Anti-obesity effects of *Diospyros lotus* leaf extract in mice with high-fat diet-induced obesity

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Received June 26, 2018; Accepted September 27, 2018

DOI: 10.3892/ijmm.2018.3941

Abstract. *Diospyros* (*D.*) *lotus* has been demonstrated to have antioxidant and anti-inflammatory properties. The purpose of the present study was to evaluate the effect of *D. lotus* leaf water extract (DLE) on high-fat diet (HFD)-induced obesity in C57BL/6 mice. The present study first investigated the effect of DLE on the lipid accumulation and triglyceride (TG) contents in 3T3-L1 cells, and the results revealed that treatment with DLE suppressed the lipid accumulation and TG level. Subsequently, the anti-obesity effects of DLE were investigated *in vivo*. Oral administration of DLE reduced the body weight gain, food efficiency ratio, and liver and visceral fat weight in mice fed with a HFD. DLE administration in these mice also reduced TG, total cholesterol, low-density lipoprotein cholesterol, glucose, insulin and leptin levels, as well as the atherogenic index. Furthermore, DLE administration decreased hepatic steatosis, as well as serum aspartate transaminase, alanine transaminase and alkaline phosphatase levels in mice fed with HFD. It was further observed that treatment of the HFD-fed mice with DLE prevented lipid peroxidation, while it recovered glutathione depletion and the activities of superoxide dismutase, catalase and glutathione peroxidase. In conclusion, the current study suggests that the anti-obesity effect of DLE may provide positive insights as a potential functional food ingredient for the prevention of obesity.

Introduction

While developing countries are fighting against the spread of infectious diseases, developed countries are currently focusing on combating non-communicable diseases, including obesity, which has exhibited a progressive rise in prevalence over the past years. However, although obesity is a disease of developed countries, its prevalence in developing countries is also on a rise (1). Thus, it can be seen as one of the most serious public health challenges of the 21st century. Obesity can actually be defined as the accumulation of excess body fat. The accumulation of fat results from a high-fat diet (HFD), caloric-dense diet, sedentary lifestyle, increased urbanization and psychosocial stress (2). Obesity is a risk factor for atherosclerosis, cancer, type 2 diabetes, dyslipidemia and metabolic syndrome. A study has even linked obesity with excess adipocyte accumulation, lung problems, and respiratory symptoms. In that study, accumulation of fat tissue in the abdominal wall and around the abdominal organs hampered the movement of diaphragm, reduced lung expansion during inspiration and also decreased lung capacity (3). In addition, the increase in adipose tissue in obesity has been linked to the production of systemic oxidative stress, through the peroxisomal and mitochondrial oxidation of fatty acids, and the overconsumption of oxygen, producing reactive oxygen species (4). Furthermore, liver damage in obesity has been reported, as diet-induced obesity is associated with liver inflammation (5).

Several drugs, including phentermine, fluoxetine, orlistat, sibutramine and rimonabant, as well as lifestyle modifications and even surgery are used for the treatment of obesity. However, the associated side effects of nausea, dizziness, insomnia, diarrhea, dyspepsia and constipation cannot be overlooked. A healthy lifestyle can be difficult to maintain, while surgery is reserved for terminal obesity cases. Thus, researchers are focusing on the need for new natural therapies and different methods of combating obesity. Plant extracts with phytochemicals including polyphenols, curcumin, resveratrol and proanthocyanidins have become increasingly popular in the past decades due to their anti-adipogenic properties (6-8). Herbal medicines have been used to control weight and for the treatment of obesity. For instance, the *Garcinia cambogia* fruit extract, containing hydroxycitric acid, has frequently been used for weight control, and has demonstrated no toxic effects (9).

Diospyros (*D.*) *lotus*, which belongs to the Ebenaceae family, is a deciduous tree native to China, Korea, Japan, Brazil, Turkey and Italy, and its fruit is widely consumed for its sedative, antidiabetic, antiseptic and antitumor properties (10). Notably, compounds isolated from *D. lotus* fruit and three new dimeric naphthoquinones isolated from *D. lotus*

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Key words: anti-obesity, *Diospyros lotus* leaf, high-fat diet, fatty liver, antioxidant

roots have been demonstrated to have antiproliferative and cytotoxic effects on a series of cancer cell lines, including COR-L23, C32, A375, CaCo-2 and mouse T-cell lymphoma cell lines (11-13). Furthermore, *D. lotus* leaves have been widely used for the treatment of muscle and joint pain of the lower back (14).

A study on the leaf extract of another species, *D. kaki*, revealed that this extract improved hyperglycemia, dyslipidemia and liver fat accumulation in type 2 diabetic db/db mice (15). Another recently published study indicated that *D. kaki* fruit in synergy with *Citrus unshiu* peel demonstrated an anti-obesity effect by inhibiting pancreatic lipase (16). However, to the best of our knowledge, no studies have examined the anti-obesity effect of *D. lotus* leaves. The present study was, therefore, conducted to investigate the anti-obesity effect of *D. lotus* leaf water extract (DLE) on adipocyte differentiation in both *in vitro* and *in vivo* studies, using a cell model and an HFD-induced obesity model in mice.

Materials and methods

Chemicals. Glucose, triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) assay kits were purchased from Asan Pharmaceutical Co., Ltd. (Seoul, Korea). Apigenin, 3-isobutyl-1-methylxanthine (IBMX) and Oil red O were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) activity colorimetric assay kits were purchased from BioVision, Inc. (Milpitas, CA, USA). Catalase (CAT), glutathione (GSH), and glutathione peroxidase (GPx) assay kits were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Superoxide dismutase (SOD) and malondialdehyde (MDA) quantification kits were purchased from Calbiochem (EMD Millipore, Billerica, MA, USA) and Cell Biolabs, Inc. (San Diego, CA, USA), respectively. A Mouse Leptin Quantikine enzyme-linked immunosorbent assay (ELISA) kit and ultrasensitive mouse insulin ELISA kit were from R&D Systems, Inc. (Minneapolis, MN, USA) and Crystal Chem, Inc. (Elk Grove Village, IL, USA), respectively. Ethanol, xylene, and paraffin wax came from Daejung Co., Ltd., (Busan, Korea), Junsei Chemical Co., Ltd., (Tokyo, Japan) and Leica Biosystems (Wetzlar, Germany), respectively. All other chemicals used were of reagent grade and purchased from Sigma-Aldrich (Merck KGaA), unless otherwise stated.

