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Lithospermum erythrorhizon Suppresses High-Fat Diet-Induced Obesity, and Acetylshikonin, a Main Compound of *Lithospermum erythrorhizon*, Inhibits Adipocyte Differentiation

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ABSTRACT: *Lithospermum erythrorhizon*, which has traditionally been used as a vegetable and to make the liquor Jindo Hongju, contains several naphthoquinone pigments, including shikonin. This study aimed to evaluate the antiobesity effects of *Lithospermum erythrorhizon* ethanol extract (LE) and elucidate the underlying mechanism. C57BL/6J mice were fed a normal or high-fat diet with or without LE supplementation for 8 weeks. LE reduced high-fat diet-induced increases in body weight, white adipose tissue mass, serum triglyceride and total cholesterol levels, and hepatic lipid levels while decreasing lipogenic and adipogenic gene expression. Furthermore, acetylshikonin suppressed adipocyte differentiation in a dose-dependent manner and significantly attenuated adipogenic transcription factor expression in 3T3-L1 cells. These findings suggest that *Lithospermum erythrorhizon* prevents obesity by inhibiting adipogenesis through downregulation of genes involved in the adipogenesis pathway and may be a useful dietary supplement for the prevention of obesity.

KEYWORDS: *Lithospermum erythrorhizon, acetylshikonin, antiobesity, mice, 3T3-L1 adipocyte*

INTRODUCTION

Obesity has become a global epidemic and is associated with numerous chronic diseases, including type 2 diabetes, dyslipidemia, atherosclerosis, hypertension, cardiovascular diseases, stroke, and certain forms of cancer.[1,2] Upregulation of adipogenic and lipogenic gene expression correlates with obesity.[3] Adipogenesis can be defined as increased fat mass due to an increase in the number and size of adipocytes, which is regulated by two crucial transcription factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα).[2] In addition, sterol regulatory element-binding protein-1c (SREBP1c) is a master regulator of genes involved in hepatic lipogenesis, such as fatty acid synthase (FAS).[4] SREBP1c and PPARγ mediate lipid accumulation in the liver induced by a high-fat diet (HFD).[5]

A variety of natural foods, including crude extracts and isolated compounds from plants, can reduce body weight gain and prevent diet-induced obesity. Therefore, numerous bioactive components from foods have been widely used to treat obesity.[6−8]

Lithospermum erythrorhizon is used in vegetable side dishes or Korean traditional distilled liquor (Jindo Hongju) and contains naphthoquinone pigments, such as shikonin derivatives. Methanol extracts of *L. erythrorhizon* have been reported to have high total phenolic contents and exhibit antioxidant activities.[9] In previous studies, shikonin derivatives have been reported to have wound-healing, antimicrobial, anti-inflammatory, and anticancer activities.[10−13] Although several pharmacological effects of *L. erythrorhizon* have been studied, the antiobesity effect and the molecular mechanisms underlying these activities of *L. erythrorhizon* have not yet been reported. This study aimed to identify the main bioactive constituents of *L. erythrorhizon* and the effect of *L. erythrorhizon* on diet-induced obesity in mice. We also examined the mRNA expression of transcriptional factors involved in adipogenesis and lipogenesis to elucidate the molecular mechanism underlying the antiobesity effect of bioactive compounds in *L. erythrorhizon*.

MATERIALS AND METHODS

Chemicals. Isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, and Oil Red O were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine calf serum, and antibiotics (penicillin and streptomycin) were purchased from Life Technologies (Burlington, ON, Canada). Antibodies against PPARγ, SREBP-1, C/EBPα, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and an antibody against FAS was purchased from Cell Signaling Technology (Danvers, MA, USA). Acetylshikonin and β-hydroxy isovaleryl shikonin were purchased from Wako (Osaka, Japan), shikonin from Calbiochem (Darmstadt, Germany), and isobutyrylshikonin and β,β-dimethylacryl shikonin from Tokyo chemical industry (Tokyo, Japan). The purity of these standards was above 95% as indicated by the manufacturer.

Sample Preparation. *L. erythrorhizon* was collected from Youngju, Kyeongbook, South Korea, and identified by Professor Y. M. Park, Department of Life Science, Cheongju University. Voucher specimens (KFRI-LE100705) were deposited in the Korea Food Research Institute. The dried roots of *L. erythrorhizon* (1.0 kg) were soaked in 80% ethanol (10 L) at room temperature for 12 h. The ethanol extract was filtered through filter paper (Whatman grade No. 2, USA) and concentrated under a vacuum at 37 °C. The concentrated extracts were then freeze-dried. Finally, the dried extracts (169.8 g) obtained from of *L. erythrorhizon* (1000 g) were stored at −20 °C until use.

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HPLC Analysis. HPLC was performed with a PU2089 HPLC system (JASCO Corporation, Tokyo, Japan). Samples were separated on an Xterra RP-C_{18} column (4.6 × 250 mm, 5 μm, Waters Corporation, Milford, MA, USA). The mobile phase consisted of solvent A (1% aqueous phosphoric acid) and solvent B (acetonitrile). The gradient eluates were filtered through a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) and degassed prior to use. The linear gradient profile was started with an initial composition 5% B to 50% B from 0 to 60 min, 70% B from 60 to 61 min and then 70% A in 61 to 85 min, followed by re-equilibrating the column to its initial conditions. The analytic condition was measured with a slight modification of a previous report.[14] The flow rate was 1 mL/min; the injection volume was 20 μL; and the wavelength at which absorbance was measured was 520 nm. Peaks were identified by comparing their standard retention times.

