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. One day after confluence, adipogenesis was induced by treating the cells for 48 h with differentiation medium I containing 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone. The medium was then replaced with differentiation medium II containing 5 μg/ml insulin in 10% FBS-DMEM; the medium was replaced with fresh medium every 2 days. SHEE dissolved in 95% ethanol was added to differentiation medium II and at every subsequent medium change for 10 days. The final concentration of ethanol was adjusted to 0.1% so as not to affect cell growth (Maeda *et al*., 2006).

The 3T3-L1 cells were then reacted with MTT (1 mg/ml) for 4 h, and absorbance was recorded at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Tokyo, Japan) (Mosmann, 1983). The percent relative activity was determined as (A1/A0)×100%, with A0 and A1 representing absorbance in the absence and presence of samples, respectively.

5. Triglyceride Analysis

The triglyceride content was measured using a triglyceride quantification kit (BioVision, Mountain View, CA, USA) (Besnard *et al*., 2009). 3T3-L1 cells were incubated in differentiation medium II supplemented with SHEE for 10 days. After culture, the cells were collected for triglyceride analysis.

6. Adiponectin Analysis

The adiponectin concentration was measured using a Quantikine ELISA mouse adiponectin immunoassay kit (R&D Systems, MN, USA) according to the manufacturer's instructions (Roffey *et al*., 2006). 3T3-L1 cells were incubated in differentiation medium II supplemented with SHEE, and the medium was collected in order to measure the adiponectin concentration every 2 days before it was replaced with fresh medium.

7. Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA (2.0 mg) was extracted using Trizol reagent (Gibco BRL, Burlington, Canada), incubated with Oligo dT (18) primers (0.5 μg/μl) at 70°C for 5 min, and reverse transcribed to cDNA in a mixture containing 5×Moloney murine leukaemia virus (MMLV) reaction buffer, 40 U RNase inhibitor (Toyobo, Osaka, Japan), 0.52 mM deoxynucleoside triphosphate (dNTP) mix (Bio-Rad, Hercules, CA, USA), and 200 U MMLV reverse transcriptase (Toyobo). PCR was performed in a mixture containing 2.5 μl of 10×PCR buffer, 2 μl of reverse transcribed template solution, 1.5 mM of $MgCl_2$, 0.5 μM of each sense and antisense primer, 0.2 mM dNTP mix (Bio-Rad), and 1 U iTaq DNA polymerase (Bio-Rad). The sequences of the primers used in this study were as follows: Adiponectin, 5′-GATGGCAGAGATGGCACTCC-3′ and 5′-CTTGCCAGTGCTGCCGTCAT-3′; PPARγ, 5′-AAAGACCCAGCTCTACAACA-3′ and 5′-TCGTAGATGACAAATGGTGA-3′; β-actin, 5'-TGTCCCTGTATGCCTCTGGT'-3′ and 5'-CTCTTTGATGTCACGCACGA-3'. All the above primers were purchased from Mission Biotech Co., Ltd. (Taipei, Taiwan). After an initial denaturation at 95°C for 1 min, amplification proceeded for adiponectin (annealing at 60°C for 1 min and extension at 72°C for 1 min), PPARγ (annealing at 58°C for 1 min and extension at 72°C for 1 min), and β-actin (annealing at 58°C for 1 min and extension at 72°C for 1 min) for 35 cycles. The products were electrophoresed on 2.0% agarose gels for 25 min and stained with ethidium bromide under UV irradiation. The image of the resulting gel was captured and analyzed by Image Master VDS (Amersham Pharmacia Biotech, Sweden). The increase in any given mRNA was semi-quantitatively determined by calculating the ratio of densitometric values of the given gene to an internal reference (fold increase in any given mRNA expression rate = the given mRNA expression rate/β-actin expression rate).

8. Statistical Analysis

All data obtained were analyzed to determine the significance of the different treatments by one-way ANOVA. Significant differences were reported at p values <0.05.