Plant material and extract preparation. *D. lotus* leaves used in the present study were harvested from Bugwi-Myeon, Jinan-Gun, Jeonbuk, Korea on June 30th, 2015. The plant was identified and authenticated by Professor Hong-Jun Kim from the College of Oriental Medicine, Woosuk University (Jeonju, Korea) and a voucher specimen (no. 2015-06-30-DLE) was deposited in our laboratory. The leaves were washed four times with distilled water, steamed for 5 min, dried at 40˚C for 12 h and then extracted (100 g) in distilled water (2,000 ml) at 100˚C for 30 min. The extract was passed through a 0.45-μm pore size filter paper (ADVANTEC, Togo, Japan). The filtered extract was concentrated by vacuum evaporation and lyophilized to obtain the dry extract (15.2 g), which was stored at -20˚C for use in further experiments.

Cell culture and Oil Red O staining. 3T3-L1 pre-adipocytes obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin in an incubator with temperature of 37˚C and 5% CO_2. To induce adipocyte differentiation, the 3T3-L1 pre-adipocytes were seeded in 6-mm cell culture dishes at a density of $5x10^4$ cells/ml. After 2 days, when the cells had reached 100% confluence, differentiation was induced by replacing the media with DMEM containing 10% FBS and 0.5 mM IBMX, 1 μM dexamethasone and 1 μg/ml insulin (designated hereafter as MDI), along with 200 or 400 μg/ml DLE, or with 10 μg/ml apigenin as the positive control. After 3 days of culture, the medium was replaced with DMEM containing serum, insulin, and DLE or apigenin, followed by replacement with fresh DMEM with the same constituents after a further 2 days of incubation (temperature 37˚C and 5% CO_2).

For Oil red O staining, cells ($5x10^5$ cells/ml) in 60 mm culture dishes were fixed with 10% formalin for 1 h and then washed with 60% isopropanol, followed by incubation in 5 ml Oil red O working solution for 5 min at room temperature. Subsequent to incubation, the cells were immediately washed five times with distilled water, and images were captured using a Leica light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Next, Oil red O was dissolved in 100% isopropanol, and the absorbance were measured by a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) at 540 nm.

TG content in 3T3-L1 adipocytes. Total TG content in 3T3-L1 adipocytes was determined using a commercial TG assay kit according to the manufacturer's protocol.

Animals and diet. The *in vivo* study involved 4-week-old male C57BL/6J mice, obtained from Orient Bio, Inc. (Iksan, Korea). The mice were fed with commercial standard laboratory diet and water *ad libitum*, and maintained in an air-conditioned room with a temperature of 22±2˚C, humidity of 50-60%, and a 12-h light-dark cycle. The animal experiment was approved by Jeonju University Institutional Animal Care and Used Committee (no. JJU-IACUC-2015-04) and the protocols were performed following the guidelines set by the institution.

Following 1 week of acclimation, the mice were randomly divided into five groups of 6 mice each, including the normal control, HFD control, HFD + 200 mg/kg DLE, HFD + 400 mg/kg DLE, and HFD + 20 mg/kg apigenin groups. The normal control group received a commercial standard diet (AIN-76A; Research Diet, Inc., New Brunswick, NJ, USA), throughout the experimental period, while the HFD model and experimental groups were fed with a diet high in fat content (Rodent Diet with 45 kcal% fat; Research Diet Inc.) for 8 weeks to induce obesity. After obesity was induced, the mice in the HFD control and experimental groups were maintained on the high-fat diet while being administered orally with DLE and Apigenin for a further 10 weeks. Apigenin was used as a positive control.

Following completion of all treatments, the mice were fasted for 12 h and then anesthetized with ether. Blood was collected by a cardiac puncture and then the mice were

euthanized. The blood samples were allowed to clot for 30 min at room temperature and centrifuged at 2,000 x g for 15 min at 4˚C. The liver and abdominal fat tissues were also completely excised and cleansed with saline. Moisture was completely removed with a filter paper, and the weights were measured using an electronic balance. The tissue samples were quickly frozen with liquid nitrogen and stored at -80˚C until used for further studies.

Weight gain, food intake and food efficiency ratio (FER). The body weight and food intake of mice were measured once per week. The FER was calculated according to the following formula: FER (%)=[body weight gain (g/day)/food intake (g/day)] x100.

Biochemical analysis. Serum glucose, TG, TC and HDL-C levels were measured using the corresponding assay kits, according to the manufacturer's protocol. Low-density lipoprotein cholesterol (LDL-C) was calculated according to previously described methods (17), using the following formula: LDL-C=[TC-HDL-C-(TG/5)]. Insulin and leptin levels in the serum samples were evaluated via commercial ELISA kits, according to the manufacturer's instructions. The atherogenic index was calculated using the following formula: (TC-HDL-C)/HDL-C. Furthermore, the activities of hepatic enzymes AST and ALT in the serum were measured using commercial assay kits, according to the manufacturer's protocol.

Histological analysis. For histological analysis, the liver tissues were fixed in 10% neutral formalin for 3 days at room temperature, washed in 4 changes of phosphate-buffered saline (1 h each), dehydrated in a series of graded ethanol (from 60-100%, 30 min each), cleared twice in xylene (30 min each) and embedded in of paraffin wax three separate times at 70˚C (30 min each). The tissues were then fixed with paraffin wax overnight at 4˚C and sectioned (5-μm) using a microtome. Next, the tissues sections were stained with hematoxylin for 3 min and eosin for 0.5 min (H&E) at room temperature.

Determination of oxidative stress and antioxidant markers. After the mice were sacrificed, liver tissues (0.3 g) were homogenized in 0.5 ml phosphate-buffered saline and then centrifuged at 2,000 x g for 15 min at 4˚C to obtain the supernatant which was stored at -70˚C for subsequent use. Hepatic MDA and GSH levels were measured using commercially available assay kits, according to the manufacturer's protocol. Liver SOD, CAT, and GPx activities were also measured as described in the corresponding kit manuals.

