

The anti-obesity effect of *Lethariella cladonioides* in 3T3-L1 cells and obese mice

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Abstract

The aim of this study was to investigate whether a water extract of *L. cladonioides* (LC) has an anti-obesity effect in 3T3-L1 cells and obese mice. Treatment of differentiated 3T3-L1 adipocytes with LC caused a significant increase in glycerol release and reduced the protein expression of the adipogenic transcription factors, PPAR γ and C/EBP α . In an animal model, obese mice were artificially induced by a high fat diet for 10 weeks. Experimental groups were treated with LC (100 mg/kg/day) by gavage for the next 10 weeks. At the end of experiment, the body weight of the LC group mice was reduced by 14.2% compared to the high fat diet (HFD) group. The treatment also decreased liver (31.0%), epididymal (18.0%) and retroperitoneal (19.3%) adipose tissue, and kidney (6.7%) weights, respectively, compared with those of the HFD group. LC prevented diet-induced increases in the serum level of TC (22.6%), TG (11.6%), and glucose (35.0%), respectively, compared with the HFD group. However, the HDL-C level was higher in the LC group (26.1%) than the HFD group. The results of this study thus suggest that LC suppressed lipid accumulation and expression of adipogenic transcription factors, and increased the amount of glycerol release. LC also indicated an anti-obese and anti-hyperlipidemic effect.

Key Words: *Lethariella cladonioides*, obesity, 3T3-L1 cells, obese mice

Introduction

Obesity results from an excess energy intake and low energy expenditure, and is associated with lifestyle related diseases such as hypertension, arteriosclerosis, diabetes, osteoarthritis, and some cancers [1]. It is characterized at the fat cell biological level by an increase in the number and/or size of adipocytes, differentiated from preadipocytes, in adipose tissue [2]. Adipose tissue is an active endocrine and paracrine organ that releases a large number of cytokines and bioactive peptides, including leptin, adiponectin, Plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, adiponectin, IL-6, and TNF- α [3]. Fat accumulation is determined by the balance between fat synthesis and fat breakdown. Lipogenesis encompasses the processes of fatty acid synthesis and the subsequent triglyceride synthesis, and takes place in both liver and adipose tissue [4]. Synthesis occurs in the cytoplasm, in contrast to degradation which occurs in the mitochondria. Many of the enzymes involved in fatty acid synthesis are organized into a multi-enzyme complex called fatty acid synthase, which includes the following enzymes: sterol regulatory element binding protein-1c (SREBP-1c), malonyl CoA decarboxylase (MCD), acetyl CoA carboxylase-1 (ACC-1), and

fatty acid synthase (FAS) [5].

Lipolysis is the breakdown of fat stored in fat cells. During this process, free fatty acids are released into the bloodstream and circulate throughout the body. Triglycerides undergo lipolysis (hydrolysis by lipases) and are broken down into fatty acids. The following enzymes induce lipolysis: carnitine palmitoyl transferase-1 (CPT-1), acyl CoA oxidase-1 (ACO-1), and uncoupling proteins (UCPs) [6].

Lipid homeostasis is thus maintained by the fine-tuning of lipogenesis and lipolysis, which are regulated by cooperative action of various enzymes in the metabolic organs, particularly adipose tissues, the liver, and muscle [7]. To prevent or control patients' overweight status, there are many different approaches, including dietary control, exercise, and anti-obesity medication. However, anti-obesity drugs have been reported to have adverse effects, including headache, stomach-ache, vomiting, and heart attack [8]. More recent drug trials have focused on screening herbal medicines that have been reported to have anti-obesity activity. These medicines generally have a natural origin, cost effectiveness, and minimal side effects [9-11]. Herbal supplements and ingredients could avert obesity and possibly lead to the prevention of lifestyle-related diseases, if they can be shown to

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be effective in reducing body weight [12]. For these reasons, many anti-obesity studies have been conducted to find a suitable low-toxicity candidate with an anti-obesity effect. *Lethariella cladonioides*, (LC), is widely used to make teas and is locally referred to as luxinxuecha and hongxuecha. It belongs to the lichen family and is mainly distributed in the western part of China and Tibet.

LC is mainly characterized by yellow and dark pink pigments, which have been identified as canarion, rubrocashmeriquinone, 7-chlororubrocashmeriquinone, 7-chlorocanarione, and atranorin [13]. LC has long been used as a Chinese traditional medicine to treat swollen and sore throats as well as dizziness and fatigue [14].

According to modern medical research, LC contains many microelements that are necessary for health promotion, and may have anti-oxidant and anti-inflammatory activity. It may also lower cholesterol, hypertension and coronary heart disease and improve mental concentration [15,16].

However, there has been no study related to its potential anti-obesity effect. This study was therefore undertaken to investigate the anti-adipogenic effects of LC. The anti-obesity possibility of LC was determined through adipocyte differentiation, and by measuring fat accumulation, glycerol release, adipogenic enzymes expression in adipocytes and lipid profiles in diet-induced obese mice.

Materials and Methods

Preparation of herbal extract

LC was extracted three times by sonication for 3 hr in distilled water, followed by filtration through filter paper. After filtration, the LC extract was concentrated and freeze-dried. The extracts were stored at -20°C until use. The LC extract showed yields in the ranges of 4.2-4.8%.

Cell culture and stimulation

3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in DMEM with 10% (v/v) BCS, 1% (v/v) penicillin-streptomycin until confluent. Two days after confluency, the cells were stimulated to differentiate with DMEM containing 10% FBS, 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), dexamethasone (1 μM), and insulin (10 $\mu\text{g}/\text{mL}$) for two days. Cells were then maintained in a 10% FBS/DMEM medium with insulin (10 $\mu\text{g}/\text{mL}$) for another two days, followed by culturing with a 10% FBS/DMEM medium for an additional four days, at which time > 90% of cells were differentiated 3T3-L1 adipocytes with accumulated lipid droplets. Differentiated 3T3-L1 cells were then treated with various concentrations of LC extract and 1 ng/mL TNF- α . The cells were maintained at 37°C with 5% CO_2 throughout the experiments.