Animal Experiments. C57BL/6 male mice (4 weeks old) were purchased from Central Laboratory Animal (Seoul, Korea). After acclimation to the commercial chow for 1 week, they were randomly divided into four diet groups (n = 10): control diet (normal), high-fat diet (HFD), high-fat diet supplemented with 0.25% LE (250 mg/kg BW) (HFD + low LE), and high-fat diet supplemented with 0.5% LE (500 mg/kg BW) (HFD + high LE). The experimental diets were based on the AIN-76 diet, and the HFD contained 25% fat (lard 200 g/kg, corn oil 50 g/kg) and 0.5% cholesterol (w/w). The mice had free access to water and diet. The animal room was maintained at 23 ± 1 °C and 53 ± 2% humidity with a 12-h light/dark cycle. Three mice were housed in each cage and fed the experimental diets for 8 weeks. Body weight was measured once a week, and food intake was recorded daily. All animal experiments were approved by the Korea Food Research Institutional Animal Care and Use Committee.

Biochemical Analysis. All mice were sacrificed after a 12-h fast. Blood was collected by orbital venipuncture and centrifuged at 800g for 15 min to isolate the serum, which was stored at −70 °C until analysis. Triglyceride (TG), total cholesterol (TC), free fatty acid (FFA), and high-density lipoprotein cholesterol (HDLC) were enzymatically analyzed with a commercial kit (Shinyang Chemical, Seoul, Korea). The hepatic lipids were extracted using the Folch method.[15] TG and TC concentrations were determined with an enzymatic assay kit (Shinyang Chemical). Serum insulin levels and leptin levels were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (Shinayagi Co., Tokyo, Japan).

Histological Analysis. For hematoxylin and eosin (H&E) staining, liver and epididymal adipose tissues were fixed in 10% formalin for 1 day and processed in a routine manner for paraffin sections. Five-micrometer-thick sections were cut and stained with H&E for microscopic examination (Leica RM2235, Wetzlar, Germany). Images were collected on a microscope (Olympus BX51, Tokyo, Japan).

Protein Extraction and Western Blot Analysis. Cells were washed with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation prior to Western blot analysis. The harvested cells were sonicated for 5 s at 40 W. Liver tissue (20 mg) was homogenized in 600 μL of lysis buffer (PRO-PREP, iNtRON Biotechnology, Sungnam, Korea). Cell and liver tissue lysates were incubated for 20 to 30 min on ice and then centrifuged at 13,000 rpm at 4 °C for 10 min. The protein concentration of the supernatant was determined with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. Total protein (30 μg per lane) was separated by 10% SDS−polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was blocked for 2 h at room temperature with Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 (Amresco Inc., Solon, OH, USA). After overnight incubation at 4 °C with primary antibodies, membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunodetection was carried out with ECL detection reagent (Amersham Biosciences, Uppsala, Sweden). All figures showing results of quantitative analysis (Image J, National Institutes of Health) include data from at least three independent experiments.

Quantitative Real-Time PCR. Total RNA was extracted from frozen liver tissue (30 mg) using the RNase kit (Nucleospin, iNtRON Biotechnology) and was used to synthesize cDNA for analysis by real-time reverse transcription-polymerase chain reaction (RT-PCR) (Step One Plus, Applied Biosystems, Carlsbad, CA, USA) using the FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the protocol provided by the manufacturer. Equal amounts of total RNA were used for each analysis. Quantitative PCR was performed as previously described.[16] The cDNA served as a template in a 20 μL reaction mixture and was processed using an initial step at 95 °C for 5 min, followed by 40 amplification cycles (95 °C for 5 s; 55 °C for 10 s; 72 °C for 15 s). The level of each mRNA was normalized to the level of β-actin. Primer sequences were as follows: SREBP-1 forward 5′-CGG CGC GGA AGC TGT-3′; SREBP-1 reverse 3′-AGT CAC TGT CTT GGT TGT TGA TGA G-5′; FAS forward 5′-ATC CTG GAA CGA GAA CAC GAT CT-3′; FAS reverse 3′-AGA GAC GTG TCA CTC CTG GAC TT-5′; PPARγ forward 3′-TCG CTG ATG CAC TGC CTA TG-5′; PPARγ reverse 5′-GAG AGG TCC ACA GAG CTG ATT-3′; C/EBPα forward 5′-GAC TTC AGC CCC TAC CTG GA-3′; C/EBPα reverse 3′-GTA GTC GTC GGC GAA GAG GT-5′; LPL (lipoprotein lipase) forward 5′-TGT AAC AAT CTG GGC TAT GAG ATC AAC-3′; LPL reverse 3′-TGC TTG CCA TCC TCA GTC CC-5′; aP2 (adipocyte binding protein 2) forward 5′-AGG CTC ATA GCA CCC TCC TGT G-3′; and aP2 reverse 3′-CAG GTT CCC ACA AAG GCA TCA C-5′.

Cell Culture and Differentiation. The 3T3-L1 mouse fibroblast cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) at 37 °C under an atmosphere of 5% CO_2. On day 3 after confluence (day 0), the cells were exposed to differentiation medium (DMEM containing 0.25 mM 3-isobutyl-1-methylzanthine, 0.25 μM dexamethasone, 1 μg/mL insulin [MDI], and 10% FBS) for 2 days. The cells were cultured for another 2 days in DMEM containing 1 μg/mL insulin and 10% FBS. Thereafter, the cells were maintained in postdifferentiation medium (DMEM containing 10% FBS), and the medium was replaced every 2 days. To evaluate the effects of LE on preadipocyte differentiation, the cells were cultured with differentiation medium in the presence of various concentrations of LE. The cells were harvested on day 8, when differentiation was complete in the control samples.