High-performance liquid chromatography (HPLC) analysis. HPLC was performed to determine the active compounds in DLE using an Agilent 1100 series system (Agilent Technologies, Inc., Santa Clara, CA, USA), equipped with a binary pump delivery system, a degasser (G1379A), an autosampler (G1313A), a diode array detector (G1315B) and Agilent Eclipse XDB-C18 column (4.6x250 mm, 5-μm particles). The mobile phase was composed of 0.5% formic acid in water (solvent A) and acetonitrile (solvent B), and the gradient elution were as follows: 0 min, 5% solvent B; 10 min, 10% solvent B; 50 min, 40% solvent B; 54 min, 100% solvent B; and then held for 10 min prior to returning to the initial conditions. Other HPLC conditions were as follows: Flow rate, 1 ml/min; UV detection wavelength, 280 nm; sample injection volume, 20 μl; and column temperature, 30˚C. All standards were identified based on retention time. The integration of each component on the chromatogram was processed using the Agilent Chemstation software (Agilent Technologies, Inc.).

Statistical analysis. The data were analyzed using the IBM SPSS statistics program (version 22; IBM Corp., Armonk, NY, USA) with one-way analysis of variance, followed by Duncan's multiple range test. A $P<0.05$ was considered to indicate a statistically significant difference. All data are presented as the mean ± standard deviation.

Results

Inhibitory effect of DLE on lipid accumulation in 3T3-L1 cells. To evaluate the effect of DLE on adipocyte differentiation, lipid accumulation was evaluated using the Oil red O staining method. As presented in Fig. 1A and B, DLE treatment significantly inhibited lipid accumulation at the doses of 200 and 400 μg/ml in 3T3-L1 adipocytes. TG accumulation was also examined in differentiated 3T3-L1 adipocytes. DLE treatment significantly diminished the TG content in 3T3-L1 adipocytes (Fig. 1C). Apigenin used as a positive control demonstrated similar effects.

DLE reduces body weight, food intake, visceral fat and liver weight in mice with HFD-induced obesity. To investigate the anti-obesity effect of DLE, mice were fed with HFD in the presence or absence of DLE (200 and 400 mg/kg), and the body, liver and visceral fat weights of the mice were measured. As presented in Table I, the final body weight gain in the HFD model group was significantly higher compared with that in the normal control group. However, body weight gain was markedly decreased by DLE administration at doses of 200 and 400 mg/kg, without significantly altering the food intake. The FER (%) was significantly higher in the HFD group as compared with that in the normal control group, whereas this ratio was significantly reduced by oral administration of DLE at doses of 200 and 400 mg/kg. Furthermore, the liver and visceral fat weights were significantly increased in the HFD group, while DLE administration at 200 and 400 mg/kg markedly decreased these weights. Apigenin administration also suppressed the body weight gain, FER, and liver and visceral fat weights in HFD-fed mice.

Effect of DLE on serum lipid and glucose levels. The effects of DLE on the serum lipid levels of HFD-fed mice were investigated at the end of the experimental period (Fig. 2A-E). Serum TG, TC, LDL-C and LDL-C/HDL-C ratio levels in the HFD group were significantly higher compared with those in the normal control group ($P<0.05$). However, oral administration of DLE at doses of 200 and 400 mg/kg led to a decrease in the level of TG, and TC, with a significant difference obtained only with the group treated with 400 mg/kg of DLE ($P<0.01$). Also, the levels of LDL-C and LDL-C/HDL-C ratio were significantly decreased with 200 and 400 mg/kg DLE treatment ($P<0.05$). Apigenin significantly reduced these levels, with the exception of TC levels (Fig. 2A, B, D, and E). HDL-C

Table I. Effect of DLE on body weight, weight gain, food intake, FER, liver weight and visceral fat weight in HFD-induced obese mice.

Group	Normal control	HFD	HFD + 200 mg/kg DLE	HFD + 400 mg/kg DLE	HFD + apigenin
Initial body weight (g)	29.83±0.99	39.53±2.49[b]	39.63±1.88	39.33±5.77	38.54±2.08
Final body weight (g)	31.13±1.00	47.13±1.43[b]	44.50±2.25	42.95±5.37	43.42±1.69[c]
Weight gain (g)	1.30±0.34	7.60±1.17[b]	4.88±0.48[d]	3.63±1.90[c]	4.88±1.31[c]
Food intake (g/day)	3.12±0.33	2.35±0.33[b]	2.14±0.21	2.42±0.36	2.43±0.32
FER (%)	3.21±1.26	29.58±5.06[b]	19.81±6.91[c]	17.27±8.52[c]	20.04±5.37[c]
Liver weight (g)	1.21±0.35	1.84±0.24[a]	1.55±0.15	1.39±0.26	1.48±0.21
Visceral fat weight (g)	0.38±0.09	2.11±0.15[b]	1.46±0.39[c]	1.34±0.26[c]	1.84±0.37

Results are presented as the mean ± standard deviation. [a]P<0.05 and [b]P<0.001 vs. the control group; [c]P<0.05 and [d]P<0.01 vs. the HFD group. The FER was calculated as follows: FER (%)=body weight gain (g/day)/food intake (g/day) x100. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; FER, food efficiency ratio.