Cell viability assay

The 3T3-L1 preadipocytes were seeded at a density of 1×10^4 cells/well in 96 well plates. The cells were then treated with varying doses (5-500 $\mu\text{g}/\text{mL}$) of LC and were observed after 24, 48, and 72 hr. After completion of the treatment, the cells were incubated with an MTT solution for 3 hr at 37°C . The supernatants were aspirated, DMSO were added to each well, and the plates were agitated to dissolve the crystal product. Absorbance was measured at 570 nm using a multi-well plate reader (Molecular Devices Corp., VERSAmax, Sunnyvale, CA, USA).

Annexin V/ PI double staining analysis

Annexin V-FITC/PI double staining of the cells was determined using the Annexin V-FITC kit (Roche Molecular Diagnostics, Mannheim, Germany). To detect normal, early apoptotic, late apoptotic, and necrotic cells induced by LC, differentiation of 3T3-L1 cells was induced, as described above.

Differentiated 3T3-L1 cells were treated with various concentrations of LC. Differentiated 3T3-L1 cells were then stained for 10 min with FITC-conjugated Annexin V-FITC and PI in a Ca^{2+} -enriched binding buffer and analyzed in the FL1 and FL2 channels of a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), using emission filters of 525 and 575 nm, respectively. The Annexin V-FITC/PI cell population was regarded as normal, while Annexin V-FITC⁺/PI⁻ cells were taken as a measure of early apoptosis, Annexin V-FITC⁺/PI⁺ as late apoptosis, and Annexin V-FITC⁻/PI⁺ as necrosis. The distribution of normal, early apoptotic, late apoptotic, and necrotic cells was calculated using CELL Quest software (BD Biosciences, San Jose, CA, USA).

Oil red O staining

Intracellular lipid accumulation was measured using Oil red O [17]. Differentiated 3T3-L1 cells were treated with various concentrations of LC. Cells were washed twice with PBS and then fixed with 10% formalin for 20 min. After the 10% formalin was removed, propylene glycol was added to each well for 3 min and washed exhaustively with PBS. Cells were then incubated with the Oil red O working solution for 3 hr. The staining of lipid droplets in 3T3-L1 adipocytes were rinsed three times with distilled water. The staining dye of cells was extracted with isopropanol and measured spectrophotometrically at 510 nm using a multi-well plate.

Glycerol release

Differentiated 3T3-L1 cells were treated with various concentrations of LC. Glycerol in the medium was measured using a free glycerol determination kit (Sigma-Aldrich Co., St. Louis, MO, USA) and glycerol standards were used for

calibration. Briefly, a free glycerol reagent solution was reconstituted with distilled water. The free glycerol reagent solution was then reacted with distilled water (blank), glycerol standard (standard), and samples for 5 min. The mixture was transferred to a 96 well plate and absorbance was determined at 540 nm using a multi-well plate reader.

Western blot analysis

As in the glycerol assay, differentiated 3T3-L1 cells were treated with various concentrations of LC. After 72 hr, cells were collected, washed in PBS and lysed in an RIPA buffer (20 mM Tris, pH 8, 150 mM NaCl, 10 mM sodium phosphate, 100 μ M sodium vanadate, 100 μ M ammonium molybdate, 10% glycerol, 0.1% nonidet P-40, 0.1% SDS, and 1 \times protease and phosphatase inhibitors) for 15 min on ice followed by centrifugation at 12,000 rpm for 20 min. The proteins were resolved by 12% SDS-PAGE and were then transferred to a nitrocellulose membrane. Blots were blocked in 5% skim milk dilute in the PBS-T buffer (PBS containing 0.5% Tween 20) for 1 hr and then incubated overnight at 4°C with polyclonal antibodies against C/EBP α , PPAR γ , and

β -actin (Cell signaling Tec., Beverly, Ma, USA). To detect the antigen bound antibodies, the blots were treated with a secondary antibody conjugated with horseradish peroxidase coupled anti-IgG. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system, and band intensity was quantified using densitometry.

Animal model and diets

Five-week-old male C57BL/6 mice (n = 40) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were randomly divided into two groups: normal diet (ND group) and high fat diet (HFD), then the mice were fed diets (Research Diets, New Brunswick, USA) containing 10% and 45% of their calories as fat with energy densities of 3.8 and 4.7 kcal/g, respectively (Table 1). After 10 weeks of dietary manipulation, obesity was obtained in the high fat diet fed group, and the HFD-fed mice were then randomly divided into three HFD groups. These three HFD groups included a group fed only with a high fat diet (HFD group), a group fed with a high fat diet + 0.05% caffeine (CA group), and a group fed with a high fat diet + 100 mg/kg/day LC (LC group). Mice were orally administered either 200 μ l of distilled water (ND and HFD group), or 200 μ l of the experimental agents dissolved in distilled water by oral gavages for 10 weeks. Caffeine was used as a positive control. Caffeine has been successfully used as an agent that cause lipolysis and a safe thermogenic agent for weight control. Animals were housed in a temperature-controlled environment with a 12 hr light/dark cycle. The mice were given water *ad libitum* during the experimental period. The feed intakes and body weights of the mice were monitored daily and weekly, respectively. The experimental protocol was approved by the Animal Care and Use Committee (IACUC) of Kyung Hee University.

