Clitorin, a Flavonoid Compound of Papaya, Ameliorates Non-Alcoholic Fatty Liver Disease in Western Diet- induced Mice and Oleic Acid-induced HepG2 Cells

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is usually correlated with metabolic diseases, such as obesity, insulin resistance, and hyperglycemia. Herein, we investigated the inhibitory effects and underlying governing mechanism of clitorin in a western diet (WD)-induced mouse model, and in oleic acid-induced HepG2 cells. Male C57BL/6 mice were fed a normal diet, WD, WD + 10 or 20 mg/kg orlistat, and WD + 10 or 20 mg/kg clitorin. HepG2 cells were treated with 1 mM oleic acid to induce lipid accumulation with or without clitorin. Clitorin administration reduced body weight gain and hepatic steatosis symptoms in WD-induced mice. Additionally, clitorin administration decreased the expression of sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer binding protein α (C/EBPα) in WD-induced mice. Moreover, clitorin administration significantly decreased the mRNA levels of liver X receptor (LXR) and acetyl-CoA carboxylase (ACC); additionally, it enhanced the mRNA levels of peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyltranserase-1 (CTP-1), as well as adenosine monophosphate-activated protein kinase (AMPK) mRNA levels, in the liver of WD-induced mice. Furthermore, clitorin treatment significantly impeded lipid accumulation in oleic acid-induced HepG2 cells. Our findings demonstrated that clitorin is a potentially efficacious candidate for NAFLD treatment.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most ubiquitous chronic liver disease in Western countries, affecting nearly 25% of adults worldwide [1]. In the United States, the number of NAFLD cases is anticipated to increase from 83.1 million in 2015 to 100.9 million in 2030 [2]. NAFLD is characterized by excessive internal fat accumulation in hepatocytes. It ranges from relatively benign nonalcoholic fatty liver to the aggressive form termed nonalcoholic steatohepatitis, typifying both fatty liver and liver inflammation [3]. NAFLD is usually correlated with metabolic diseases, such as obesity, insulin resistance, hyperglycemia, and hypertension. Although considerable progress has been achieved with regard to drug development for NAFLD, no suitable therapeutic agent has yet been approved [2]. Therefore, there is a critical need to develop optimal therapeutic agents for NAFLD.

Hepatic steatosis can be stimulated by increased *de novo* lipogenesis and decreased fatty acid oxidation [4]. When the high-fat diet feeding, peroxisome proliferator-activated receptor γ (PPARγ) is the early-induced lipogenic transcription factor in the liver [5]. Hepatic lipid synthesis is also modulated by several important transcription factors, including the liver X receptor (LXR) and sterol regulatory element–binding protein 1c (SREBP1c) [6]. As a major transcription factor, SREBP1 has been reported to control key enzymes involved in fatty acid biosynthesis, such as acetyl-CoA carboxylase (ACC) [6]. ACC catalyzes a master rate-controlling step in *de novo* lipogenesis and fatty acid oxidation, that is, the synthesis of malonyl-CoA, which is both an intermediate in fatty acid synthesis and an allosteric inhibitor of carnitine palmitoyltranserase-1 (CTP-1) [7]. Peroxisome proliferator-activated receptor α (PPARα) is closely associated with the transcription of genes related to hepatic beta-oxidation, including *CPT-1* [8]. This beta-

oxidation provides energy in the form of ATP, and the activity of adenosine monophosphate-activated protein kinase (AMPK) hinders fat accumulation by inducing lipolysis and beta-oxidation in adipose tissue and liver [9].

Papaya (*Carica papaya* L.) is a fruit crop that is widely grown in tropical and sub-tropical regions. Traditionally, papaya plants are used to treat various ailments such as asthma, ulcers, eczema, diabetes, helminth infections, and fever [10]. Papaya plants have been reported to possess therapeutic potential for metabolic disorders, such as diabetes mellitus type 2, causing alterations in both glycemic metabolism and lipid metabolism, oxidative stress, and in models of arterial hypertension [10,11]. Previous profiling indicates that four flavonoids, including manghaslin, clitorin, rutin, and nicotiflorin, were identified in papaya plants [12,13]. Among them, we focused on clitorin, a kaempferol glycoside, because it has only been reported antioxidant effects [14]. Based on these findings, the present study was designed to provide basic data to delineate the pharmacological effects of clitorin on the alleviation of NAFLD in western diet (WD)-induced mice and oleic acid-induced HepG2 cells.