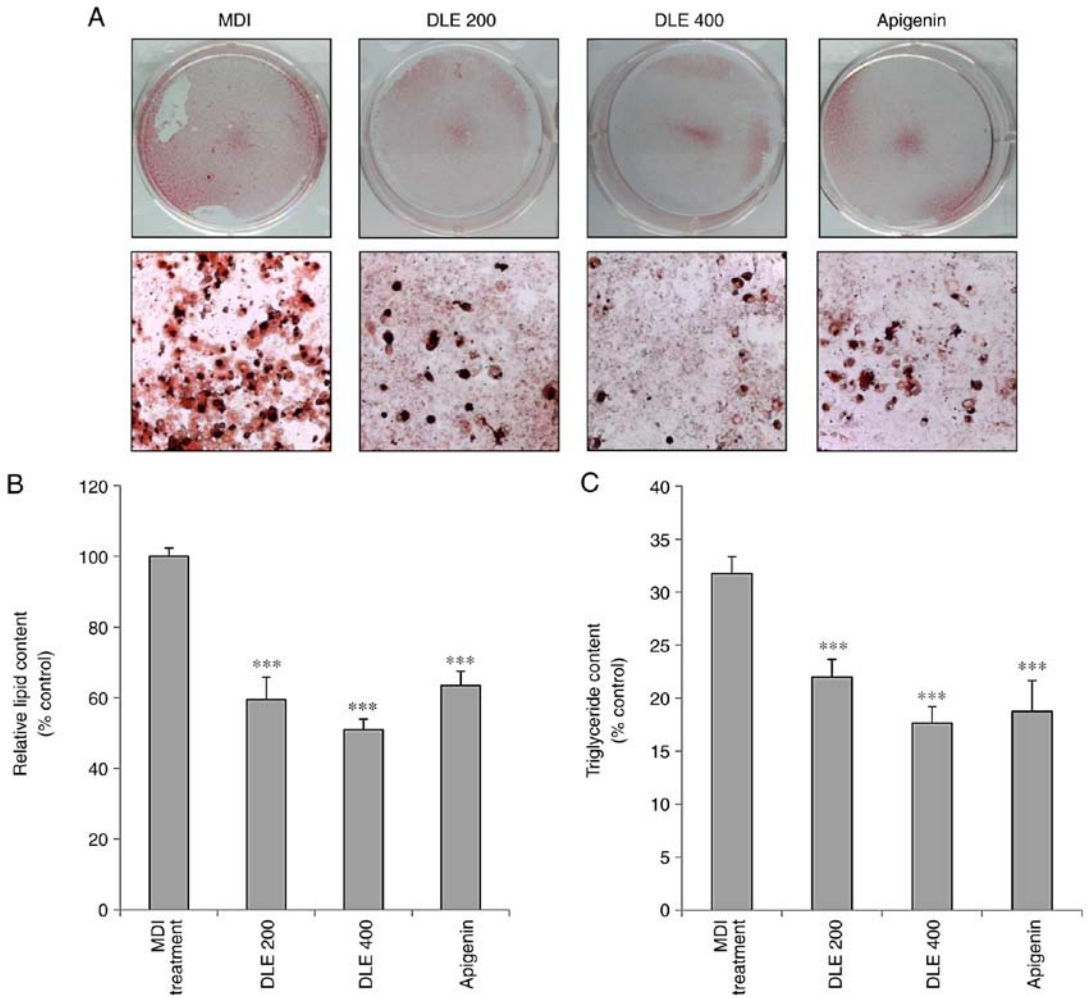

Figure 1. (A) Images of dishes and microscopic view (magnification, x100) of lipid accumulation in the control and experimental adipocytes treated with or without DLE and apigenin. (B) Oil red O stain was dissolved using isopropyl alcohol, and the lipid contents were measured using a microplate reader. (C) Adipocyte cell lysates were used to quantify triglycerides levels. Results are presented as the mean ± standard deviation. ***P<0.001 vs. the MDI (control) treatment. DLE, *Diospyros lotus* leaf water extract; MDI, 0.5 mM IBMX, 1 μM dexamethasone and 1 μg/ml insulin.

levels were markedly increased in the HFD group, while DLE and apigenin administration slightly increased HDL-C levels (Fig. 2C). However, there was no significant difference in the HDL-C levels between the HFD group and the DLE or apigenin-treated groups. Furthermore, the serum glucose levels were significantly increased in the HFD group compared

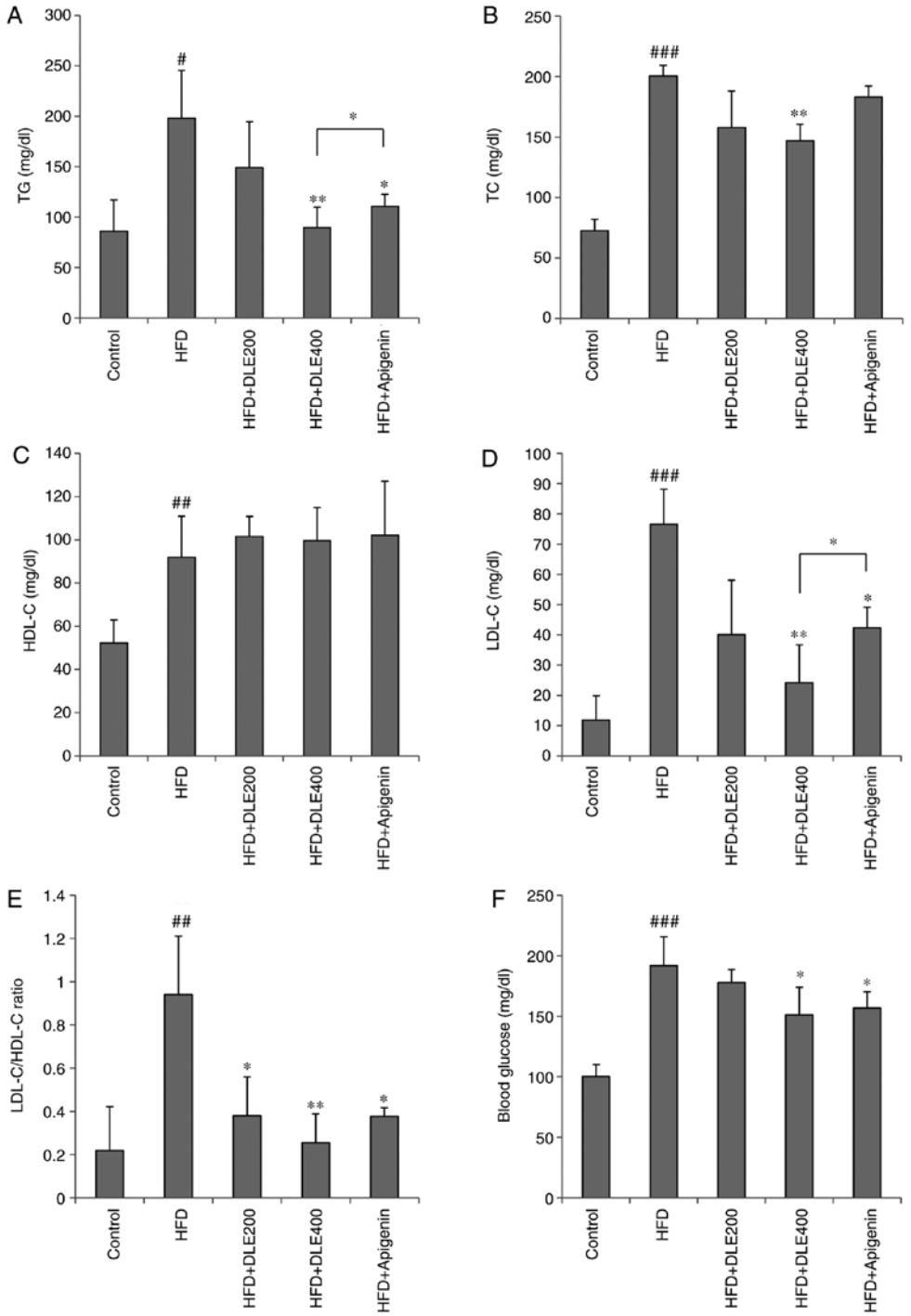

Figure 2. Effect of DLE on lipid profile and glucose levels in the serum of mice with HFD-induced obesity. At the end of the experimental period, serum lipid profiles and glucose levels were measured. (A) TG, (B) TC, (C) HDL-C, (D) LDL-C, (E) LDL-C/HDL-C ratio, and (F) glucose levels. Results are presented as the mean ± standard deviation. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 vs. the control group; *P<0.05 and **P<0.01 vs. the HFD group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