RESULTS AND DISCUSSION

1. Analysis of the Fucoxanthin Content of SHEE

The most common method for extracting fucoxanthin from brown seaweed is liquid solvent extraction using acetone (Maeda *et al*., 2009; Hii *et al*., 2010), methanol (He *et al*., 2009; Heo *et al*., 2008), or chloroform/methanol (Maeda *et al*., 2005). However, these methods lead to the discharge of potentially hazardous solvents that are environmentally harmful, have poor effects on the health of the operational staff, and can also damage the functional properties of the extracts. Therefore, replacing the extraction solvent with a solvent that has better safety profile is important. In this study, we used ethanol to extract *S. hemiphyllum*, and the yield was 2.65% (g/g dry weight). After purification, SHEE was found to contain 7.56 mg of fucoxanthin per gram dry matter. HPLC analysis of SHEE revealed a fucoxanthin peak, which had the same retention rate (11.6 min) as that for fucoxanthin standard (Fig. 1). Ethanolic extracts of fucoxanthin-rich seaweed have been used as nutraceuticals in body fat-lowering agents and their antiobesity effect on C57BL/6J mice has also been demonstrated (Jeon *et al*., 2010). Ethanol is also a suitable cosolvent if supercritical carbon dioxide is used for fucoxanthin extraction (Roh *et al*., 2008). Thus, using ethanol as the extraction solvent for *S. hemiphyllum* is a safe and effective means of obtaining fucoxanthin-containing extract.

2. Effects of SHEE on Adipocyte Proliferation

3T3-L1 cells were used in our study because of their widespread use as a model for biological research of adipose cells (Pajvani and Scherer, 2003). The data showed that proliferation of 3T3-L1 cells was not influenced by incubation in differentiation medium II supplemented with 250 (contained 2.86 μM fucoxanthin), 500 (contained 5.74 μM fucoxanthin), or 750 (contained 8.61 μM fucoxanthin) μg/ml SHEE or 15 μM fucoxanthin standard for 10 days (data not shown). Maeda *et al*. (2006) also demonstrated that 15 μM fucoxanthin was not cytotoxic to 3T3-L1 cells even after 5 days of incubation. The concentrations of SHEE and fucoxanthin used in this study were non-toxic.

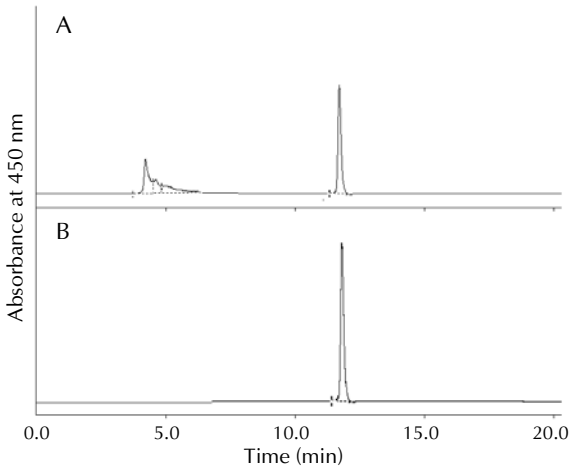

Fig. 1 HPLC chromatograms of SHEE (A) and fucoxanthin standard (B). The detection wavelength was 450 nm.

3. Effects of SHEE on Lipid Accumulation

Next, we examined the effects of SHEE on adipocyte differentiation and corresponding lipid acquisition in 3T3-L1 cells. The intracellular triglyceride content in control 3T3-L1 cells was 1.62 ± 0.05 mM whereas that in 3T3-L1 cells treated with 250, 500, and 750 μg/ml SHEE and 15 μg/ml fucoxanthin was decreased to 1.59 ± 0.02, 1.44 ± 0.08, 1.26 ± 0.06, and 1.21 ± 0.07 mM, respectively. At concentrations of 750 μg/ml SHEE and 15 μg/ml fucoxanthin, the intracellular triglyceride content was significantly reduced compared with control cells ($p < 0.05$) (Fig. 2). The mechanism by which fucoxanthin inhibits adipocyte differentiation has been demonstrated previously; some of the fucoxanthin incorporated into the 3T3-L1 cells is converted into

fucoxanthinol metabolite, which suppresses 3T3-L1 cell differentiation through downregulation of PPARγ (Maeda *et al*., 2006). Jeon *et al*. (2010) and Maeda *et al*. (2009) also reported that supplementation with fucoxanthin obtained from brown seaweed significantly reduced WAT weight in animal models. Although the sulfated polysaccharides from brown seaweed also inhibit the early stage of differentiation of 3T3-L1 cells (Kim *et al*., 2009, 2010), their anti-obesity effects have not been determined *in vivo*. Moreover, phlorotannin-containing methanol extract from the brown seaweed *Ascophyllum nodosum* increases cellular concentrations of myristoleic acid, palmitoleic acid, oleic acid, and total monounsaturated fatty acids (He *et al*., 2009). Therefore, we believe that fucoxanthin was the key substance in SHEE responsible for reducing adipogenesis of the 3T3-L1 cells.