Results

Clitorin reduced the total body weight and weight gain in the WD-induced NAFLD mouse model

A representative photograph of the experimental mice revealed that the 20 mg/kg clitorin-administered group mice were slightly smaller than those in the WD group (Fig. 1A). When mice were fed a WD for 12 weeks, we observed a significant difference in the change in total body weight and weight gain between the CON and WD groups. The clitorin-administered group mice displayed significantly lower total body weight and weight gain than the WD group mice (Fig. 1B and 1C). However, we did not observe any differences in food intake among the WD-fed groups (Fig. 1D). These data clearly showed that clitorin administration reduces body weight gain in WD-induced mice.

Clitorin ameliorated liver steatosis in the WD-induced NAFLD mouse model

Because NAFLD participants manifested higher serum levels of TC and TG than those without NAFLD [15], we first investigated the impact of clitorin on serum TC and TG levels. Compared to the corresponding levels recorded in the CON group, we detected significant increases in the serum TC and TG levels in the WD group; furthermore, the marked increases in serum TC and TG levels were all lower in the clitorin-administered groups (Fig. 2A and 2B). Second, the liver weight and liver index (mg/body weight) in the WD group were significantly higher than the corresponding parameters in the CON group. In contrast, clitorin administration significantly reversed these changes compared to the parameters observed in the WD group (Fig. 2C and 2D). Moreover, we observed smaller sized and fewer hepatocytic lipid vacuoles in the livers of the clitorin-administered group mice than in the WD group mice (Fig. 2E). Finally, compared to the corresponding levels observed in the WD group, serum ALT and AST levels also tended to decrease in the clitorin-administered mice; however, these were not statistically significant (Fig. 2F and 2G). Altogether, these data indicated that the NAFLD mouse model, utilized in the present study, was an

optimal and completely established one; additionally clitorin administration demonstrated a protective effect against NAFLD in mice challenged with WD.

Clitorin regulated adipogenesis, lipogenesis, and fatty acid oxidation in the WD-induced NAFLD mouse model liver

To investigate the mechanisms suppressing lipid accumulation in the livers of WD mice administered with clitorin, adipogenic and lipogenic transcriptional expression profiles were further examined. In the liver, the protein expression levels of *SREBP1*, as well as *PPARγ* and *C/EBPα*, were higher in the WD group; however, this was rescued by clitorin administration (Fig. 3A). qRT-PCR analysis revealed that the upregulated SREBP1, PPARγ, and C/EBPα protein expression levels in the livers of WD mice coincided with increases in *SREBP1*, *PPARγ*, and *C/EBPα* mRNA levels. Notably, the marked upregulation of these genes was strongly suppressed in the livers of clitorin-administered mice compared to the corresponding expression profiles in the WD group (Fig. 3B, 3C, and 3D). Since high rates of hepatic lipogenesis and lipid oxidation are distinguishing features of NAFLD in rodents and humans, we next assessed the impact of clitorin on lipogenesis and fatty acid oxidation genes. The mRNA levels of both *LXR* and *ACC*, hepatic lipogenesis genes, were significantly inhibited in the clitorin-administered groups (Fig. 3E). Furthermore, the mRNA levels of *CPT-1* and *PPARα*, fatty acid oxidation genes, were significantly elevated in the clitorin-administered groups (Fig. 3E). We also found that the AMPK mRNA level, which was eliminated in the WD group, was augmented by clitorin administration (Fig. 3E). These data supported the concept that clitorin counteracts WD-induced NAFLD by regulating lipogenesis and fatty acid oxidation genes in the liver.

WD: western diet; NAFLD: nonalcoholic fatty liver disease; SREBP1 sterol regulatory element binding protein 1; PPARγ: peroxisome proliferator activated receptor γ; C/EBPα: CCAAT/enhancer binding protein α; ACC: acetyl-CoA carboxylase; AMPK: adenosine monophosphate-activated protein kinase; LXR: liver X receptor

Clitorin improved oleic acid-induced steatosis in HepG2 Cells

The MTT assay was used to examine the effects of various concentrations of clitorin on cell viability. Clitorin treatment (0-200 μM) did not induce cytotoxicity for 24 h in HepG2 cells (Fig. 4A). Accordingly, we designated three doses of clitorin at 50, 100, and 200 μM for further study. Oil Red O staining showed a significant increase in lipid droplets in 1 mM oleic acid-treated cells compared to the lipid droplet concentration in non-treated cells. However, this lipid accumulation was significantly decreased by high concentrations of clitorin in oleic acid-induced HepG2 cells (Fig. 4B and 4C).