with the control. However, the glucose levels were decreased in HFD-fed mice following treatment with 200 and 400 mg/kg DLE, with significant difference obtained only with 400 mg/kg treatment group. Apigenin treatment demonstrated a similar effect with DLE 400 mg/kg treatment (Fig. 2F).

Effect of DLE on the atherogenic index. The atherogenic index was determined using the values of TC and HDL-C. As presented in Fig. 3, the atherogenic index was significantly higher in the HFD group as compared with that in the normal control group. However, this increase in the atherogenic index was significantly reduced by oral administration of DLE at doses of 200 and 400 mg/kg. Apigenin had a similar effect, although DLE at the dose of 400 mg/kg significantly decreased the atherogenic index in comparison with apigenin (P<0.05).

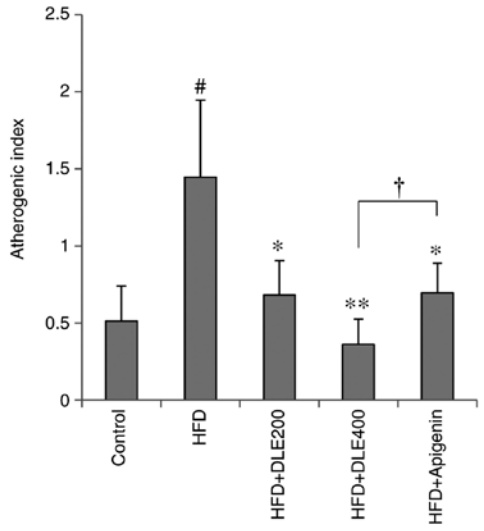

Figure 3. Effect of DLE on atherogenic index in the serum of mice with HFD-induced obesity. Atherogenic index was calculated by the following formula: (TC-HDL-C)/HDL-C. Results are presented as the mean ± standard deviation. #P<0.05 vs. the control group, *P<0.05 and **P<0.01 vs. the HFD group. †P<0.05 vs. the Apigenin group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

Effect of DLE on serum insulin and leptin levels. The serum insulin and leptin levels were also determined. As presented in Fig. 4A, serum insulin was increased significantly in the HFD group, while oral administration of DLE at the doses of 200 and 400 mg/kg decreased the serum insulin levels with a significant difference obtained with the 400 mg/kg DLE-treated group (P<0.05). The serum leptin levels were also significantly increased in the HFD group (P>0.001), whereas they were decreased in the DLE-treated group at doses of 200 and 400 mg/kg (Fig. 4B). However, only the 400 mg/kg treated group exhibited a significant difference. Apigenin administration also reduced the elevation of serum insulin and leptin levels similar to the effect of DLE at both doses, in mice fed with the high-fat diet.

Effect of DLE on liver function parameters. Changes in serum liver function parameters, including AST, ALT and ALP levels, are indicated in Fig. 5. The levels of the liver function parameters were significantly increased in the HFD group when compared with those in the normal control group. However, DLE administration at both doses, significantly decreased the ALT and ALP levels in HFD-fed mice, except for AST levels which demonstrated a significant difference only in the 400 mg/kg-treated group. Similar to the DLE 200 and 400 mg/kg treated, apigenin treatment also resulted in a reduction of these parameters in the mice with HFD-induced obesity, with no significant differences among the group.

Effect of DLE on liver histology. Next, the study evaluated the effect of DLE on liver morphology by H&E staining. As presented in Fig. 6, the lipid droplets were increased significantly in the HFD group in comparison with those observed in the normal control group. However, DLE at 400 mg/kg and apigenin administration significantly decreased the lipid droplets in the mice with HFD-induced obesity.

DLE treatment reduces oxidative stress in HFD-induced obese mice. MDA is a known marker of lipid peroxidation involved in oxidative stress. In the present study, hepatic MDA levels were significantly increased in the HFD group. However, DLE at both doses and apigenin administration significantly reduced the MDA levels (Fig. 7). There was no statistically significant difference between the DLE and apigenin groups. In addition, the activities of antioxidant enzymes SOD, CAT and GPx were markedly diminished, while the hepatic GSH antioxidant was significantly reduced in the HFD group, compared with the normal control group. However, DLE administration at doses of 200 and 400 mg/kg resulted in a significant increase in SOD and CAT activities, while GPx activities and GSH levels were significantly increased only with 400 mg/kg treatment, compared with the HFD group levels. Apigenin administration demonstrated similar effects as those observed by treatment with 400 mg/kg DLE (Fig. 8).

Chemical composition of DLE. HPLC analysis was performed to characterize the active compounds in DLE. The results of the HPLC analysis revealed that gallic acid (peak 1), myricitrin (peak 2) and astragalin (peak 3) were the active compounds in DLE (Fig. 9).

Discussion

Previous studies by Uddin *et al* (10), Rauf *et al* (11,12,14), and Loizzo *et al* (13) have verified various functions of *D. lotus*, including its antioxidant, anti-inflammatory, antiseptic, antidiabetic, antitumor and sedative properties. In the current study, in order to elucidate the potential of DLE as a food ingredient for preventing obesity, we focused on the regulation of adipogenesis and lipogenesis *in vitro* and *in vivo*. The *in vitro* results revealed that DLE treatment ameliorated lipid and TG accumulation in 3T3-L1 adipocytes, indicating that DLE was able to inhibit adipocyte differentiation. Next, the present study demonstrated the anti-obesity effects of DLE in HFD-induced obesity in mice. The results indicated that DLE inhibited HFD-induced obesity by preventing the increase of body weight and visceral fat weight, the alteration of lipid profile and liver function parameters, and the increase in insulin and leptin levels, as well as by improving the antioxidant defense system. According to the findings of a previous study (18), apigenin was used as a positive control, and it was also able to reverse these conditions in mice with HFD-induced obesity.