Table 1. Composition of experimental diets

Product	Normal diet ¹⁾		High fat diet ²⁾	
	gm%	kcal%	gm%	kcal%
Protein	19.2	20	24	20
Carbohydrate	67.3	70	41	35
Fat	4.3	10	24	45
Total kcal/gm	3.85	100	4.73	100
Ingredient	gm	kcal	gm	kcal
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1,260	72.8	291
Maltodextrin 10	35	140	100	400
Sucrose	350	1,400	172.8	691
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	177.5	1,598
Mineral Mix S10026 ³⁾	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H ₂ O	16.5	0	16.5	0
Vitamin Mix V10001 ⁴⁾	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	1,055.05	4,057	858.15	4,057

* Typical analysis of cholesterol in lard = 0.95 mg/gram

¹⁾ ND, normal diet (Cholesterol 18 mg/kg diet)

²⁾ HFD, high fat diet (Cholesterol 196.5 mg/kg diet)

³⁾ Mineral mix S10026: Magnesium oxide; Mg 0.5 mg, Magnesium sulfate; S 0.33 mg, Sodium chloride; Na 1.0 mg, Cl 1.6 mg, Chromium KSO₄; Cr 2.0 mg, Cupric carbonate; Cu 6.0 mg, Potassium iodate; I 0.2 mg, Ferric citrate; Fe 45.0 mg, Manganous carbonate; Mn 59.0 mg, Sodium selenate; Se 0.18 mg, Zinc carbonate; Zn 29.0 mg, Sodium fluoride; F 0.9 mg, Ammonium molybdate; Mo 1.6 mg

⁴⁾ Vitamin mix V10001: nicotinic acid; 30 mg, calcium pantothenate; 16 mg, pyridoxine-HCl; 7 mg, thiamin-HCl; 6 mg, riboflavin; 6 mg, folic acid; 6 mg, biotin; 0.2 mg, vitamin E acetate; 50 IU, vitamin B₁₂; 10 μ g, vitamin A palmitate; 4,000 IU, vitamin D₃; 1,000 IU, menadione Na bisulfate; 0.5 mg

Collection of serum and tissue samples

Feed was removed 12 hr before sacrificing. Blood samples were collected from each mouse by orbital venipuncture and were incubated on ice for 1hr. Serum was separated from whole blood by centrifugation at 3,000 rpm for 20 min at 4°C and kept at -80°C until it could be analyzed. The liver, kidneys, spleen, testes, and adipose tissue were removed, rinsed with PBS, wiped with a paper towel, weighed quickly, frozen in liquid nitrogen, and stored at -80°C until needed for assayed.

Analysis of serum and liver lipid profiles

Serum triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), and glucose were measured through an enzymatic method using a commercial kit (Asan Pharm Co., Seoul, Korea). Total lipids were extracted from the livers using Folch's method [18]. The extracted samples were dried under nitrogen gas, resolved in isopropanol containing 10% Triton X-100, and

analyzed for lipid components. Hepatic TG and TC concentrations were determined using the same enzyme assay kit used for serum analysis.

Statistical analysis

Each experiment was performed in triplication. The results are expressed as the mean \pm SD. Statistical analysis was performed using SPSS 15.0 for Windows. Analysis of variance was performed using ANOVA procedures. Significant differences ($P < 0.05$) between the means were determined by Duncan's multiple range tests.

Results

The effects of *L. cladonioides* on cell viability in cultured 3T3-L1 adipocytes

To evaluate the effects of LC on the growth of 3T3-L1 adipocytes, the cells were treated with escalating concentration (5-500 $\mu\text{g/mL}$) for 24, 48, and 72 hr (Fig. 1). Treatment of LC with 5 $\mu\text{g/mL}$ (11.7% at 24 hr), 10 $\mu\text{g/mL}$ (14.8% at 48 hr and 7.3% at 72 hr) significantly reduced the cell viability of 3T3-L1 adipocytes. LC treatment inhibited cell viability in a dose dependent manner. However, the extent of inhibition did not exceed IC_{50} . The results of our data demonstrate that LC dosage did not have toxicity in LC treated (5-500 $\mu\text{g/mL}$) 3T3-L1 adipocytes.

The Effects of *L. cladonioides* on apoptosis in differentiated 3T3-L1 adipocytes

To quantify the modes of cell death (apoptosis or/and necrosis) induced by LC, differentiated 3T3-L1 adipocytes were treated with LC for 72 hr, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. As shown in Fig. 2, flow cytometric analysis demonstrated that treatment of cells with LC did not affect the number of normal cells and apoptosis cells (early and late apoptotic cells) regardless of the treatment dosage.

The inhibitory effect of *L. cladonioides* on lipid accumulation in differentiated 3T3-L1 adipocytes

The inhibitory effect of LC on lipid accumulation was evaluated according to the duration of treatment after we induced adipogenesis by adding adipogenic hormone mixture containing various concentrations of LC for 72 hr (Fig. 3).

As a positive control, TNF- α (1 ng/mL) was used. Obesity leads to infiltration of the expanded adipose tissue by macrophages and increased levels of proinflammatory cytokines. The first indication of increased cytokine release in obesity was provided by the identification of increased expression of TNF- α , a

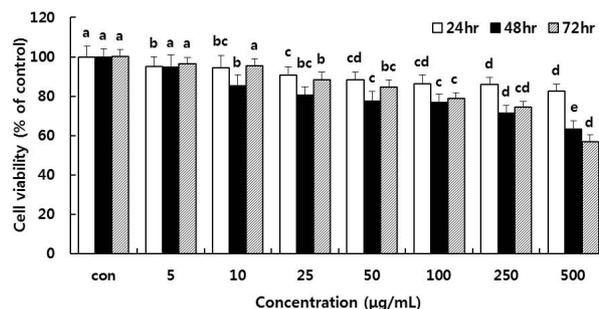


Fig. 1. The effect of *L. cladonioides* on cell viability in 3T3-L1 cells. Differentiated 3T3-L1 cells were incubated with *L. cladonioides* at the indicated various concentrations for 24, 48, and 72 hr. Data are expressed as percent growth rate of cells cultured in the presence of *L. cladonioides*, compared with untreated control cells, taken as 100% (mean \pm SD). Statistical analysis was performed using a one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