Discussion

Obesity directly contributes to the abundance of hepatic TG accumulation, which is linked to NAFLD progression [16]. Immoderate exposure to a high-fat diet has been determined as a key attribute in an increasing number of NAFLD patients, which is demonstrated by the fact that the prevalence of NAFLD in

obese patients is reported to be up to 90% [17]. Consequently, a high-fat diet is widely used to construct NAFLD animal models [18]. In the present study, clitorin administration significantly reduced the body weight gain in a WD-induced NAFLD mouse model (Fig. 1). Moreover, clitorin administration significantly reduced the liver index (mg/body weight) and notably decreased lipid droplet concentration without hepatic toxicity (ALT and AST) in the livers of WD-induced mice; this demonstrates that it suppresses hepatic steatosis (Fig. 2). Orlistat was used as a positive control because it has been reported that orlistat effectively alleviates steatosis and may serve as a viable treatment option for NAFLD [19].

The hepatic effect of PPARγ appears to be steatogenic; hepatocyte-specific *PPARγ* knockout mice showed a remarkable decrease in the number of hepatic lipid vacuoles, as well as downregulation of *de novo* lipogenesis activators [20]. Conversely, PPARγ overexpression in the liver induced by HFD feeding leads to lipid accumulation, which is the initiation step in the development of NAFLD [5]. CCAAT/enhancer binding proteins (C/EBPs), including C/EBPα and SREBP1, are also considered key regulators of adipogenesis. SREBP1 plays an important role in the regulation of *de novo* lipogenesis in the liver [21]. SREBP1c levels are enhanced in the fatty livers of obese, insulin-resistant, and hyperinsulinemic *ob*/*ob* mice [7]. In addition, SREBP1c expression is also elevated in patients with NAFLD; additionally, in concordance with its lipogenic role, hepatic triglyceride levels are higher in SREBP1c-overexpressing transgenic mice [22]. Thus, SREBP1, PPARγ, and C/EBPα are crucial transcription factors that upregulate the expression of genes modulating fat accumulation in the liver. Clitorin administration remarkably downregulated the protein and mRNA expression of *SREBP1*, *PPARγ*, and *C/EBPα* in the livers of WD-induced NAFLD mice (Fig. 3A, 3B, 3C, and 3D).

LXRs are involved in hepatic lipogenesis via direct regulation of SREBP1c [23], which positively modulates ACC expression [24]. ACC catalyzes a key rate-limiting step in fatty acid biosynthesis, and is also associated with the control of fatty acid oxidation by the synthesis of malonyl-CoA, an inhibitor of CPT-1 [4]. CPT-1 is the rate-limiting enzyme in fatty acid oxidation [25]. Indeed, inhibition of the liver-specific isoform ACC1 in mice ameliorated hepatic triglyceride levels in mice by simultaneously suppressing fatty acid biosynthesis and augmenting fatty acid beta oxidation in the liver [4]. CPT-1 is also linked to PPARα expression. PPARα activation gives rise to the transcription of *CPT-1*, a target gene that is responsible for beta-oxidation, as it allows fatty acids to reach the mitochondrial matrix [8]. Among the three PPAR isotypes, PPARα, PPARβ/δ, and PPARγ, PPARα is the most abundant isotype in hepatocytes and is related to numerous aspects of lipid metabolism [26] and high fatty acid oxidation rates [27]. Ineffective PPAR-α sensing leads to diminished energy burning, resulting in hepatic steatosis and steatohepatitis [28]; it is thus inferred that it can potentially prevent NAFLD. Therefore, targeting lipogenesis and beta-oxidation genes is considered a promising therapeutic approach to control NAFLD. AMPK, a major energy sensor of the cell, downregulates ACC activity to suppress lipid biosynthesis [29]. In addition, AMPK regulates hepatic and adipose lipid metabolism by modulating lipogenesis, lipolysis, gluconeogenesis, and adipogenesis; AMPK inhibits *de novo* lipogenesis by downregulating *PPARγ*, *C/EBPα*, and *SREBP1*; furthermore, it promotes fatty acid oxidation by upregulating *CPT-1a* [30]. Our results showed that clitorin administration

significantly decreased the mRNA levels of *LXR* and *ACC*, which are lipogenic genes. It also enhanced the mRNA levels of *PPARα* and *CTP-1*, beta-oxidation genes, as well as *AMPK* mRNA levels in the livers of WD-induced NAFLD mice (Fig. 3E).

HepG2 cells have been widely used to model NAFLD *in vitro*, and steatosis in this cell line can be induced by treatment with oleic acid [31–33]. Several studies have also used HepG2 cells to evaluate their effects on the management and prevention of NAFLD [34–36]. Hence, we used an oleic acid-induced HepG2 cell model to evaluate the effect of clitorin on NAFLD *in vitro*. Consistent with *in vivo* experiments, our results showed that clitorin treatment significantly diminished lipid accumulation in oleic acid-induced differentiated HepG2 cells (Fig. 4).