Obesity is associated with numerous diseases, including atherosclerosis, cancer, type 2 diabetes, dyslipidemia and metabolic syndrome (3). Several studies have demonstrated that HFD causes elevation of body weight, liver weight and fat mass, as well as increased TG, TC, HDL-C, LDL-C and glucose levels in the serum (19-21). In the present study, DLE clearly decreased body weight gain, liver weight, visceral fat accumulation and FER levels, while it also reduced serum TG, TC, LDL-C, and glucose levels, with the exception of HDL-C levels. TG, TC, and LDL-C are associated with fat accumulation and are major risk factors for dyslipidemia (22). The ratio of LDL-C to HDL-C is associated with cardiovascular disease and dyslipidemia (19,23). The TC/HDL-C and TG/HDL-C molar ratios are associated with coronary heart disease (24). Furthermore, the atherogenic index is used as a predictor

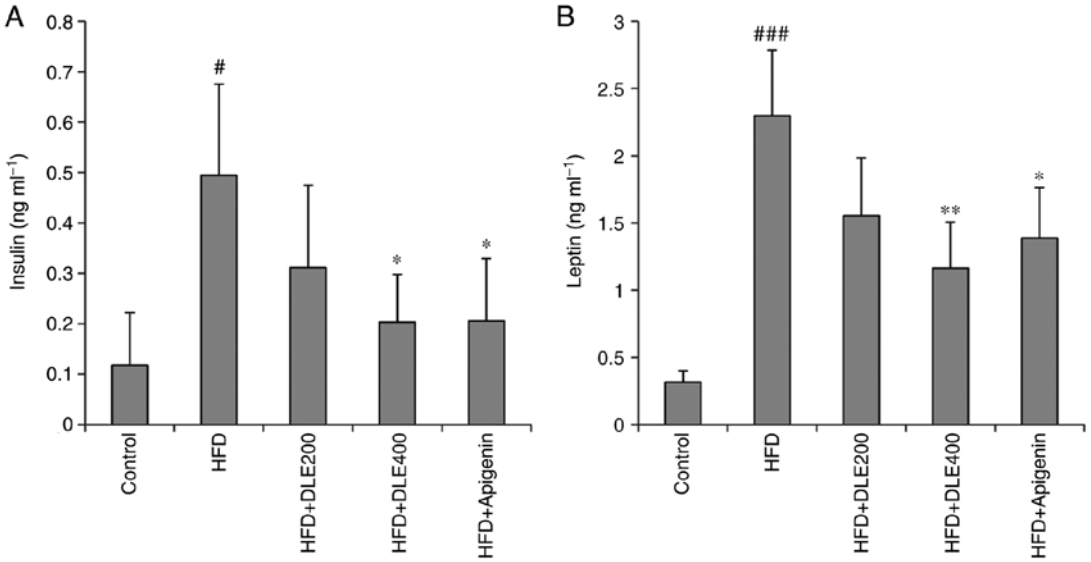

Figure 4. Effect of DLE on (A) insulin and (B) leptin levels in the serum of mice with HFD-induced obesity. Results are presented as the mean ± standard deviation. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. the normal control; *P<0.05 and **P<0.01 vs. the HFD group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet.

of atherosclerosis and coronary artery disease (25,26). The increase in cholesterol is associated with the risk of fatty liver and atherosclerosis (5). The results of the current study indicated that these levels were lower in the DLE-treated mice in comparison with the untreated HFD-fed group. Therefore, the presented results imply that DLE was able to decrease the levels of the lipid profile, and ameliorate cardiovascular disease, atherosclerosis and dyslipidemia in HFD-induced obesity.

Obesity is also known to cause insulin resistance, which serves a key role in type 2 diabetes. Previous studies have indicated that in mice fed with HFD, an increase in fasting blood glucose and insulin levels was induced, while plant extracts were demonstrated to suppress the HFD-induced increase in these levels in obese animals (27-29). In the present study, the HFD also led to an increase in serum glucose and insulin levels and this increase in glucose and insulin levels were markedly decreased when mice fed with the HFD were administered DLE.

Leptin is secreted by adipocytes and is a hormone involved in energy balance and in the regulation of glucose metabolism. It serves a role in reducing appetite and increasing energy consumption (30,31). It has been reported that leptin levels in the serum are increased in HFD-induced obese mice (32,33), causing leptin resistance (34). In the present study, it was proven that DLE reduced serum leptin levels in HFD-induced obese mice. Therefore, the results suggested that DLE may ameliorate insulin and leptin resistance induced by HFD in obese mice.

Serum AST, ALT and ALP levels have been widely used as major markers of liver damage. It has been reported that HFD administration increased serum AST, ALT and ALP levels in obese animals (35-37). To assess liver function in the current study, these markers were measured in the serum of mice with HFD-induced obesity. The results revealed that DLE administration reduced the levels of AST, ALT and ALP, suggesting that DLE attenuated the liver damage caused by HFD. The author's previous study has also demonstrated similar activities in acetaminophen-induced acute liver damage in mice (38).

It has been reported that obesity also induces hepatic steatosis, characterized by excessive fat accumulation in the liver, and exhibits liver cell injury caused by oxidative stress (27,37). The data of the current study indicated that DLE diminished hepatic steatosis caused by HFD, corresponding with the reduction of liver parameters. Furthermore, HFD-induced obesity is known to cause oxidative damage through lipid peroxidation, the depletion of endogenous antioxidants and the reduction of the activities of antioxidant enzymes, such as SOD, CAT and GPx (19,39,40). In the present study, HFD feeding of mice resulted in oxidative damage as seen by the reduction of the major endogenous antioxidant GSH, the decrease in SOD, CAT and GPx activities, and the induction of lipid peroxidation products. However, DLE administration not only significantly prevented lipid peroxidation, but also recovered GSH levels, and SOD, CAT and GPx activities in the liver. These results suggest that DLE may ameliorate HFD-induced hepatic steatosis through inhibition of oxidative stress. In previous studies, plant extracts have been reported to protect against obesity-induced oxidative damage in mice (41,42). Our previous study also demonstrated that DLE protects against UVB-induced oxidative damage and acetaminophen-induced oxidative liver damage in mice (38,43). Therefore, it can be suggested that DLE may be a potent antioxidant used for preventing the oxidative stress associated with HFD-induced obesity.