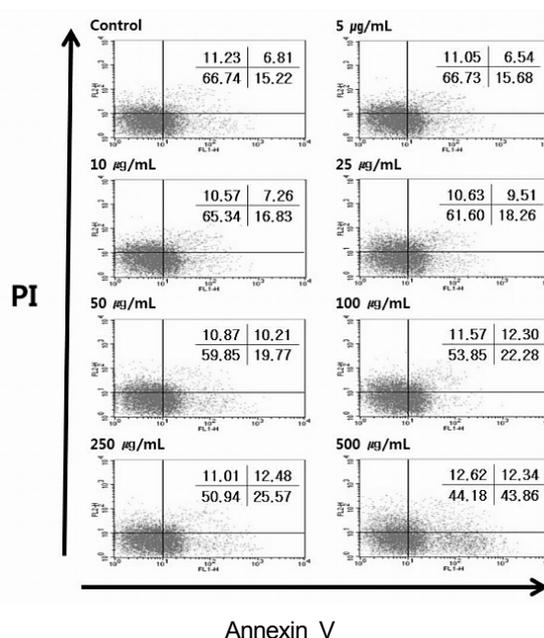


Fig. 2. The effect of *L. cladonioides* on apoptosis/necrosis in 3T3-L1 cells. Differentiated 3T3-L1 cells were incubated with *L. cladonioides* at the indicated various concentrations for 72 hr by Annexin V/PI double staining. Annexin V FITC/PI⁻ (Normal cells), Annexin V FITC⁺/PI⁺ (Early apoptotic cells), Annexin V FITC⁻/PI⁺ (Late apoptotic cells), and Annexin V FITC⁻/PI⁻ (Necrotic cells). Statistical analysis was performed using one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

proinflammatory cytokine in the adipose tissue of the obese mice. TNF- α is expressed in and secreted by adipose tissue, its levels correlating with the degree of adiposity and the associated level of insulin resistance. Targeting TNF- α and/or its receptors has been suggested as a promising treatment for insulin resistance and type 2 diabetes [19]. In this study, treatment with LC 50 $\mu\text{g/mL}$ significantly reduced lipid accumulation (13.7% reduction, $P < 0.05$) in differentiated 3T3-L1 adipocytes in a dose dependent manner.

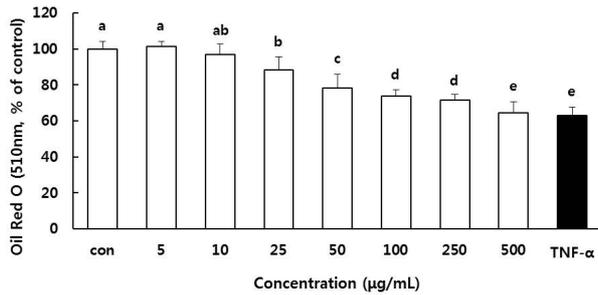


Fig. 3. Effect of *L. cladonioides* on lipid accumulation in 3T3-L1 cells. Differentiated 3T3-L1 cells were treated with various concentrations of *L. cladonioides*. As a positive control, TNF- α (1 ng/mL) was used. After 72 hr, lipid accumulation was evaluated using Oil Red O staining. Each experiment was performed in triplicate. Values are means \pm SD. Statistical analysis was performed using one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

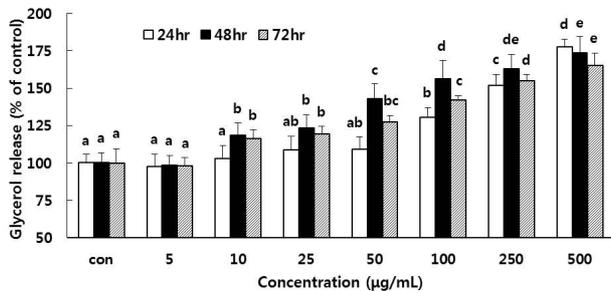


Fig. 4. The effect of *L. cladonioides* on glycerol release in 3T3-L1 cells. Differentiated 3T3-L1 cells were treated with various concentrations of *L. cladonioides*. Cell supernatants were collected after 24, 48, and 72 hr and free glycerol release was assayed. Each experiment was performed in triplicate. Values are mean \pm SD. Statistical analysis was performed using a one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

These results suggest that LC controls lipid accumulation in differentiated 3T3-L1 adipocytes.

The effects of L. cladonioides on lipolysis in differentiated 3T3-L1 adipocytes

Generally, triglyceride hydrolysis proportionally release glycerol and free fatty acid from adipocytes, and this glycerol release is a marker of cellular lipolysis [20]. Therefore, we examined whether LC could increase glycerol release in differentiated 3T3-L1 adipocytes. Differentiation of 3T3-L1 adipocytes was clearly induced, as mentioned above. After serum starvation, differentiated 3T3-L1 adipocytes were treated with LC. Cell supernatants were collected after 24, 48, and 72 hr, and free glycerol release was then assayed. Treatment of LC at 50 µg/mL (55% at 24 hr), 50 µg/mL (29% at 48 hr), and 10 µg/mL (17% at 72 hr) all significantly increased the glycerol release ($P < 0.05$) of differentiated 3T3-L1 adipocytes (Fig. 4). These results thus demonstrate that the lipolytic effect observed in response to LC treatment was partially associated with the release of glycerol.