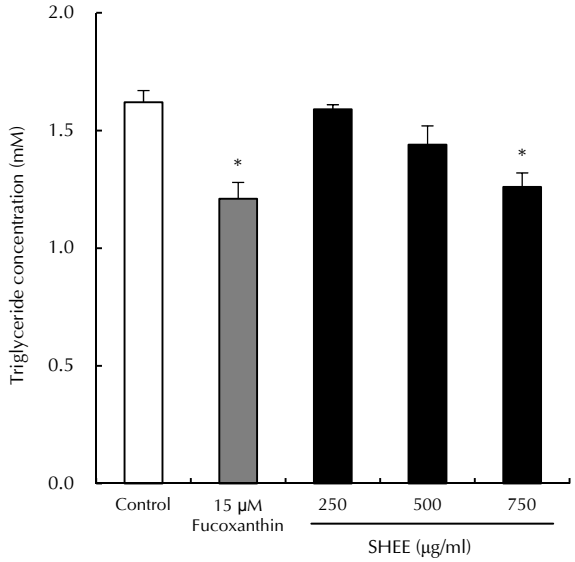

Fig. 2 Effects of SHEE and 15 μM fucoxanthin on intracellular triglyceride content by 3T3-L1 cells treated in differentiation medium II for 10 days (n=3). *Significantly different from the control (P< 0.05).

4. Effects of SHEE on Adiponectin Secretion during Adipocyte Differentiation

Adiponectin is an adipocytokine, which has antiatherogenic, antiinflammatory, antidiabetic (Pajvani *et al*., 2003), and antiobesity (Fruebis *et al*., 2001) effects. In recent years, brown seaweeds have proved to be a rich source of chemical diversity for developing novel therapeutic agents. Kim *et al*. (2008) demonstrated that sargaquinoic acid and sargahydroquinoic acid from the brown seaweed *Sargassum yezoense* increased gene expression of adiponectin. Thus, adiponectin could be an important target for the treatment of diseases.

To determine the extent of variation of adiponectin secretion in 3T3-L1 cells during different differentiation periods, 3T3-L1 cells were incubated in differentiation medium II supplemented with 750 μg/ml SHEE for 2, 4, 6, 8, and 10 days. The medium was collected before being replaced with fresh medium every 2 days and before measuring the adiponectin concentration. The adiponectin concentration was 1.41 ± 0.17 μg/ml after 2 days of incubation and did not remarkably change until after 4 days of incubation (compared with each control). However, the adiponectin concentration was significantly increased to 3.10 ± 0.27, 3.13 ± 0.27, and 3.23 ± 0.18 μg/ml after 6, 8, and 10 days of incubation, respectively (Fig. 3). Thus, adiponectin secretion was markedly higher when 3T3-L1 cells were incubated with SHEE for 10 days. Furthermore, we examined the effects of supplementation of differentiation medium II with 250, 500, and 750 μg/ml SHEE and 15 μM fucoxanthin on adiponectin secretion over 10 days. Adiponectin secretion was significantly increased with 500 and 750 μg/ml SHEE and 15 μM fucoxanthin, from 2.10 ± 0.24 μg/ml (control) to 2.91 ± 0.12, 3.23 ± 0.21, and 3.34 ± 0.24 μg/ml, respectively (Fig. 4). Next, we examined the mRNA levels of adiponectin using RT-PCR. The fold increase for the control was regarded as 1; 500 and 750 μg/ml SHEE and 15 μM fucoxanthin caused significant 1.46 ± 0.12, 1.87 ± 0.15, and 2.25 ± 0.12 fold decreases in mRNA levels compared with the control (Fig. 5). The increase in adiponectin secretion was in line with the increase in adiponectin mRNA expression in the SPEE-treated 3T3-L1 cells. The use of fucoxanthin was also found to increase the plasma adiponectin concentration in mice (Woo *et al*., 2009). Therefore, we concluded that SPEE was able to improve adiponectin secretion by suppressing adiponectin gene expression.

