Euphorbia supina extract results in inhibition of high-fat-diet-induced obesity in mice

SARMILA NEPALI1, DO-KUK KIM2, HOON-YEON LEE2, HYEON-HUI KI1,2, BO-RAM KIM2, SUNG-WOO HWANG2, MIN PARK2, DAE-KI KIM1 and YOUNG-MI LEE2

1Department of Immunology and Institute of Medical Sciences, Medical School, Chonbuk National University, Jeonju, Jeollabuk 54907; 2Department of Oriental Pharmacy, College of Pharmacy and Wonkwang-Oriental Medicines Research Institute, Wonkwang University, Iksan, Jeollabuk 54538, Republic of Korea

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Abstract. The present study was undertaken to investigate the anti-obesity effect of a 50% ethanol extract of Euphorbia supina (ESEE) in high-fat-diet (HFD)-induced obese C57BL/6J mice. Mice were fed a HFD with or without ESEE (2, 10, or 50 mg/kg) or with Garcinia cambogia (positive control) for 6 weeks. ESEE supplementation significantly reduced body, epididymal white adipose tissue (eWAT), and organ weights (P<0.05). ESEE also reduced hepatic steatosis and improved serum lipid profiles. In addition, ESEE significantly reduced serum leptin levels and increased adiponectin levels, and significantly downregulated the mRNA and protein levels of proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein-α (C/EPBα) in eWAT and liver tissues (all P<0.05). These results suggested that ESEE supplementation protects against HFD-induced obesity by downregulating PPARγ and C/EPBα, and that ESEE may be beneficial for the prevention and treatment of obesity and associated diseases.

Introduction

Obesity is a major health concern in developed countries, and is caused by an imbalance between energy intake and expenditure. Obesity is also known to be associated with various metabolic diseases, including hypertension, type 2 diabetes, cardiovascular diseases, atherosclerosis and various cancers (1-4). Adipocytes are the major cellular components of fat tissue, and excessive fat tissue growth, hypertrophy and adipocyte differentiation are fundamental characteristics of obesity (5). The accumulation of lipid droplets in adipocytes has an important role in lipid metabolism and regulation (6), and the differentiation of preadipocytes is regulated by transcription factors, such as peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein-α (C/EPBα) (7,8).

Numerous methods, including diet control, exercise and medication, have been suggested to prevent and treat obesity (9). However, many clinically available drugs have side effects such as constipation, anorexia, dizziness, and insomnia, which remain issues of concern (10). To avoid the side effects of pharmacological agents, there is a need for substitute therapies with minimum side-effects, perhaps based on herbal or natural products (11).

Euphorbia supina (E. supina) belongs to the Euphorbiaceae family and is used in traditional medicine to treat a variety of diseases, such as bronchitis, jaundice, hemorrhage, and several gastrointestinal diseases (12,13). It has also been reported that E. supina contains various biologically active compounds like tannins, terpenoids and polyphenols (14), and polyphenols with antioxidant properties (15). Furthermore, a recent study revealed that E. supina polyphenol mixtures inhibit the invasion and metastasis of breast cancer cells (16). However, to the best of our knowledge, no scientific evidence has yet been presented regarding the anti-obesity effect of E. supina. The purpose of the present study was to investigate the anti-obesity effect of E. supina and the mechanism underlying its inhibitory effect on adipogenesis.

Materials and methods

Materials and reagents. TRIZol reagent and SuperScript III kit were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Mouse primary antibodies against PPARγ (sc-7273), C/EPBα (sc-166258) and β-actin (sc-47778), and goat-anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2030) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies were diluted to 1:1,000 and secondary antibodies at 1:5,000. Garcinia cambogia (GC) extract was purchased from Prakruti Products Pvt. Ltd. (Karnataka, India).
Preparation of the ethanol extract of E. supina (ESEE). E. supina was obtained from Gungangbogam (Jechon, Korea). The dried and powdered fruit of E. supina (500 g) were extracted using 50% ethanol for 2 h under mantle-reflux at 80°C. Following filtration, and solvent removal on a rotary vacuum evaporator (N-000; EYELA; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) ESEE was obtained as a powder (17). The yield of ESEE obtained using this procedure was 83.5 g (16.7% wt/wt). ESEE was stored at 4°C until required.