MTT Assay. 3T3-L1 cells were plated in each well of a 96-well plate and were allowed to adhere and spread for 24 h. The cells were combined with various concentrations of samples and cultured for 48 h at 37 °C. Ten microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma-Aldrich) was added into each well and incubated for 4 h. Then, 100 μL of DMSO was added to dissolve the purple crystals, which are the products of MTT substrates. The absorbance at 540 nm was determined in each well with a 96-well plate reader.

Oil Red O Staining and Cell Quantification. After differentiation was induced, cells were stained with Oil Red O (0.2% Oil Red O in 60% isopropanol). The cells were washed twice with PBS, fixed with 10% formalin for 1 h, dried, and stained with Oil Red O for 10 min. The cells were washed with 70% ethanol in water and then dried. The lipid content of the stained cells was visualized by microscopy (Olympus IX71). The stained lipid droplets were dissolved in isopropanol and quantified by measuring absorbance at 490 nm.

Statistical Analysis. Group results were compared by analysis of variance (ANOVA) followed by Duncan's test using SAS 10.0 software. Data are expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered significant.

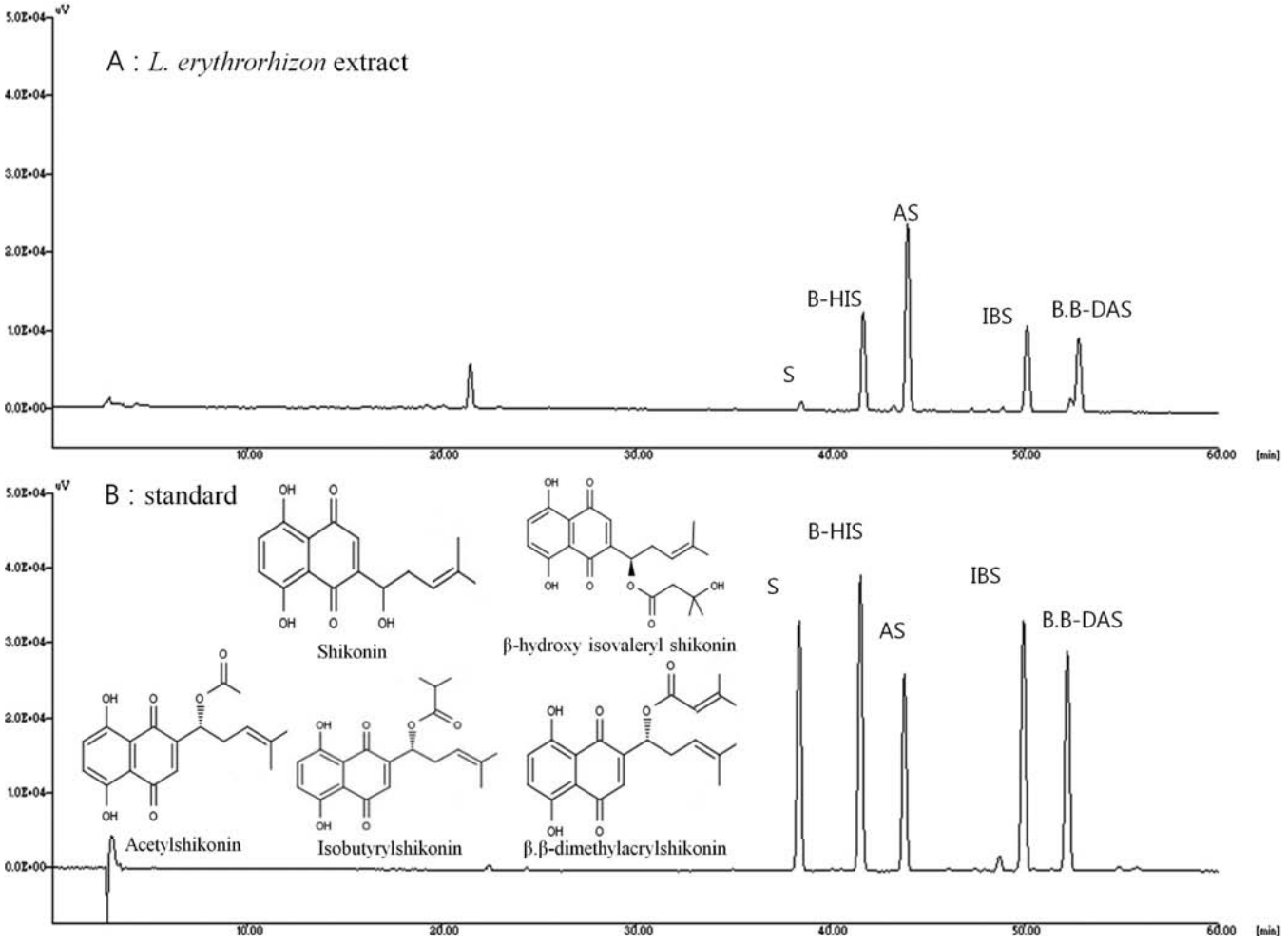

Figure 1. HPLC analysis of *Lithospermum erythrorhizon* extract. Peaks: S, shikonin; B-HIS, *β*-hydroxy isovaleryl shikonin; AS, acetylshikonin; IBS, isobutyrylshikonin; B,B-DAS, *β*,*β*-dimethylacryl shikonin.

RESULTS

Shikonin Derivative Contents of LE. HPLC analysis was applied to separate shikonin derivatives, the main bioactive components of LE. Shikonin, *β*-hydroxy isovaleryl shikonin, acetylshikonin, isobutyrylshikonin, and *β*,*β*-dimethylacryl shikonin were identified by their retention times of 62, 65.32, 66.66, 70.79, and 73.12 min, respectively (Figure 1). The total amount of shikonin derivatives in LE was 16 mg/g LE. Two compounds, *β*-hydroxy isovaleryl shikonin (3.0 ± 0.07 mg/g LE) and acetylshikonin (9.8 ± 0.13 mg/g LE), were identified as the major shikonin derivative constituents of LE (Table 1).