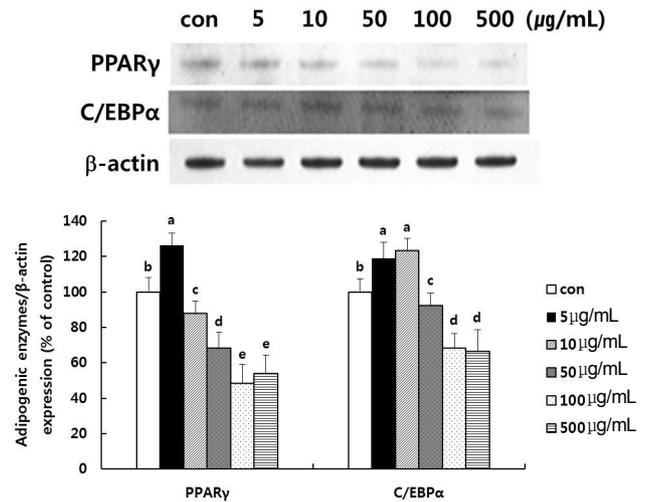


Fig. 5. The effect of *L. cladonioides* on the expression of the adipogenic enzyme in 3T3-L1. Differentiated 3T3-L1 cells were treated with various concentrations of *L. cladonioides*. After 72 hr, cells were collected and they were washed in PBS and lysed in RIPA buffer. Each experiment was performed in triplicate. Values are mean \pm SD. Statistical analysis was performed using one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

The effects of L. cladonioides on expression of adipogenic enzymes in differentiated 3T3-L1 adipocytes

PPAR γ is known as a key station protein that is expressed early in the adipocyte differentiation of 3T3-L1 cells and prior to C/EBP α . The effects of LC on the protein expression of PPAR γ and C/EBP α in differentiated 3T3-L1 adipocytes are shown in Fig. 5. The protein expression of the adipogenic transcription factors, PPAR γ and C/EBP α , were decreased in differentiated 3T3-L1 adipocytes treated with LC 10 µg/mL (15.2%, $P < 0.05$) and 50 µg/mL (6.9%, $P < 0.05$), respectively. Thus, LC mediated differentiation of 3T3-L1 adipocytes by inhibiting both adipogenic transcription factors PPAR- γ and C/EBP- α expression.

The effects of L. cladonioides on food intake and body and organ weight in obese mice

The experimental mice in the LC group appeared healthy, showing no pathological signs or abnormalities during the experimental period. Table 2 shows the body and organ weights and serum and hepatic lipid profiles of mice fed with the experimental supplements for 10 weeks. Total food intake among the HFD-fed groups was not significantly different. As expected, the body weight change was higher in the high-fat diet fed groups than the normal (ND) group. Supplementing CA and LC to the high-fat diet group significantly suppressed body weight gain at the end of the experiment: compared with that of the high fat diet (HFD) group the caffeine (CA) and *Lethariella cladonioides* (LC) group showed a weight reduction of 35.1%, and 14.2%, respectively (Fig. 6). The CA group showed significantly lower

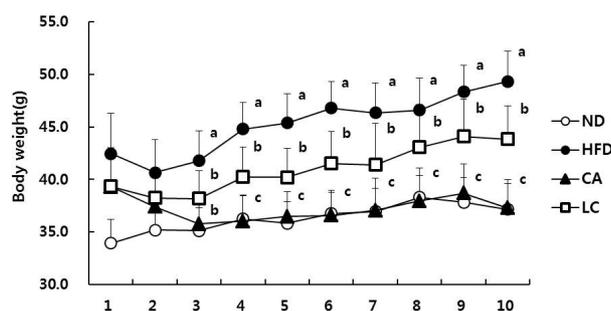
Table 2. Body weight, food intake and food efficiency ratio of experimental groups

	ND	HFD	CA	LC
Body weight (g)				
Initial	21.69 ± 0.89	19.61 ± 1.59	19.09 ± 0.73	18.51 ± 0.71
Final	37.15 ± 2.48	47.69 ± 2.20 ^a	37.31 ± 2.68 ^c	42.60 ± 3.17 ^b
Total food intake (g/day)	2.74 ± 1.11	2.49 ± 0.59	2.34 ± 0.68	2.34 ± 0.55
FER ¹ (%)	0.47	0.98	0.64	0.87

ND, Normal diet; HFD, high fat diet; CA, caffeine; LC, *L. cladonioides*

¹FER, food efficiency ratio = [body weight gain(g)/total food intake(g) × 10²]

The values for the normal diet group were measured, but excluded from statistics. Data were presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range tests. Letters with different superscripts in the same row are significantly different ($P < 0.05$) among groups by one-way ANOVA.

**Fig. 6.** Comparisons of body weight in DIO mice treated with *L. cladonioides*.

ND, Normal diet; HFD, high fat diet; CA, caffeine; LC, *L. cladonioides*. Values for the normal diet group were measured, but excluded from statistics. Data were presented as the mean ± SD. Statistical analysis was performed using a one-way ANOVA with repeated measures followed by Duncan's multiple range tests. Letters with different superscripts are significantly different ($P < 0.05$) among groups by one-way ANOVA.

Table 3. Organ weights of experimental groups

	ND	HFD	CA	LC
Organ weight (g)				
Liver	1.55 ± 0.24	2.64 ± 0.59 ^a	1.17 ± 0.12 ^c	1.77 ± 0.42 ^b
EWAT ¹	2.05 ± 0.32	1.98 ± 0.24 ^a	1.36 ± 0.22 ^c	1.69 ± 0.35 ^b
RWAT ²	0.94 ± 0.18	2.85 ± 0.46 ^a	2.07 ± 0.27 ^b	2.31 ± 0.24 ^b
Kidney	0.41 ± 0.03	0.42 ± 0.02 ^a	0.37 ± 0.04 ^b	0.39 ± 0.04 ^b
Testis	0.20 ± 0.01	0.23 ± 0.02 ^a	0.21 ± 0.03 ^b	0.22 ± 0.02 ^b
Spleen	0.08 ± 0.02	0.12 ± 0.02 ^a	0.07 ± 0.004 ^c	0.10 ± 0.02 ^b

ND, Normal diet; HFD, high fat diet; CA, caffeine; LC, *L. cladonioides*

¹EWAT, epididymal white adipose tissue

²RWAT, retroperitoneal white adipose tissue.