Taken together, clitorin administration reduced the adipogenic genes, including SREBP1, PPARγ, and C/EBPα in WD-induced NAFLD mice liver. In addition, it significantly decreased the mRNA levels of lipogenic genes *LXR*, and *ACC*; additionally it enhanced the mRNA levels of beta-oxidation genes *PPARα* and *CTP*-1, as well as *AMPK* mRNA levels, in the liver of WD-induced mice. The present study is the first to report on the positive impact of clitorin on NAFLD. Furthermore, our findings provide basic data, which lead to deeper understanding of the pharmacological effects of clitorin on the improvement of NAFLD in WD-induced mice and oleic acid-induced HepG2 cells.

Materials And Methods

Chemicals and reagents

Oil Red O powder, oleic acid, and methyl alcohol were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Minimum Essential Medium (MEM), fetal bovine serum (FBS), and penicillin were purchased from Life Technologies Inc. (Grand Island, NY, USA). Orlistat was purchased from Tokyo Chemical Inc. (Tokyo, Japan). The Research Diets (New Brunswick, NJ, USA) provided 45% of the WD (D-12451). Antibodies against PPARγ (cat. no. sc-7273), C/EBPα (cat. no. sc-365318), SREBP1 (cat. no. sc-13551), and β-actin (cat. No. sc-47778) were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Preparation of clitorin

Clitorin, a compound derived from *Carica papaya* L., was identified by Professor Agung Nugroho (Lambung Mangkurat University, Indonesia). The leaves of *Carica papaya* were collected from a papaya farm near Pelaihari City, South Kalimantan Province. The collected leaves were dried completely at 40°C. The dried powder of *C. papaya* leaf (750 g) was extracted thrice with MeOH (6 L) under reflux at 70°C for 5 h. Thereafter, clitorin was isolated and purified from the fraction. Freeze-dried samples were dissolved in dimethyl sulfoxide.

Experimental animal care protocols and treatment cycles

Six-week-old male C57BL/6J mice were procured from Daehan Biolink (Daejeon, Republic of Korea). The mice were maintained under conditions of controlled temperature (22 ± 2°C) and humidity (55 ± 9 %), with a 12-h light/dark cycle. After a week of adjustment, the mice were fed 45 % WD for 7 weeks, except for the normal diet group (CON). After 7 weeks, the mice were divided into five groups of six mice each: WD group, WD + treatment group with 10 or 20 mg/kg orlistat as a positive control, and WD + treatment group with 10 or 20 mg/kg clitorin. Orlistat and clitorin were orally administered to the mice once daily for 4 weeks. Mice in the CON and WD groups were administered water as a vehicle. The mice were allowed free access to water and food, and their body weight and food intake were measured every week. The livers of the mice were excised, cleaned with phosphate-buffered saline (PBS), weighed, and directly stored at −80°C. All protocols were performed under the Ethical Committee for Animal Care and the Use of Laboratory Animals, Sangji University (approval document no. 2017-22).

Serum analysis

During blood sample collection, the animals were already under the influence of terminal anesthesia. Blood samples were collected via cardiac puncture. The samples were centrifuged at 1000 × g for 20 min to obtain the serum samples. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), and total cholesterol (TC) were measured by enzymatic methods using commercially available kits (BioVision; Milpitas, CA, USA).

Histological analysis

The liver tissues from the mice in each group were fixed in 10% formalin, embedded in paraffin, and cut into 8 μm sections. Certain sections were stained with hematoxylin and eosin (H&E) for histological examination. Stained liver sections were observed for the evaluation of lipid droplet. All observations were performed using an Olympus SZX10 microscope (Olympus, Tokyo, Japan).

Western blot analysis

Fresh liver tissues were homogenized using PRO-PREP® (Intron Biotechnology, Gyeonggi-do, Republic of Korea), a protein extraction solution. The same amount (15–30 μg) of protein sample was separated on an 8 %–12 % sodium dodecyl sulfate polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 2.5 % skim milk solution for 30 min, incubated with PPARγ (1:1000), C/EBPα (1:1000), SREBP1 (1:1000), and β-actin (1:2500) primary antibodies overnight at 4°C, followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2500) for 2 h at 25°C. The membranes were washed thrice for 10 min with Tris-buffered saline containing Tween 20 and visualized by enhanced chemiluminescence using Amersham Imager 680 (GE Healthcare Bio-Sciences AB, Sweden).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was performed as previously described [37]. Briefly, the liver was homogenized, and total RNA was isolated using the Easy-Blue® reagent according to the manufacturer's instructions (Intron Biotechnology; Seongnam, Republic of Korea). Total RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, CA, USA) and thermocycler (Gene Amp® PCR system 9700; Applied Biosystems). qPCR analysis was conducted using a Step One Plus® Real-time PCR system (Applied Biosystems). Gene expression was determined using the comparative threshold cycle method. *GAPDH* was used as an internal control. Sequences of mouse oligonucleotide primers are presented in Table 1.