Reduction of Body Weight, Adipose Tissue Weight, and White Adipose Tissue Size by LE. Figure 2 shows the effect of LE on body weight, tissue weights, and white adipose tissue morphology of C57BL/6J mice fed a HFD for 8 weeks.

Table 1. Content of Shikonin Derivatives in Ethanol Extract of *Lithospermum erythrorhizon*[a]

L. erythrorhizon compds	composition (mg/g)
β-hydroxy isovaleryl shikonin	2.99 ± 0.07
acetylshikonin	9.79 ± 0.13
isobutyrylshikonin	1.81 ± 0.07
β,*β*-dimethylacryl shikonin	1.41 ± 0.05

[a]Values are means ± SEM.

HFD-fed mice had body weights 23% higher than control diet-fed mice ($P < 0.05$) (Figure 2A). In contrast, the LE groups had body weights 10% lower than HFD-fed mice ($P < 0.05$). Food intake did not significantly differ among the HFD groups (data not shown). White adipose tissue weights also significantly increased in HFD-fed mice compared to control mice (Figure 2B). LE supplementation significantly reduced the gain of adipose tissue mass caused by HFD. Histological analysis showed that adipocytes of the HFD group were larger than those of the control group and that adipocytes of the LE groups were smaller than those of the HFD group (Figure 2C). These results demonstrate that LE reduces HFD-induced gain of body weight and adipose tissue mass in mice.

Effects of LE on Serum Lipid, Insulin, and Leptin Levels. The effect of LE on serum lipids, leptin, and insulin levels is shown in Table 2. The LE groups possessed significantly reduced serum TG, TC, and FFA levels as compared with HFD-fed mice. There was no significant difference in HDL cholesterol between the HFD group and the LE groups, whereas the ratio of HDL/TC was higher in mice fed high levels of LE than in HFD-fed mice ($P < 0.05$).

Serum leptin and insulin levels were higher in the HFD-fed mice compared to the control diet-fed mice, but LE supplementation reduced serum leptin levels by 40−53% and insulin levels by 24−32% in a dose-dependent manner.

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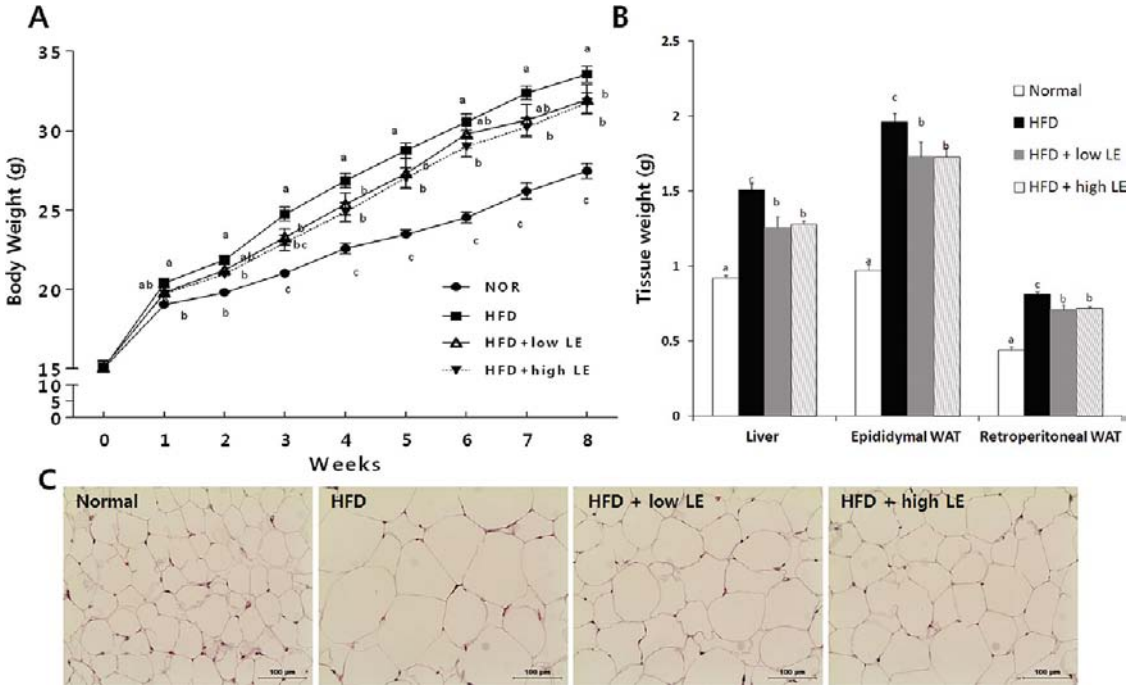

Figure 2. Effects of *Lithospermum erythrorhizon* extract on body weight (A), tissue weight (B), and white adipose tissue morphology (C) in high-fat diet-fed mice. Original magnification, ×200. HFD + low LE, high-fat diet supplemented with 0.25% *L. erythrorhizon* extract; HFD + high LE, high-fat diet supplemented with 0.5% *L. erythrorhizon* extract.