Animal treatment. Male C57BL/6 mice (n=36; weight, 20.4±1.03 g; age, 5 weeks) were purchased from Samtako Bio Korea (Samtako Bio Korea, Osan, Korea). Experimental procedures were conducted according to a protocol approved by the institutional animal care committee of Chonbuk National University. Mice were housed at 22±2°C, 50±5% humidity and provided a normal diet and water ad libitum. Following a 1-week acclimation period, mice were divided into six groups (n=6 each), as follows: i) the normal diet (ND) group, fed with normal standard diet containing 14% fat, 21% protein and 65% carbohydrate (5L79; Orient Bio, Inc., Seongnam, Korea); ii) the high-fat diet (HFD) group, fed with HFD containing 60% fat, 20% protein and 20% carbohydrate (D12492; Research Diets, Inc., New Brunswick, NJ, USA); iii) the HFD+ESEE (2) group, fed with HFD and treated with ESEE (2 mg/kg/daily); iv) the HFD+ESEE (10) group, fed with HFD and treated with ESEE (10 mg/kg/daily); v) the HFD+ESEE (50) group, fed with HFD and treated with ESEE (50 mg/kg/daily); vi) the HFD+GC group, fed with HFD and treated with GC (200 mg/kg/daily). ESEE or GC was administered daily via oral gavage for 6 weeks. Body weights and amounts of food consumed were determined weekly.

Determination of abdominal fat volume by micro-computed tomography (micro-CT). Following the 6-week treatment period, mice were starved for 6 h prior to sacrifice and anesthesia via intraperitoneal injection of ketamine (75 mg/kg; Yuhan Corporation, Seoul, Korea) and rompun (15 mg/kg; Bayer Korea Ltd., Seoul, Korea). Images were acquired using a Skyscan-1076 micro-CT scanner (Bruker microCT, Kontich, Belgium). CT was performed using a pixel size of 35 µm, a source voltage of 50 kVp, and a source current of 200 µA. The X-ray detector contained a 12-bit, water-cooled charge-coupled device camera with a resolution of 4,000x2,300 pixels and a scintillator. Images were acquired at 0.6 degrees and an exposure time of 0.46 sec using a 1-mm aluminum energy filter. Mice were sacrificed following micro-CT scanning and abdominal fat volumes were measured using an Olympus SP-500 UZ camera (Olympus Corporation, Tokyo, Japan). Following micro-CT, under anesthesia, mice were euthanized via cardiac puncture. Blood was collected (0.8-0.9 ml) via cardiac puncture followed by removal of organs such as liver, kidney and spleen. Following exsanguination, mortality was confirmed by incising the heart and ensuring that no respiratory movement occurred for ≥3 min.

Hematoxylin and eosin (H&E) staining. Liver tissue and epididymal white adipose tissue (eWAT) were fixed using 10% neutral buffered formalin at room temperature for 8 h, embedded in paraffin wax, and cut serially into 10-µm sections. Sections were stained with H&E at room temperature (hematoxylin, 4 min; eosin, 2 min) and histologic alterations were observed and photographed under a light microscope (magnification, x100; Olympus CX21; Olympus Corporation) (18).

Biochemical analysis. To evaluate hepatic steatosis, levels of hepatic total triglycerides (TG) and cholesterol (TC) were determined by homogenizing liver tissues in a chloroform/methanol mixture (2:1, v/v). TG and TC concentrations were then measured using commercially available kits (TG; AM 1575-K; TC; AM 202-K; Asan Pharmaceutical Co., Ltd., Seoul, Korea) as detailed previously (19). In addition, rapid enzymatic assay kits were used to measure serum levels of TC, TG and high-density lipoprotein cholesterol (HDL-c; AM 203-K; Asia Pharmaceutical Co., Ltd., Seoul, Korea), and low density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (20) as follows: LDL-c=TC-HDL-c-cholesterol (TG/5).

ELISA kits were used to measure leptin (ADI-900-019A; Enzo Life Sciences Inc., Farmingdale, NY, USA) and adiponectin (47-ADPMS-E01; R&D Systems, Inc., Minneapolis, MN, USA). HFD-induced liver damage was assessed by measuring the serum enzyme activities of alanine transaminase (ALT) and aspartate aminotransferase (AST) using the ALT/AST cassette test kit (Alere Cholestech LDX® System; Alere, Inc., Waltham, MA, USA) (18).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from eWAT and liver tissues using TRIzol RNA Isolation Reagent, according to the manufacturer's instructions. The synthesis of cDNA was performed with 2 µg RNA using the SuperScript III First Strand Synthesis kit protocol. qPCR was performed using the ABI Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.). PCR was performed using the following conditions: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing at 60°C for 1 min, as detailed previously (21). Primer sequences were as follows: GAPDH, forward 5'-CATGGGCTTCCGTGTTTC-3' and reverse 5'-CTTGGTCTCCATGTTAGC-3'; PPARγ, forward 5'-GATGGAAGACCACTCGATT-3' and reverse 5'-AACCATGGTGCTGCTTTT-3'; and C/EBPα, forward 5'-TTGTGTTTGGATTATTCGCGT-3' and reverse 5'-CCAAGAAGTGGTGGACAGAT-3'. The comparative quantification cycle method was used to measure the relative quantification of each gene and mRNA levels were normalized with GAPDH, as detailed previously (6).