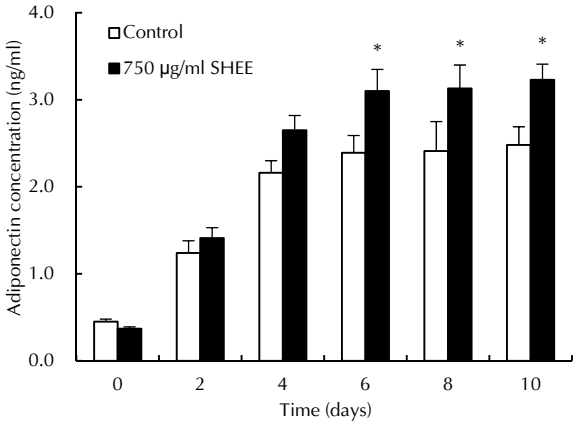

Fig. 3 Effects of 750 μg/ml SHEE and 15 μM fucoxanthin on adiponectin secretion by 3T3-L1 cells during adipocyte differentiation. *Significantly different from the control (P< 0.05).

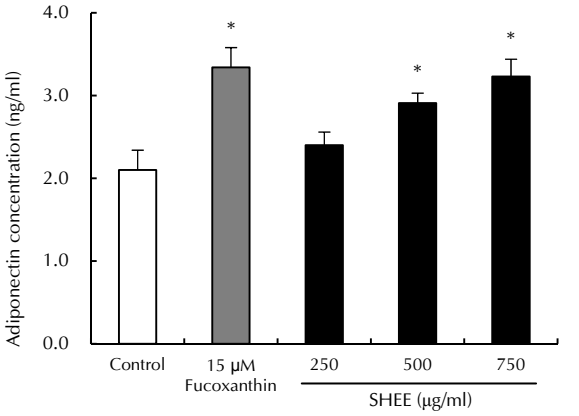

Fig. 4 Effects of SHEE and 15 μM fucoxanthin on adiponectin secretion by 3T3-L1 cells treated in differentiation medium II for 10 days (n=3). *Significantly different from the control (P< 0.05).

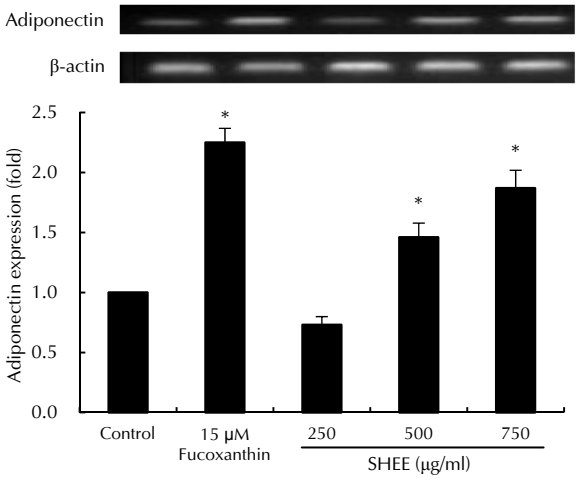

Fig. 5 Effects of SHEE and 15 μM fucoxanthin on the mRNA expression of adiponectin by 3T3-L1 cells treated in differentiation medium II for 10 days (n=3). *Significantly different from the control (P< 0.05).