Table 2. Effect of *Lithospermum erythrorhizon* Extract on Serum Lipid, Leptin, and Insulin Levels in Mice Fed a High-Fat Diet[a]

parameter	control	HFD	HFD + low LE	HFD + high LE
triglyceride (mg/dL)	84.5 ± 3.46 a	84.4 ± 4.99 a	64.0 ± 2.87 b	58.9 ± 3.28 b
total cholesterol (mg/dL)	131 ± 4.07 c	214 ± 4.91 a	192 ± 4.66 b	183 ± 5.73 b
HDL cholesterol (mg/dL)	108 ± 1.68 c	168 ± 4.31 a	155 ± 4.00 a	162 ± 6.07 a
HDL/TC (%)	83.6 ± 2.88 ab	78.1 ± 0.68 b	80.6 ± 2.13 ab	85.1 ± 2.46 a
free fatty acid (μEq/L)	1418 ± 75.96 a	1419 ± 48.1 a	1167 ± 82.36 b	1101 ± 57.65 b
leptin (ng/mL)	9.47 ± 1.34 c	51.7 ± 4.36 a	30.8 ± 5.84 b	23.8 ± 2.45 b
insulin (pg/mL)	345 ± 52.60 b	558 ± 47.34 a	419 ± 77.72 ab	377 ± 52.04 b

[a]Values are means ± SEM. Values within a column labeled with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test. HFD + low LE, high-fat diet supplemented with 0.25% *L. erythrorhizon* extract; HFD + high LE, high-fat diet supplemented with 0.5% *L. erythrorhizon* extract; HDL/TC, (HDL cholesterol/total cholesterol) × 100.

Effect of LE on Lipids, Histology, and Lipogenic Gene Expression in the Liver. We next examined the effect of LE on hepatic lipid levels in HFD-fed mice (Figure 3). Total hepatic lipid levels were increased in the HFD group compared to the control group. However, LE supplementation significantly decreased the degree to which total hepatic lipid levels were elevated by HFD. Hepatic TG and TC levels in the HFD group were also significantly higher than those in the control group (Figure 3A). Compared to the HFD group, hepatic TG and TC levels in LE-fed mice were significantly lower. In addition, liver weight was significantly lower in the LE groups than in the HFD group (Figure 2B). H&E staining of liver tissue revealed that LE reduced lipid droplets that accumulated in hepatocytes due to HFD (Figure 3B).

To clarify the mechanism underlying the inhibitory effect of LE on hepatic fat accumulation by HFD, we analyzed hepatic mRNA expression of several genes known to be closely related to lipogenesis using quantitative real-time PCR. Hepatic mRNA expression of SREBP-1, PPARγ, FAS, and LPL was significantly upregulated in the HFD group compared to the control group. However, mRNA expression of these genes was significantly downregulated by LE supplementation (Figure 3C).

Effects of LE on Adipogenic Protein and mRNA Expression in White Adipose Tissue. To determine whether the antiobesity effects of LE are accompanied by changes in adipogenic gene expression, we assessed the protein and mRNA levels of adipogenic transcription factors and enzymes in epididymal adipose tissue (Figure 4). HFD induced protein expression of the key transcription factors PPARγ and C/EBPα. However, protein levels of these transcription factors were lower in the LE group compared with the HFD group (Figure 4A). In addition, protein levels of the adipogenesis target gene aP2 were decreased in the LE group compared with the HFD group. Furthermore, consistent with the protein results, LE supplementation significantly reduced expression of PPARγ, C/EBPα, LPL, and aP2 at the mRNA level (Figure 4B).

Effect of Acetylshikonin on 3T3-L1 Preadipocyte Differentiation. To elucidate whether the main compound of LE, acetylshikonin, plays a role in the antiadipogenic effect of LE, we evaluated the effect of acetylshikonin on adipogenic

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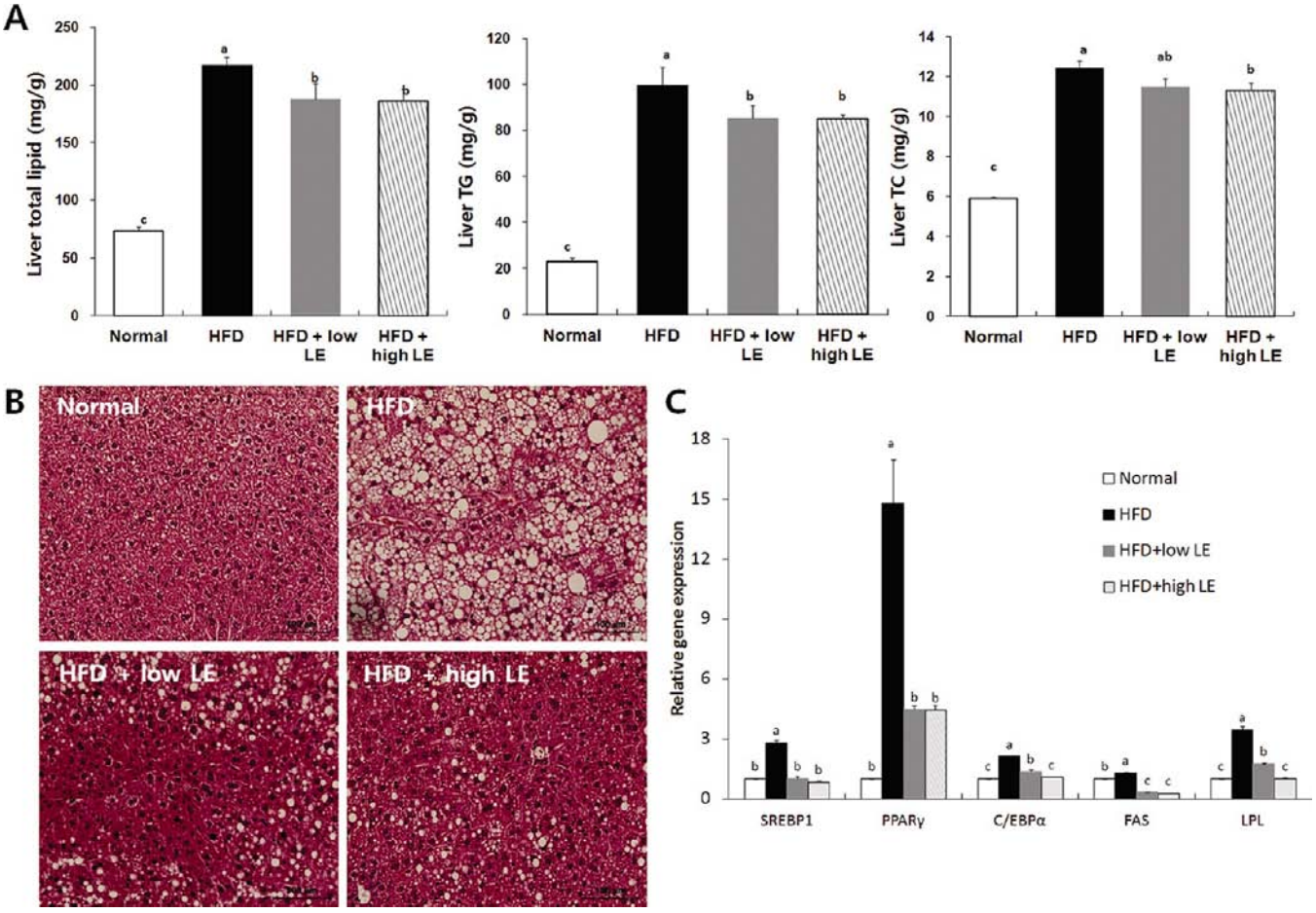