Western blot analysis. eWAT and liver tissues were lysed in ice-cold radioimmunoprecipitation assay buffer (sc-24948; Santa Cruz Biotechnology, Inc., CA, USA) for 40 min and centrifuged (12,000 x g) for 20 min at 4°C, as detailed previously (21). Protein quantification was determined via bicinechonic acid assay and protein samples (20 µg/lane) were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, NEPALI et al: ESEE REDUCES HFD-INDUCED OBESITY IN MICE 2953
USA), which were then blocked with 5% skimmed milk in tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Membranes were then probed with primary antibodies at 4°C overnight, washed with TBST 4 times, incubated at room temperature with HRP-conjugated secondary antibody for 45 min, and rewashed with TBST 3 times. Proteins were visualized using an enhanced chemiluminescence detection kit (EMD Millipore, Bedford, MA, USA) and a Fusion FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France).

Ultra-pressure liquid chromatography (UPLC). UPLC was performed using an ACQUITY UPLC BEH C18 column (2.1x50 mm, 1.7 µm) and a photodiode array detector (Waters Corporation, Milford, MA, USA), as detailed previously (17). Elution was performed at a flow rate of 0.15 ml/min using distilled water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) in gradient mode (B 5% from 0-1 min, B 5-95% from 1-16 min, B 95-100% from 16-18 min, and B 100% from 18-26 min at a flow rate of 0.15 ml/min at 25°C. Detection was performed at a wavelength of 330 nm.

Statistical analysis. Data are presented as the mean ± standard error of the mean, and were analyzed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was used to measure the significant difference followed by Tukey’s post hoc test for comparison of means. P<0.05 was considered to indicate a statistically significant difference.

Results

ESEE reduces body weight gain in HFD-fed mice. To investigate the anti-obesity effect of ESEE, mice were fed HFD with or without ESEE (2, 10 and 50 mg/kg, oral gavage daily) or with GC (200 mg/kg, oral gavage daily) for 6 weeks. Food intake and body weights were recorded once weekly. At the end of the 6-week treated period, HFD mice had a mean body weight gain of 41.2%, whereas ND mice exhibited a gain of 18.7%. Mean percentage body weight gain of HFD mice was significantly reduced by ESEE (50 mg/kg) supplementation (Fig. 1A and B). In addition, no significant difference in dietary intake was observed among the HFD-fed and ESEE administered groups (Fig. 1C), but 50 mg/kg ESEE significantly reduced the food conversion efficiency as compared with HFD-fed mice (Fig. 1D). These results indicate that ESEE induced adipose weight loss, and reduced body weight gain and the percentage of food efficiency.

ESEE reduces fat deposition in organs in HFD-fed mice. Micro-CT analysis results demonstrated that orbital fat volume percentage was significantly lower in ESEE (50 mg/kg) supplementation (Fig. 2A and B). In addition, no significant difference in dietary intake was observed among the HFD-fed and ESEE administered groups (Fig. 1C), but 50 mg/kg ESEE significantly reduced the food conversion efficiency as compared with HFD-fed mice (Fig. 1D). These results indicate that ESEE induced adipose weight loss, and reduced body weight gain and the percentage of food efficiency.
ESEE suppresses histological changes in the eWAT and liver tissues of HFD-fed mice. Increases in lipid accumulations in the liver and eWAT are commonly observed in HFD-fed obese mice (17). H&E staining revealed enlarged or hypertrophic eWAT in the HFD group, and smaller eWAT sizes in ESEE supplemented mice (Fig. 3A). In addition, the histological study revealed enlarged hepatocytes, as evidenced by excessive vacuolation in the HFD group as compared with the ND group. As presented in Fig. 3B, ESEE markedly reduced vacuolization and lipid droplet numbers in the liver tissues of HFD mice. Hepatic steatosis was determined by measuring hepatic TG and TC levels. The present results demonstrated that ESEE (10 and 50 mg/kg) supplementation significantly reduced HFD-induced levels of TG and TC in liver tissues (Fig. 3C and D). Serum ALT and AST levels were also measured, which are clinical markers of liver damage. The results indicated that ESEE significantly reduced the HFD-induced serum levels of AST and ALT compared with the HFD group (Fig. 3E and F). These data indicate that ESEE inhibited HFD-induced hepatic steatosis and serum liver enzymes.