HPLC analysis revealed that DLE contains myricitrin, gallic acid and astragalin. In a previous study, we reported that ethanol extracts of *D. lotus* leaf contained myricitrin as the main flavonoid compound (43). It has been demonstrated that plant extracts containing myricitrin exhibit anti-obesity effects in HFD-induced obese mice (4). A recent study has demonstrated that myricitrin protects against diabetic cardiomyopathy in mice (44). In addition, oral administration of gallic acid in HFD-induced obese mice ameliorated impaired glucose and lipid homeostasis (45). A previous

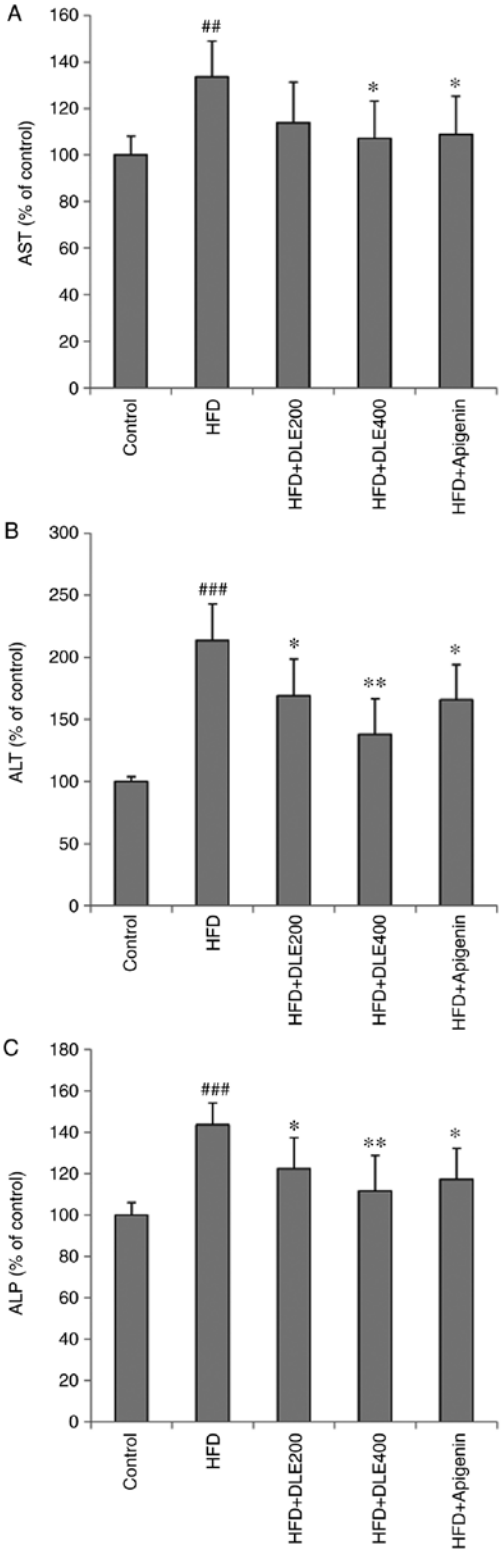

Figure 5. Effect of DLE on liver function parameters levels in the serum of mice with HFD-induced obesity. (A) AST, (B) ALT and (C) ALP levels. Results are presented as the mean ± standard deviation. ##P<0.01 and ###P<0.001 vs. the control group; *P<0.05 and **P<0.01 vs. the HFD group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase.

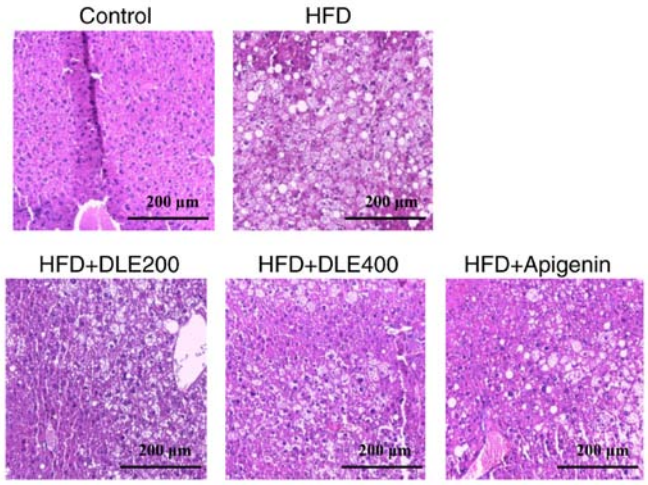

Figure 6. Effect of DLE on hepatic morphology in HFD-induced obese mice, examined by hematoxylin and eosin staining (magnification, x100; scale bar, 200 μm). DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet.

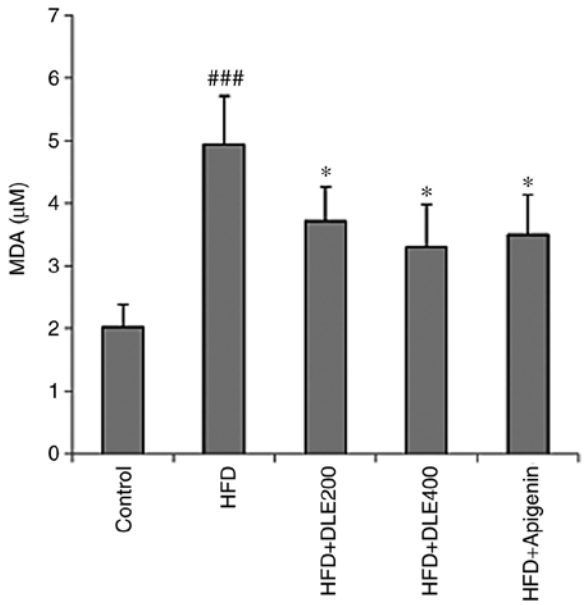

Figure 7. Effect of DLE on lipid peroxidation levels in the liver tissue of mice with HFD-induced obesity. MDA levels in the different groups are shown. Results are presented as the mean ± standard deviation. ###P<0.001 vs. the control group; *P<0.05 vs. the HFD group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; MDA, malondialdehyde.

study has also reported that astragalin stimulate lipolysis in the visceral adipose tissue of mice (46). Therefore, the anti-obesity effect of DLE may be attributed to myricitrin, gallic acid and astragalin flavonoids. However, this needs to be confirmed by further studies.