The values from the normal diet group were measured, but excluded from statistics. Data were presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range tests. Letters with different superscripts in the same row are significantly different ($P < 0.05$) among groups by one-way ANOVA.

weights of liver, epididymal and retroperitoneal adipose tissues, kidney, testis, and spleen, in comparison with those of the HFD group. Moreover, compared with the HFD mice, significant decreases were observed in the liver (31.0%), epididymal (18.0%) and retroperitoneal adipose tissues (19.3%), kidney (6.7%), testis (6.0%), and spleen (17.1%) weights in the LC group (Table 3). Thus, LC may induce body and organ weight loss, without influencing food intake.

Table 4. Serum triglyceride, total cholesterol, HDL cholesterol and glucose of experimental groups

	ND	HFD	CA	LC
Serum (mg/dl)				
Triglyceride	92.1 ± 2.2	137.6 ± 26.6 ^a	95.0 ± 11.2 ^c	121.7 ± 9.2 ^b
Total cholesterol	144.2 ± 21.1	259.8 ± 71.6 ^a	141.6 ± 32.9 ^c	201.0 ± 42.0 ^b
HDL-cholesterol	102.7 ± 13.6	92.4 ± 20.0 ^a	117.5 ± 9.6 ^b	113.4 ± 13.8 ^b
Glucose	249.2 ± 21.5	313.7 ± 67.0 ^a	196.4 ± 29.5 ^b	203.9 ± 32.5 ^b
Liver (mg/g liver)				
Triglyceride	213.1 ± 28.4	252.3 ± 43.8 ^a	103.6 ± 14.1 ^c	174.4 ± 10.7 ^b
Total cholesterol	40.3 ± 7.5	45.8 ± 7.2 ^a	33.2 ± 2.6 ^c	37.0 ± 2.9 ^b

ND, Normal diet; HFD, high fat diet; CA, caffeine; LC, *L. cladonioides*

Values for the normal diet group were measured, but excluded from statistics. Data were presented as the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple range tests. Letters with different superscripts in the same row are significantly different ($P < 0.05$) among groups by one-way ANOVA.

The effects of *L. cladonioides* on the serum and hepatic levels of lipid profiles

The fasting TG level of the LC group (11.6%) was significantly decreased compared to that of the HFD group, and that of the HSF group was significantly greater than that of the CA group. TC and glucose levels were significantly decreased by 22.6% and 35.0%, respectively, in the LC group compared with those of the HFD group (Table 4). On the other hand, HDL cholesterol level was significantly increased in the LC group (26.1%) compared to that in the HFD group and was not significantly different than that of the CA group. Similar to the serum level, hepatic TG and TC levels were significantly decreased in the LC group compared to those of the HFD group, by 30.9% and 19.1%, respectively. These results suggest that LC positively improved circulating and hepatic lipid levels.

Discussion

A variety of naturally occurring herbal extracts have been found to have beneficial effects on health, and these compounds have drawn attention because of their relative safeness and accumulating evidence in support of their beneficial physiological properties *in vitro* and *in vivo*.

LC has been found to be a useful natural substance that contains many microelements. It has been reported to assist in alleviating hypertension, hyperlipemia, swollen throat, and fatigue [15,16,21].

However, the anti-obesity effects of LC extract *in vitro* and *in vivo* have not yet been properly investigated. In this study, the anti-obesity effects of LC were investigated through the differentiation of 3T3-L1 adipocytes and body weight, and serum lipid profile measuring in diet-induced obese mice. The 3T3-L1 preadipocyte line has been proven to undergo complete differentiation into fully differentiated adipocytes [22]. We focused on observing the balance between lipogenesis and lipolysis, which is the mechanism of proposed anti-obesity. The present study provided evidence that LC treatment after a time interval of adipocyte differentiation is sufficient to prevent lipid accumula-

tion in differentiated 3T3-L1 adipocytes and that it prevented body weight gain in mice.

In the *in vitro* experiment, treatment of preadipocytes with LC during the cell proliferative stage inhibited cell viability in a dose dependent manner with no apparent toxicity. Flow cytometric analysis demonstrated that treatment of differentiated 3T3-L1 adipocytes with LC did not affect the proportion of normal cells and apoptosis cells including cells in the early apoptosis stage. This data suggests that adipose tissue mass could not be decreased by the deletion of adipocytes via apoptosis in treatment with LC. The accumulation of triglyceride in differentiated 3T3-L1 adipocytes was decreased in cells treated with LC as compared with those in untreated cells. The results of our data thus demonstrate that LC had an inhibitory effect on lipid accumulation in differentiated 3T3-L1 adipocytes. Triglyceride hydrolysis proportionally release glycerol and free fatty acid from adipocytes, and this glycerol release was significantly increased after the treatment of LC on differentiated 3T3-L1 adipocytes, suggesting that the anti-adipogenic effects were partially mediated by increased lipolysis. Other natural compounds, such as conjugated linoleic acid (CLA), epigallocatechin gallate (EGCG), and curcumin have also induced lipolysis in adipocyte models [23-25].