ESEE improves biochemical parameters of blood in HFD-fed mice. HFD-fed mice exhibited significantly higher serum levels of TC, low-density lipoprotein cholesterol (LDL-c) and TG, and significantly lower levels of high-density lipoprotein cholesterol (HDL-c) than mice in the ND group. ESEE significantly inhibited TC (50 mg/kg), LDL-c (10 and 50 mg/kg) and TG (50 mg/kg) increases, and increased HDL-c levels (10 and 50 mg/kg) vs. HFD-fed mice (Fig. 4A-D). Furthermore, ESEE significantly inhibited leptin levels and increased adiponectin levels. At a dose of 50 mg/kg, ESEE decreased mean leptin levels by 64.7% (Fig. 4E) and increased mean adiponectin levels by 51.1% compared with the HFD group (Fig. 4F).

ESEE inhibits transcription factors of adipogenesis in HFD-fed mice. RT-qPCR and western blotting were performed to measure the mRNA and protein expressions, respectively, of transcription factors of adipogenesis, such as PPARγ and C/EBPα. The results demonstrated that ESEE (10 and 50 mg/kg) significantly inhibited the mRNA and protein expressions of PPARγ and C/EBPα in eWAT tissues (Fig. 5) and liver tissues (Fig. 6), as compared with the HFD group.

Chemical components of ESEE by UPLC. UPLC assay demonstrated that corilagin and ellagic acid were the main components of ESEE (Fig. 7). However, other peaks observed were unidentified and require further study.
Figure 3. Effect of ESEE on histological changes and lipid accumulations in eWAT and liver tissues of HFD-induced obese mice. Histological changes were determined via H&E staining in (A) eWAT and (B) liver tissue sections (magnification, x100) of mice from the (a) ND, (b) HFD, (c) HFD+GC, (d) HFD+ESEE (2), (e) HFD+ESEE (10) and (f) HFD+ESEE (50)-fed mice. Lipid accumulation in liver was assessed by measuring hepatic (C) TG and (D) TC levels. Serum levels of (E) AST and (F) ALT were measured using commercial kits. Results are presented as the mean ± the standard error of the mean (n=6). *P<0.05 vs. ND; †P<0.05 vs. HFD; ‡P<0.01 vs. ND. ESEE, ethanol extract of Euphorbia supina; HFD, high-fat diet; eWAT, epididymal white adipose tissue; ND, normal diet; (2), 2 mg/kg; (10), 10 mg/kg; (50), 50 mg/kg; GC, Garcinia cambogia; TG, total triglycerides; TC, total cholesterol; AST, aspartate aminotransferase; ALT, alanine transaminase.
Figure 4. Effect of ESEE on blood biochemical parameters in HFD-fed mice. Enzymatic methods were used to measure serum levels of (A) triglyceride (B) total cholesterol (C) HDL-c, and (D) LDL-c. ELISA was used to measure serum levels of (E) leptin and (F) adiponectin. Results are presented as the mean ± the standard error of the mean (n=6). #P<0.05 vs. ND; *P<0.05 and **P<0.01 vs. HFD. ESEE, ethanol extract of Euphorbia supina; HFD, high-fat diet; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; ND, normal diet; (2), 2 mg/kg; (10), 10 mg/kg; (50), 50 mg/kg; GC, Garcinia cambogia.

Figure 5. Effect of ESEE on PPARγ and C/EBPα expressions in eWAT tissues of HFD-induced obese mice. Reverse transcription-quantitative polymerase chain reaction was performed to measure mRNA levels of (A) PPARγ and (B) C/EBPα. Western blotting was used to determine the protein expressions of (C) PPARγ and C/EBPα. (D) Relative band intensities of blots from three experiments. Results are presented as the mean ± the standard error of the mean (n=6). #P<0.05 vs. ND; *P<0.05 and **P<0.01 vs. HFD. ESEE, ethanol extract of Euphorbia supina; HFD, high-fat diet; PPARγ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT/enhancer-binding protein-α; ND, normal diet; (2), 2 mg/kg; (10), 10 mg/kg; (50), 50 mg/kg; GC, Garcinia cambogia.
Discussion

The induction of obesity in animals via HFD supplementation is the most popular means of mimicking obesity in human, and a number of studies have been conducted using HFD-induced obese mouse models, in which HFD supplementation increases abdominal fat and body weights (22). Previous studies have reported crude plant extracts and natural
compounds isolated from them induce weight loss and prevent diet-induced obesity (17,23). Although, *E. supina* is known to have potent anti-oxidant, anti-aging and anti-cancer effects, its effects on obesity have not been previously studied. Therefore, the present study was designed to investigate the effects of ESEE in HFd-fed obese mice.