Table 1
Real-Time PCR primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
PPARγ	ATCGAGTGCCGAGTCTGTGG	GCAAGGCACTTCTGAAACCG
SREBP1	GGCTATTCCGTGAACATCTCCTA	ATCCAAGGGCAGTTCTTGTG
C/EBPα	GGAACTTGAAGCACAATCGATC	TGGTAAAGGTTCTCA
LXRα	CAGGAGACCAGGGAGGCAAC	GCAGGGCTGTAGGCTCTGCT
ACC	TTTTCGATGTCCTCCCAAACTTT	GCTCATAGGCGATATAAGCTCT
CPT-1	CTCAGTGGGAGCGACTCTTCA	GGCCTCTGTGGTACACGACAA
PPARα	CAGGAGAGCAGGGATTTGCA	CCTACGCTCAGCCCTCTTCAT
AMPK	GGTGGATTCCCAAAAGTGCT	AAGCAGTGCTGGGTCACAAG
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG

Cell culture and differentiation of HepG2 cells

The human hepatoma cell line HepG2 (No. 88065) was obtained from the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). HepG2 cells were grown in MEM containing 10 % FBS and 100 mg/L penicillin. The cells were maintained under a humidified atmosphere of 5 % CO_2 at 37°C. For induction of hepatocyte differentiation, cells were seeded at a density of 2×10^5 cells per well into 6-well plates with oleic acid (1 mM) and incubated for 48 h. After incubation, the cells were treated with different concentrations of clitorin (50, 100, and 200 μM) for 24 h.

Cell viability assay

HepG2 cells were seeded into a 96-well plate at a concentration of 1×10^6 cells per well for 24 h. After incubation, the cells were treated with different concentrations of clitorin (0–200 μM) for 24 h. After treatment, the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL) and incubated again for 4 h. The supernatant from the plates was discarded, and the

purple formazan product was dissolved in dimethyl sulfoxide. The absorbance was measured at 540 nm using an Epoch microplate spectrometer (Biotek, Winooski, VT, USA).

Oil red O staining of HepG2 cells

After cell differentiation with oleic acid, the cells were washed with PBS and fixed with 10% formaldehyde in PBS at 25°C for 1 h. Cells were then washed thrice with distilled water and stained with Oil Red O working solution (3 mg/mL in 60 % isopropanol) at 25°C for 2 h. The cells were rinsed thrice with distilled water and photographed using an Olympus SZX10 microscope. Next, the Oil Red O dye was eluted with isopropanol to determine the intracellular lipid content and was measured using an Epoch® microvolume spectrophotometer at 520 nm.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of triplicate experiments. Statistically

significant values were compared using ANOVA and Dunnett's post hoc test, and p-values < 0.05

were considered statistically significant. Statistical analysis was performed using SPSS statistical

analysis software (version 19.0, IBM SPSS, Armonk, NY, USA).

Abbreviations

adenosine monophosphate-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), CCAAT/enhancer binding protein α (C/EBPα), carnitine palmitoyltranserase-1 (CTP-1), liver X receptor (LXR), Nonalcoholic fatty liver disease (NAFLD), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor γ (PPARγ), sterol regulatory element-binding protein 1 (SREBP1), western diet (WD)

Declarations

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Author Contribution staement: D.C.C. and H.J.A. conceived and designed the experiments. D.C.C. and Y.J.P. wrote the manuscript. D.C.C. conducted the experiments. A.N. supplied the clitorin used in experimental analyses, which is derived from the papaya plant. Y.J.P., Y.M.K., and H.J.A. substantially contributed to the analysis and interpretation of data and revised the manuscript. All authors read and approved the final manuscript.