We examined the protein expressions of adipogenic transcription factors by LC after adipocyte differentiation. The induction of adipocytes differentiation involves the cooperative interplay of members of the PPAR γ and C/EBP α [26,27]. Once transcription of the PPAR γ and C/EBP α gene has been initiated, its continued expression is assured through transcriptional autoactivation. This mechanism is believed to be responsible, at least in part, for maintaining expression of adipocyte genes transactivated by PPAR γ and C/EBP α in the terminally differentiated state. Treatment with LC, except at the 5 μ g/mL dose, reduced the protein expression of the adipogenic transcription factors (PPAR γ and C/EBP α). Given the fact that the level of Oil O red was not changed at the dose of 5 μ g/mL, it is of interest to find the underlying mechanism for the increase in PPAR γ level at a low level. It may be that the initial low-dose treatment of LC might have acutely stimulated the synthesis of PPAR γ . Putting these results together, it is suggested that the anti-adipogenic effects of LC were mediated by reduced lipogenesis.

In vivo, we conducted animal studies to further assess the effects of LC on body fat metabolism and its potential role in the prevention and treatment of obesity. High fat diets are used widely and have been accepted in nutritional experiments as a good strategy to induce over-weight conditions and fat deposition in animals [28]. Thus, the period of high fat feeding induced a marked weight increase in mice. As a positive control, caffeine was used because it has been demonstrated in literature to reduce the body and adipose tissue weight in animal modelling [29]. For instance, Zheng *et al.* [30] have shown that a 0.05% caffeine intake for 10 weeks reduced the weight of intraperitoneal adipose tissues in mice fed a normal diet. Similarly, Yun *et al.* [31] reported that an intake of 0.025% caffeine for eight weeks

reduced the body weight and epididymal adipose tissue weight of mice fed a high fat diet. A possible mechanism by which caffeine affects lipolysis involves inhibiting the phosphodiesterase-induced degradation of the intracellular cAMP [32]. Presently, possible evidence for the metabolic response to caffeine from mechanisms involving catecholaminergic stimulation of adipocyte or other cellular metabolisms seems less convincing [33,34]. In the results of this study, a high fat diet for 10 weeks resulted in obesity in mice, which was associated with increased body weight and fat mass with the development of hyperlipidemia. However, supplementation of caffeine or *Lethariella cladonioides* significantly reduced body weight and liver, epididymal and retroperitoneal adipose tissue, and spleen weight. Additionally, the triglyceride was more reduced in the CA and LC groups than in the HFD group. *Lethariella cladonioides* has phenolic compounds, such as canarione, thamnolic acid, usnic acid and *etc.* Polyphenolics, as compared with vitamin E, have been found to exert powerful antioxidant effects, to inhibit lipid peroxidation by acting as chain breaking peroxy radical scavengers, to reduce plasma, inhibit hepatic lipid synthesis, and to increase cholesterol elimination via bile acids [35].

This is in agreement with Wei *et al.* [36], who reported that an ethanol extract of LC possesses strong antioxidant activity *in vitro* and hypolipidaemic activities in high fat diet mice.

As expected, total cholesterol was higher in HFD group than the normal (ND) group. This increase in total cholesterol was suppressed by both CA and LC treatment. Similarly, the glucose level was lower in the CA and LC groups than the HFD group. On the other hand, HDL-cholesterol was higher in the CA and LC group than the HFD group. These results suggest that LC exerts body weight loss and hypolipidemic effects in HFD fed mice. A high calorie diet is normally related to an increase of the synthesis of fatty acids in the liver and the delivery of free fatty acids to the liver [37]. It may also decrease β -oxidation of free fatty acids in the liver. Therefore, we investigated the lipid profiles, including triglyceride levels and total cholesterol, in the liver. Hepatic triglyceride and total cholesterol were lower in the CA and LC groups than the HFD group.

In conclusion, the results of this study showed that LC suppressed lipid accumulation and increased the amount of glycerol release into the medium. It also suppressed adipogenic gene expression in 3T3-L1 adipocytes and decreased body weight and modulated the lipid metabolism in obese mice. Although the exact mechanisms by which these effects take place remain to be elucidated, this study's results provide a basis for proposing that LC may have future application in the treatment of obesity.

References

1. Poulos SP, Dodson MV, Hausman GJ. Cell line models for differentiation: preadipocytes and adipocytes. *Exp Biol Med* (Maywood) 2010;235:1185-93.
2. Qureshi K, Abrams GA. Metabolic liver disease of obesity and