Figure 3. Effects of *Lithospermum erythrorhizon* extract on hepatic levels of total lipid, triglycerides, and total cholesterol (A), hepatic morphology (B), and mRNA expression of lipogenic genes in the liver (C). Bars represent means ± SEM (n = 10). The means for bars labeled with different letters are significantly different ($P < 0.05$). Original magnification, ×200. HFD + low LE, high-fat diet supplemented with 0.25% *L. erythrorhizon* extract; HFD + high LE, high-fat diet supplemented with 0.5% *L. erythrorhizon* extract.

differentiation of 3T3-L1 preadipocytes. We performed an MTT assay to analyze the viability of 3T3-L1 preadipocyte cells treated with acetylshikonin for 48 h. Acetylhikonin did not show any effects on cell viability and cytotoxicity (Figure 5A). Oil Red O staining of adipocytes revealed that acetylshikonin (at 0.12, 0.25, and 0.50 μM) treatment inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner. Furthermore, expression of the adipogenic genes PPARγ, C/EBPα, LPL, and aP2 were significantly decreased at the mRNA level by acetylshikonin treatment. Thus, acetylshikonin inhibits adipocyte differentiation of 3T3-L1 cells by regulating the adipogenesis pathway.

DISCUSSION

Many studies have shown that *L. erythrorhizon* has numerous biological effects, including antitumor, anti-inflammatory, and antimicrobial activities.[11,17,18] However, the antiobesity effect of *L. erythrorhizon* had not yet been reported in vivo. Here, we identified possible active compounds in LE and examined the effect of LE on HFD-induced obesity.

In the present study, LE supplementation significantly reduced body weight gain, adipocyte enlargement, and adipose tissue weight gain in obese mice. Also, LE significantly lowered serum lipid, leptin, and insulin levels. These results clearly demonstrate that LE reduces HFD-induced weight gain in mice.

Obesity is characterized by hyperplasia and hypertrophy of adipocytes.[19] Adipogenesis is the formation of new adipocytes from precursor cells and is associated with obesity.[2] PPARγ and C/EBPα are key transcription factors in adipogenesis and mediate the transcription of terminal adipocyte differentiation marker genes.[20] In the present study, LE downregulated the expression of C/EBPα and PPARγ in white adipose tissue in HFD-fed mice. Furthermore, we also observed that mRNA levels of aP2 and LPL were significantly downregulated by LE supplementation. PPARγ and C/EBPα activate downstream adipocyte-specific genes, such as aP2 and LPL.[21] Expression of aP2 is regulated by adipocyte differentiation and is well-known to increase fatty acid transport to adipose tissue.[22] Lipoprotein lipase (LPL) controls the uptake of fatty acids into the liver and promotes TG deposition in adipose tissue.[23] Taken together, our findings demonstrate that LE prevents weight gain by inhibiting adipogenesis.

The consumption of a HFD results in hepatic fat accumulation, obesity, and metabolic syndrome.[24] We also evaluated the effects of LE on HFD-induced hepatic fat accumulation. LE supplementation attenuated the increase of total lipid, TC, and TG levels in the liver caused by HFD. In previous studies, *L. erythrorhizon* extracts reduced LDL oxidation and cholesterol biosynthesis in HepG2 cells.[25]