In conclusion, the present study demonstrated that DLE administration restored the body, liver and visceral fat weights, as well as the TG, TC, HDL-C, LDL-C, glucose, insulin, leptin, AST, ALP and ALP levels in mice with HFD-induced obesity. In addition, it was also revealed that DLE was able to reverse the obesity-induced the hepatic steatosis, lipid peroxidation, and GSH depletion, as well as activities of antioxidant enzymes, including SOD, CAT, and GPx in the liver of obese mice. These data suggest that DLE may be an effective ingredient for the treatment and prevention of HFD-induced obesity.

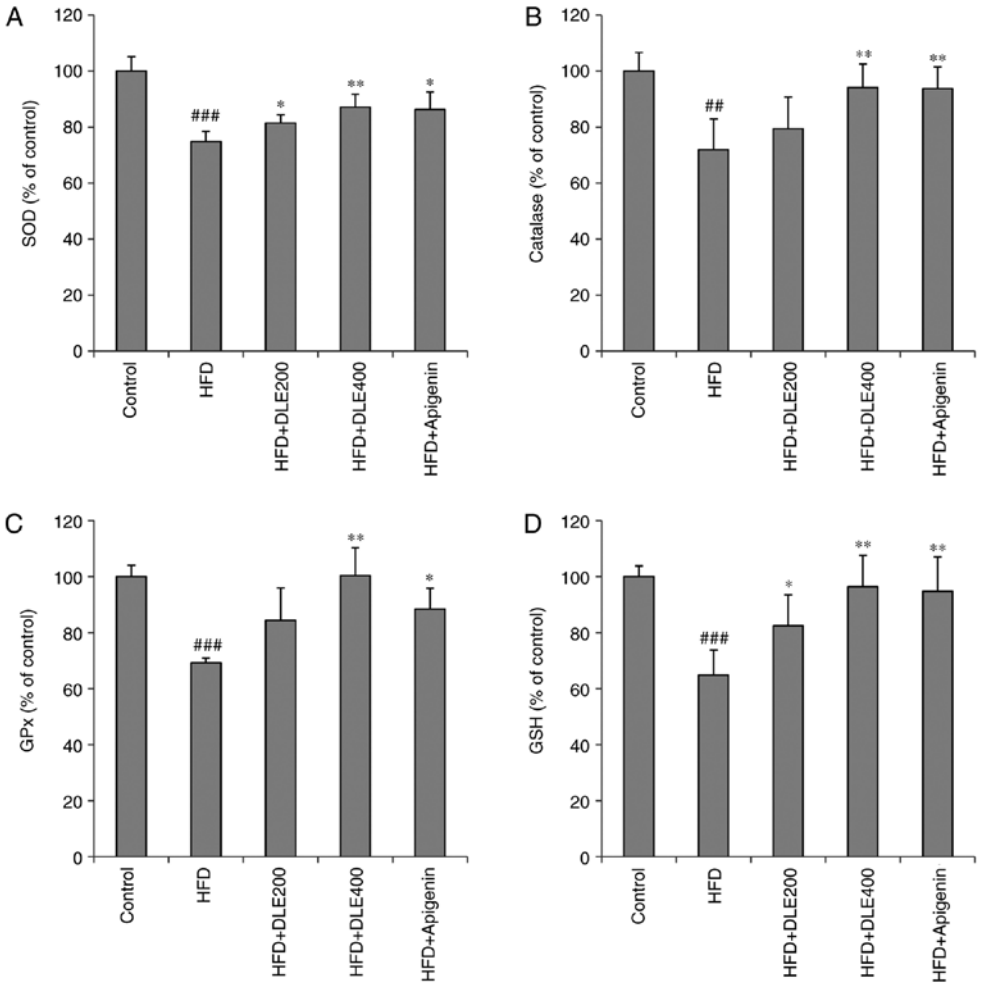

Figure 8. Effect of DLE on antioxidant defense system in liver tissue of mice with HFD-induced obesity. (A) SOD, (B) CAT and (C) GPx activities, and (D) GSH levels. Results are presented as the mean ± standard deviation. ##P<0.01 and ###P<0.001 vs. the control group; *P<0.05 and **P<0.01 vs. the HFD group. DLE, *Diospyros lotus* leaf water extract; HFD, high-fat diet; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione.

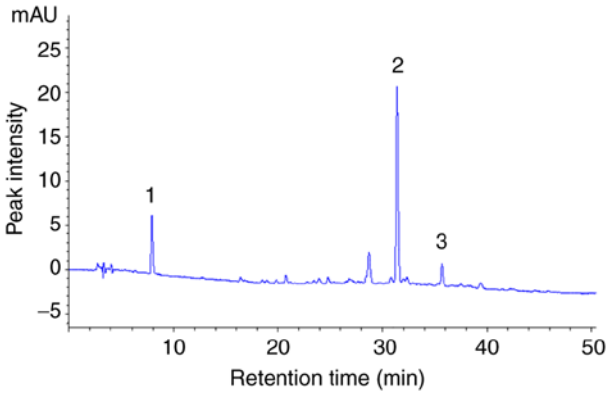

Figure 9. Phytochemicals identified in the *Diospyros lotus* leaf water extract. Comparative chromatograms of *D. lotus* leaf extracts obtained by high-performance liquid chromatography at 280 nm. Peak numbers represents the following compounds: Peak 1, gallic acid; peak 2, myricitrin; peak 3, astragalin.

Acknowledgements

The authors would like to thank Mr. Denis Nchang Che (Department of Food Science and Technology, Chonbuk National University, Jeonju, Republic of Korea) for grammar editing and Mr. Jae Young Shin (Department of Health Management, Jeonju University, Jeonju, Republic of Korea) for animal care.

Funding

The present study was financially supported by the Ministry of SMEs and Startups, Korea, under the 'Regional Specialized Industry Development Program (R&D, Project number R0006168)' supervised by the Korea Institute for Advancement of Technology.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BMK, BOC and SIJ designed the research. BMK and BOC performed the study. BMK, BOC and SIJ analyzed the data and wrote the manuscript. SIJ supervised the research project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocols were performed following approval from Jeonju University Institutional Animal Care and Use Committee (no. JJU-IACUC-2015-04).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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