- role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol* 2007;13:3540-53.
3. Lee WJ, Koh EH, Won JC, Kim MS, Park JY, Lee KU. Obesity: the role of hypothalamic AMP-activated protein kinase in body weight regulation. *Int J Biochem Cell Biol* 2005;37:2254-9.
 4. Kersten S. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep* 2001;2:282-6.
 5. Shi Y, Burn P. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nat Rev Drug Discov* 2004;3:695-710.
 6. Chen N, Bezzina R, Hinch E, Lewandowski PA, Cameron-Smith D, Mathai ML, Jois M, Sinclair AJ, Begg DP, Wark JD, Weisinger HS, Weisinger RS. Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. *Nutr Res* 2009;29:784-93.
 7. Meier U, Gressner AM. Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem* 2004;50:1511-25.
 8. Park YS, Yoon Y, Ahn HS. Platycodon grandiflorum extract represses up-regulated adipocyte fatty acid binding protein triggered by a high fat feeding in obese rats. *World J Gastroenterol* 2007;13:3493-9.
 9. Pittler MH, Schmidt K, Ernst E. Adverse events of herbal food supplements for body weight reduction: systematic review. *Obes Rev* 2005;6:93-111.
 10. Naik GH, Priyadarsini KI, Satav JG, Banavalikar MM, Sohoni DP, Biyani MK, Mohan H. Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. *Phytochemistry* 2003;63:97-104.
 11. Slanc P, Doljak B, Kreft S, Lunder M, Janes D, Strukelj B. Screening of selected food and medicinal plant extracts for pancreatic lipase inhibition. *Phytother Res* 2009;23:874-7.
 12. Saito M, Ueno M, Ogino S, Kubo K, Nagata J, Takeuchi M. High dose of *Garcinia cambogia* is effective in suppressing fat accumulation in developing male Zucker obese rats, but highly toxic to the testis. *Food Chem Toxicol* 2005;43:411-9.
 13. Obermayer W. On the identity of *Lethariella sinensis* wei & jiang, with new reports of tibetan *Lethariella* species. *Bibl Lichenol* 2001;78:321-6.
 14. Jiang B, Zhao QS, Yang H, Hou AJ, Lin ZW, Sun HD. Constituents from *Lethariella cladonioides*. *Fitoterapia* 2001;72: 832-3.
 15. Kinoshita K, Togawa T, Hiraishi A, Nakajima Y, Koyama K, Narui T, Wang LS, Takahashi K. Antioxidant activity of red pigments from the lichens *Lethariella sernanderi*, *L. cashmeriana*, and *L. sinensis*. *J Nat Med* 2010;64:85-8.
 16. Niu DL, Harada H, Wang LS, Zhang YJ, Yang CR. Chemotaxonomic study of the *Lethariella cladonioides* complex (lichenized Ascomycota, Parmeliaceae). *Lichenologist* 2011;43: 213-23.
 17. Ramirez-Zacarias JL, Castro-Muñozledo F, Kuri-Harcuch W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 1992;97: 493-7.
 18. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497-509.
 19. Tzanavari T, Giannogonas P, Karalis KP. TNF-alpha and obesity. *Curr Dir Autoimmun* 2010;11:145-56.
 20. Moussalli C, Downs RW, May JM. Potentiation by glucose of lipolytic responsiveness of human adipocytes. *Diabetes* 1986;35: 759-63.
 21. Hauck M, Jürgens SR, Willenbruch K, Huneck S, Leuschner C. Dissociation and metal-binding characteristics of yellow lichen substances suggest a relationship with site preferences of lichens. *Ann Bot* 2009;103:13-22.
 22. Cowherd RM, Lyle RE, McGehee RE Jr. Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* 1999;10:3-10.
 23. Wolfram S, Wang Y, Thielecke F. Anti-obesity effects of green tea: from bedside to bench. *Mol Nutr Food Res* 2006;50:176-87.
 24. Basu A, Sanchez K, Leyva MJ, Wu M, Betts NM, Aston CE, Lyons TJ. Green tea supplementation affects body weight, lipids, and lipid peroxidation in obese subjects with metabolic syndrome. *J Am Coll Nutr* 2010;29:31-40.
 25. Shehzad A, Ha T, Subhan F, Lee YS. New mechanisms and the anti-inflammatory role of curcumin in obesity and obesity-related metabolic diseases. *Eur J Nutr* 2011;50:151-61.
 26. Farmer SR. Regulation of PPARgamma activity during adipogenesis. *Int J Obes (Lond)* 2005;29 Suppl 1:S13-6.
 27. Fajas L, Fruchart JC, Auwerx J. Transcriptional control of adipogenesis. *Curr Opin Cell Biol* 1998;10:165-73.
 28. Chun MR, Lee YJ, Kim KH, Kim YW, Park SY, Lee KM, Kim JY, Park YK. Differential effects of high-carbohydrate and high-fat diet composition on muscle insulin resistance in rats. *J Korean Med Sci* 2010;25:1053-9.
 29. Hursel R, Westerterp-Plantenga MS. Thermogenic ingredients and body weight regulation. *Int J Obes (Lond)* 2010;34:659-69.
 30. Zheng G, Sayama K, Okubo T, Juneja LR, Oguni I. Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. *In Vivo* 2004;18:55-62.
 31. Yun JW, Shin ES, Cho SY, Kim SH, Kim CW, Lee TR, Kim BH. The effects of BADGE and caffeine on the time-course response of adiponectin and lipid oxidative enzymes in high fat diet-fed C57BL/6J mice: correlation with reduced adiposity and steatosis. *Exp Anim* 2008;57:461-9.
 32. Murosaki S, Lee TR, Muroyama K, Shin ES, Cho SY, Yamamoto Y, Lee SJ. A combination of caffeine, arginine, soy isoflavones, and L-carnitine enhances both lipolysis and fatty acid oxidation in 3T3-L1 and HepG2 cells in vitro and in KK mice in vivo. *J Nutr* 2007;137:2252-7.
 33. Mori S, Satou M, Kanazawa S, Yoshizuka N, Hase T, Tokimitsu I, Takema Y, Nishizawa Y, Yada T. Body fat mass reduction and up-regulation of uncoupling protein by novel lipolysis-promoting plant extract. *Int J Biol Sci* 2009;5:311-8.
 34. Jung RT, Shetty PS, James WP, Barrant MA, Callingham BA. Caffeine: its effect on catecholamines and metabolism in lean and obese humans. *Clin Sci (Lond)* 1981;60:527-35.
 35. Chumark P, Khunawat P, Sanvarinda Y, Phornchirasilp S, Morales NP, Phivthong-Ngam L, Ratanachamnong P, Srisawat S, Pongrapeeporn KU. The in vitro and ex vivo antioxidant properties, hypolipidaemic and antiatherosclerotic activities of water extract of *Moringa oleifera* Lam. leaves. *J Ethnopharmacol* 2008;116:439-46.
 36. Wei AH, Zhou DN, Ruan JL, Cai YL, Xiong CM, Li MX. Characterisation of phenols and antioxidant and hypolipidaemic activities of *Lethariella cladonioides*. *J Sci Food Agric* 2012;92: 373-9.
 37. Frayn KN, Maycock PF. Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability. *J Lipid Res* 1980;21:139-44.