5. Effects of SHEE on mRNA Expression of PPARγ

PPARγ plays an important role in physiological and pathophysiological events, as well as in the regulation of lipid metabolism (Rosen and Spiegelman, 2001). Moderate reductions in PPARγ activity can decrease the triglyceride content of WAT, enhance fatty acid combustion, and decrease lipogenesis, thereby protecting against obesity and insulin resistance (Yamauchi *et al*., 2001). To investigate the mechanisms underlying SHEE-induced inhibition of adipogenesis, the expression of PPARγ mRNA in 3T3-L1 cells treated with SHEE was analyzed by RT-PCR. The PPARγ/β-actin mRNA expression in the control was regarded as 1. With 15 μM fucoxanthin, the PPARγ mRNA expression level was significantly decreased 0.30 ± 0.04-fold relative to the control. PPARγ mRNA expression was significantly decreased 0.44 ± 0.09-fold by 750 μg/ml SHEE relative to the control (Fig. 6). However, not all brown seaweed extracts induce strong activation of PPARγ. The transcriptional activation of PPARγ of the brown seaweeds *Sargassum confusum, Sargassum fusiforme, Sargassum thunbergii, Sargassum horneri, Sargassum macrocarpum,* and *Sargassum micracanthum* is less than 50% (Kim *et al*., 2008). Thus, fucoxanthin was considered to be the major substance in SHEE responsible for suppressing adipogenesis (Maeda *et al*., 2006). Adiponectin expression can be regulated by PPARγ transcriptional activity (Hsu and Yen, 2007; Gustafson *et al*., 2003).

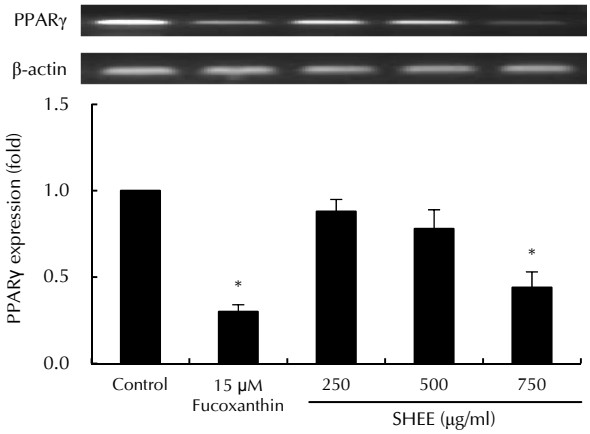

Fig. 6 Effects of SHEE and 15 μM fucoxanthin on the mRNA expression of PPARγ by 3T3-L1 cells treated in differentiation medium II for 10 days (n=3). *Significantly different from the control (P< 0.05).

Our data showed that SHEE had a beneficial effect of reducing lipid accumulation and increasing adiponectin secretion from 3T3-L1 cells in a dose-dependent manner as a result of downregulation of PPARγ expression. This finding agrees with previous reports that flavonoids and phenolic acids inhibit intracellular triglycerides and PPARγ expression and upregulate the expression of adiponectin (Hsu and Yen, 2007). This in turn leads to an increase in β-oxidation, which could also be partly responsible for lipid reduction (Fruebis *et al*., 2001). Thus, dietary SHEE may be a useful natural compound for preventing obesity.

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半葉馬尾藻之藻褐素對抑制 3T3-L1 細胞脂肪新生之影響

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摘　要

肥胖為熱量攝取與消耗失衡之主要臨床表徵，是引發糖尿病、高血壓、高三酸甘油脂血症及心血管疾病的危險因子之一，本研究係探討半葉馬尾藻 (*Sargassum hemiphyllum*) 酒精萃取物對抑制 3T3-L1 脂肪細胞脂質新生之影響。藻褐素 (Fucoxanthin) 是褐藻中的類胡蘿蔔素之一，其在半葉馬尾藻酒精萃取物中的含量為 7.56 mg/g (以乾重計)。半葉馬尾藻酒精萃取物在 250、500 和 750 μg/ml 濃度下，不會對 3T3-L1 脂肪細胞產生毒性，且在 750 μg/ml 濃度下，與控制組 (1.62 ± 0.05 mM) 相比能顯著減少脂肪細胞內的脂質堆積 (1.26 ± 0.06 mM)。此外，在 250、500 和 750 μg/ml 濃度下，可顯著提升脂締素 (Adiponectin) 的分泌量及其 mRNA 表現量，在 750 μg/ml 濃度下亦可有效降低過氧化小體增生活化受體 γ (Peroxisome proliferator-activated receptor γ, PPARγ) 的 mRNA 表現量至 0.44 ± 0.09 倍。從上述結果推測，富含藻褐素之半葉馬尾藻酒精萃取物藉由降低 PPARγ 表現量，進而達到抑制 3T3-L1 脂肪細胞脂質新生的作用。

關鍵詞：半葉馬尾藻、藻褐素、3T3-L1 脂肪細胞、脂締素、過氧化小體增生活化受體 γ

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