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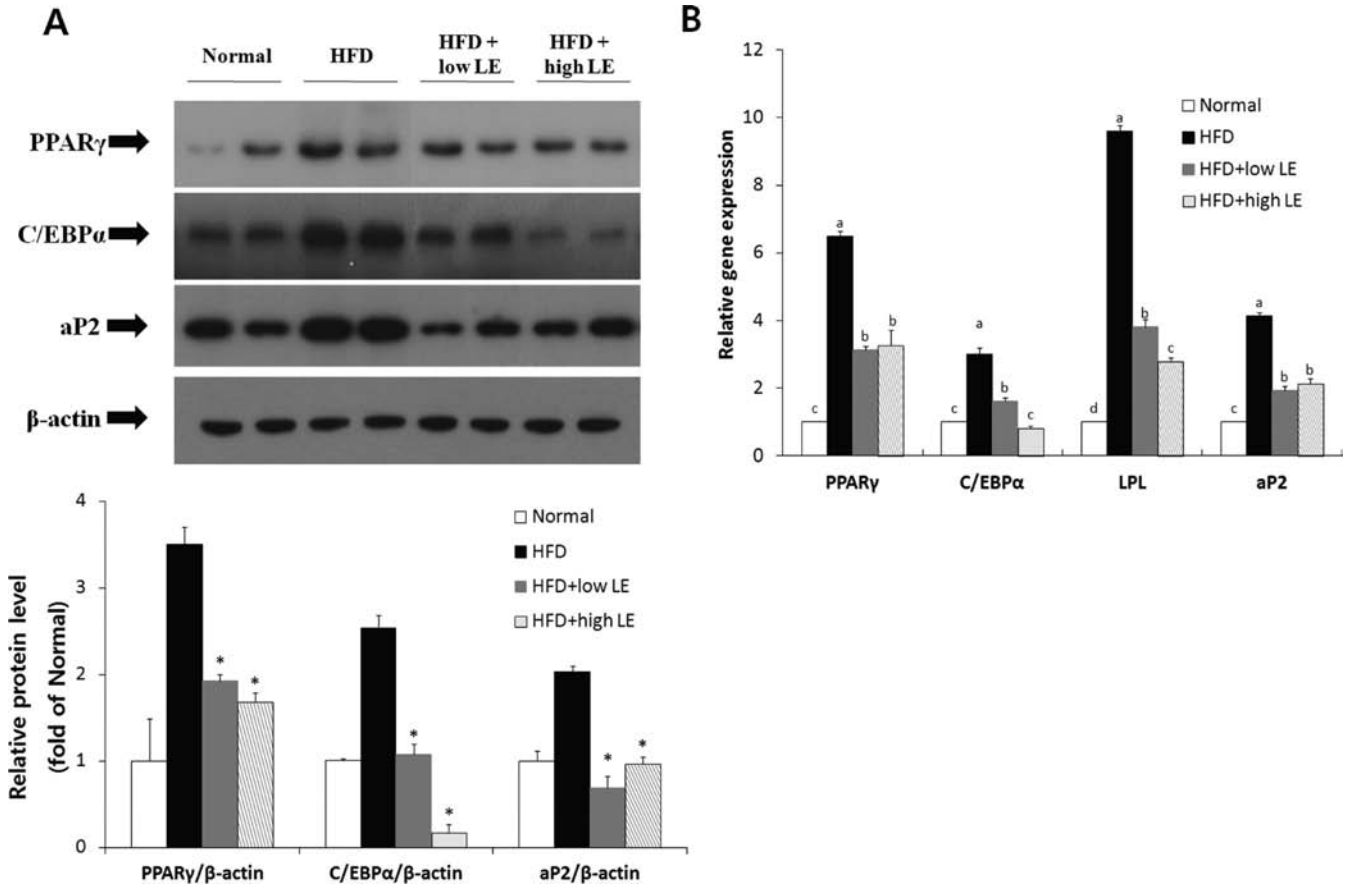

Figure 4. Effects of *Lithospermum erythrorhizon* extract on protein (A) and mRNA (B) expression of adipogenic genes in white adipose tissue. Bars represent means ± SEM (n = 3). The means for bars labeled with different letters are significantly different ($P < 0.05$). The band density was analyzed by ImageJ software (National Institutes of Health). HFD + low LE, high-fat diet supplemented with 0.25% *L. erythrorhizon* extract; HFD + high LE, high-fat diet supplemented with 0.5% *L. erythrorhizon* extract.

Histological examination of liver tissue revealed a marked accumulation of fat droplets, indicating fatty liver disease, in HFD-fed mice; however, hepatic fat accumulation was prevented by LE supplementation.

To gain more mechanistic insight into the antiobesity effect of LE, we examined the expression of lipogenic genes in the liver. LE supplementation significantly reduced mRNA levels of lipogenic genes, such as PPARγ, C/EBPα, and SREBP-1. These data suggest that the LE effects are mediated primarily by downregulating the transcription factor SREBP-1 and its target, FAS, in the liver. As the master regulator of hepatic triglyceride synthesis, SREBP-1 regulates the expression of target genes involved in fatty acid synthesis.[26]

To clarify which components of LE can regulate adipogenesis and lipogenesis in HFD-fed mice, we analyzed the shikonin derivative constituents of LE by HPLC. It has been reported that the main active components of *L. erythrorhizon* are the phenolic compounds, especially naphthoquinone compounds (shikonin derivatives). Also, shikonin derivatives found in LE inhibit the activities of human acyl-CoA:cholesterol acyltransferase (ACAT)-1 and ACAT-2. [27]

LE contained four different shikonin derivatives: β-hydroxy isovaleryl shikonin, acetylshikonin, isobutyrylshikonin, and β,β-dimethylacryl shikonin. Among them, acetylshikonin was the predominant LE component. It has not been reported that acetylshikonin has antiobesity effects, even though it has been reported to have anticancer and antifungal effects. [28] Therefore, to examine whether the antiobesity effect of LE is due to acetylshikonin, we examined the inhibitory effect of acetylshikonin on the differentiation of 3T3-L1 preadipocytes. Our results showed that differentiation of 3T3-L1 cells was clearly attenuated by acetylshikonin (Figure 4). Moreover, acetylshikonin inhibited the expression of adipogenic transcription factors, such as PPARγ and C/EBPα. Thus, acetylshikonin-induced downregulation of adipogenic transcriptional target genes of LPL and aP2 may be mediated by lower PPARγ and C/EBPα expression. These results were in accordance with the results of Lee et al.,[29] which showed that shikonin could inhibit adipocyte differentiation in 3T3-L1 cells. Taken together, these results demonstrate that the inhibitory effect of LE on HFD-induced obesity may be partially attributed to shikonin derivatives. Therefore, further study is needed.