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Figures

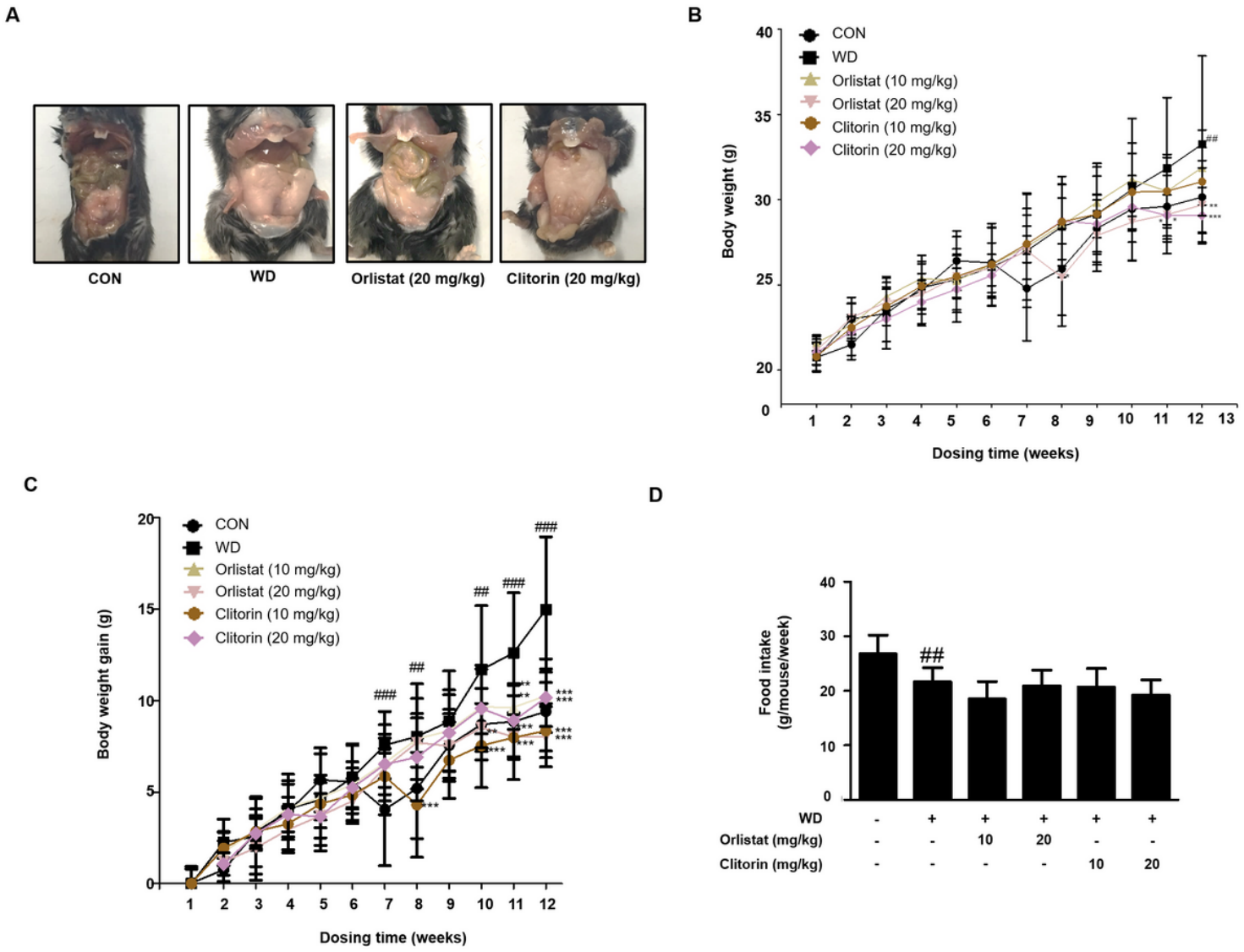

Figure 1

Effect of clitorin on total body weight, weight gain, and food intake in the WD-induced NAFLD mouse model. The WD-induced mice were administered orlistat (10 or 20 mg/kg) or clitorin (10 or 20 mg/kg) for 4 weeks, whereas control mice were fed a normal diet. (A) Macroscopic body images of the mice in each group were taken at the end of the 13-week experimental period. (B) Body weight, (C) weight gain, and (D) food intake were recorded every week. The values are represented as the mean ± SD (n=6 per group). ##P < 0.01, ###P < 0.001 vs. CON group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. WD group; significance was determined using two-way ANOVA followed by a Bonferroni post hoc test, and one-way ANOVA followed by Dunnett's post hoc test. WD: western diet; NAFLD: nonalcoholic fatty liver disease