To the best of our knowledge, this is the first study to report the anti-obesity effects of ESEE in an HFd-fed obese mice model. ESEE was observed to reduce body weight gain and fat accumulation in organs versus HFd-fed mice. Furthermore, ESEE did not affect food intake levels but did reduce the food conversion efficiencies, which indicated that ESEE treatment reduced the abilities of animals to convert food into body mass. ESEE also suppressed weight increases of eWAT, and of organs including the liver, spleen and kidneys versus HFd-fed mice. These findings are in accordance with those of a previous study, in which a capsicoside G-rich fraction from pepper exhibited anti-obesity effects in HFd-induced obese mice (23).

Obesity is associated with the development of fatty liver (24), and an imbalance between food consumption and combustion leads to hepatic steatosis (25). Therefore, H&E staining was performed to measure the histological morphologies of adipose and liver tissues. ESEE was observed to inhibit adipocyte hypertrophy and the accumulation of lipid droplets in the liver. Furthermore, ESEE reduced hepatic steatosis as evidenced by reductions in the levels of hepatic TG and TC. In addition, ESEE reduced HFd-induced serum markers of fatty liver, including AST and ALT. These findings were similar to those reported for the anti-obesity effect of Gomisin N in HFd-induced obese mice (26). ESEE reduced HFd-induced increases in serum lipid profiles, such as TC, TG and LDLc, and increased serum levels of HDLc, which were in accordance with its observed suppressive effect on HFd-induced fatty liver and hyperlipidemia in HFd-fed mice. WAT synthesizes leptin, which serves important roles in the suppression of food intake and the control of body weight (27), and adiponectin, which regulates glucose levels and serves an important role in hepatic fatty acid oxidation (28,29). Therefore, the levels of leptin and adiponectin in ESEE treated HFd-fed mice were examined. The present results demonstrated that ESEE significantly reduced serum leptin levels and increased serum adiponectin levels in HFd-fed mice. These findings concur with those of a previous study, which demonstrated that *Triticum aestivum* sprouts have an anti-obesity effect in HFd-fed mice (17).

PPARγ and C/EBP-α are important transcription factors of adipogenesis (30). PPARγ is expressed predominantly in adipose tissues and serves an important role in adipocyte differentiation, lipid storage, and glucose homeostasis (31,32). PPARγ binds with C/EBP-α promoter region and this binding induces the expression of C/EBP-α, which directly controls adipocyte differentiation (33). In the present study, ESEE significantly inhibited the mRNA and protein expressions of PPARγ and C/EBP-α, which was previously detected in green tomato extract-treated HFd-induced obese mice (34).

Chemical analysis by UPLC in the present study showed that corilagin and ellagic acid were the main components of ESEE, but other observed peaks were not identified. Many studies have reported the anti-inflammatory, anti-cancer and hepatoprotective effects of corilagin (35-37). Previous studies have also reported that ellagic acid improved hepatic steatosis and suppressed serum resistin levels in obese mice (38,39), and that ellagic acid suppresses lipid accumulation by inhibiting adipogenesis and cell proliferation (40). In a previous study, it was demonstrated that the anti-obesity effect of *Geranium thunbergii* was due to the presence of components including corilagin and ellagic acid (41). According to the present findings, corilagin and ellagic acid are the major components if ESEE, and therefore, it is hypothesized that these compounds were at least partially responsible for the observed anti-obesity effects of ESEE.

The present study has some limitations that should be noted. First, ESEE was administered at various doses to HFd-fed mice but not ND-fed mice. Second, the effects of corilagin and ellagic acid on HFd-induced obesity were not investigated.

To the best of our knowledge, the present study was the first to demonstrate that ESEE effectively inhibits adipogenesis, and that ESEE contains anti-adipogenic molecules that downregulate transcription factors for adipogenesis, such as C/EBPα and PPARγ. These findings suggest that ESEE possesses anti-obesity effects, and that it may be a promising agent for the treatment of obesity and its metabolic syndrome.

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**Competing interests**

The authors declare that they have no competing interests.

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