In conclusion, the results of the present study suggest that LE has antiobesity activity that is mediated by suppressing lipogenic genes in the liver and adipogenic transcription factors in white adipose tissue. Also, the antiobesity effects of LE in the present study appear to be partially attributed to acetylshikonin. Thus, *L. erythrorhizon* may be a beneficial dietary supplement to prevent obesity. This is the first time the antiadipogenic effect of acetylshikonin has been reported. Further studies are needed to demonstrate the effect of other bioactive compounds in *Lithospermum erythrorhizon*.

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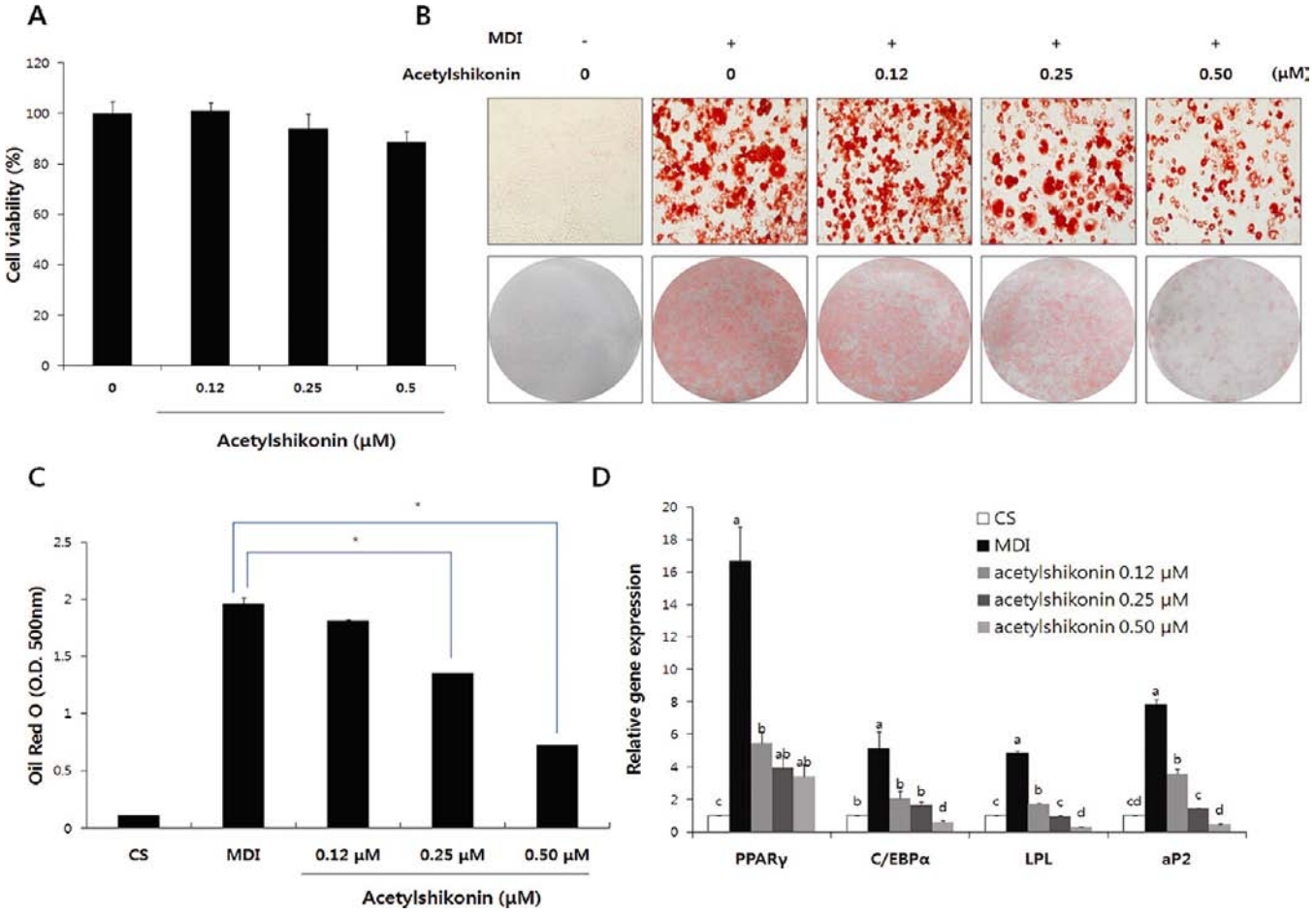

Figure 5. Effect of acetylshikonin on adipocyte differentiation and adipogenic gene expression. (A) The dose-dependent cytotoxic effect of acetylshikonin after 48 h treatment measured by MTT assay. (B) Differentiation of 3T3-L1 preadipocytes was induced with MDI in the absence or presence of acetylshikonin (0.12, 0.25, and 0.50 μg/mL). On day 8, cytoplasmic triacylglycerol was stained with Oil Red O. (C) Lipid accumulation was assessed by quantifying optical density at 500 nm. Results are expressed as mean ± SEM (n = 3; *P < 0.05). (D) Cell lysates were prepared on day 8, and PPARγ, C/EBPα, aP2, and LPL expression was analyzed at the mRNA level. C/EBPα, CCAAT/enhancer binding protein α; PPARγ, peroxisome proliferator-activated receptor γ; FAS, fatty acid synthase; SREBP-1, sterol regulatory element-binding protein.

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Notes

The authors declare no competing financial interest.

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