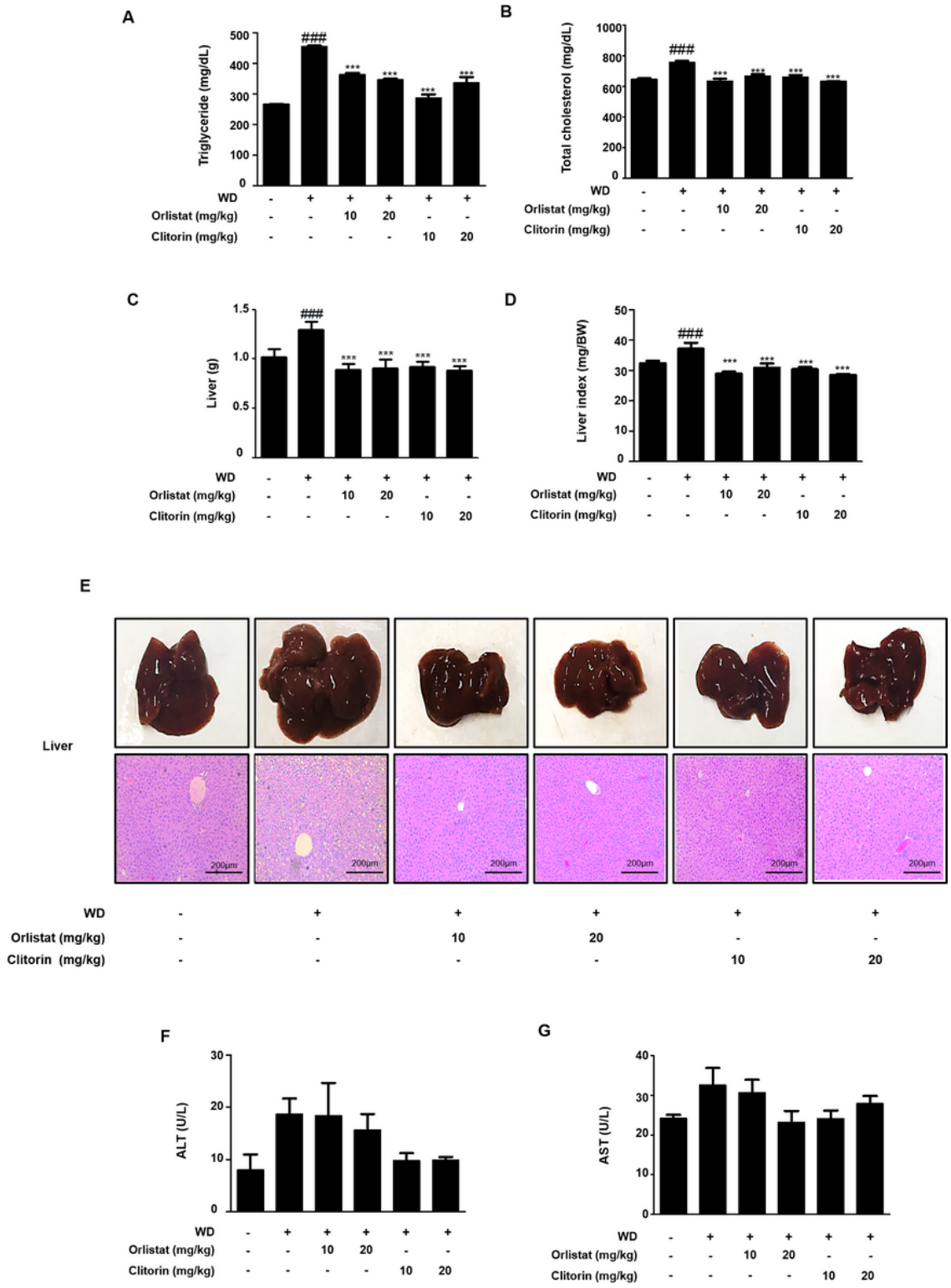

Figure 2

Effect of clitorin on liver steatosis in the WD-induced NAFLD mouse model. The levels of (A) serum TG and (B) serum TC were determined using enzymatic methods. (C) The weight of liver tissue and (D) relative liver weight ratio (mg/body weight) were measured at the end of the experimental period. (E) Morphology and H&E staining images are shown. The liver tissues from representative mice in each group were fixed, embedded in paraffin, and stained with H&E solution. Images are shown at an original

magnification of 100 ×. The levels of (F) serum ALT and (G) serum AST were determined using enzymatic methods. The values are represented as the mean ± SD (n=6 per group). ###P < 0.001 vs. CON group; ***P < 0.001 vs. WD group; significance was determined using one-way ANOVA followed by Dunnett's post hoc test. WD: western diet; NAFLD: nonalcoholic fatty liver disease; TG: triglyceride; TC: total cholesterol; H&E: hematoxylin and eosin

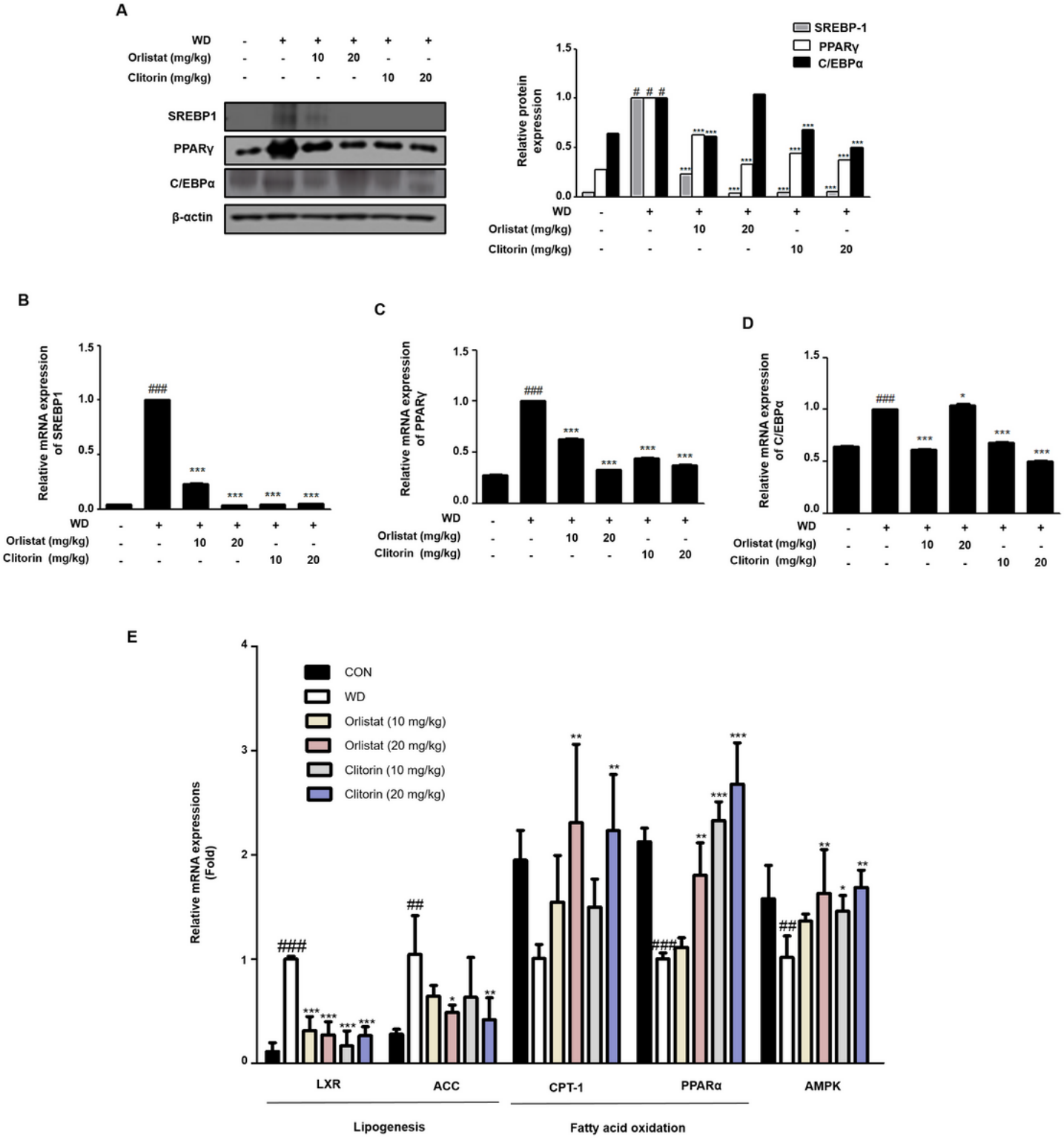

Figure 3

Effect of clitorin on adipogenic, lipogenic, and fatty acid oxidation-related genes in the WD-induced NAFLD mouse model liver. The protein levels of (A) SREBP1, PPARγ, and C/EBPα were determined using western blot analysis. Densitometric analysis was performed using ImageJ ver. 1.50i. The mRNA levels of (B) SREBP1, (C) PPARγ, and (D) C/EBPα were determined using qRT-PCR analysis. (E) The mRNA levels of LXR, ACC, CPT-1, PPARα, and AMPK were determined using qRT-PCR analysis. The values are represented as the mean ± SD (n=6 per group). ##P < 0.01 and ###P < 0.001 vs. CON group; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. WD group; significance was determined using one-way ANOVA followed by Dunnett's post hoc test. WD: western diet; NAFLD: nonalcoholic fatty liver disease; SREBP1 sterol regulatory element binding protein 1; PPARγ: peroxisome proliferator activated receptor γ; C/EBPα: CCAAT/enhancer binding protein α; ACC: acetyl-CoA carboxylase; AMPK: adenosine monophosphate-activated protein kinase; LXR: liver X receptor

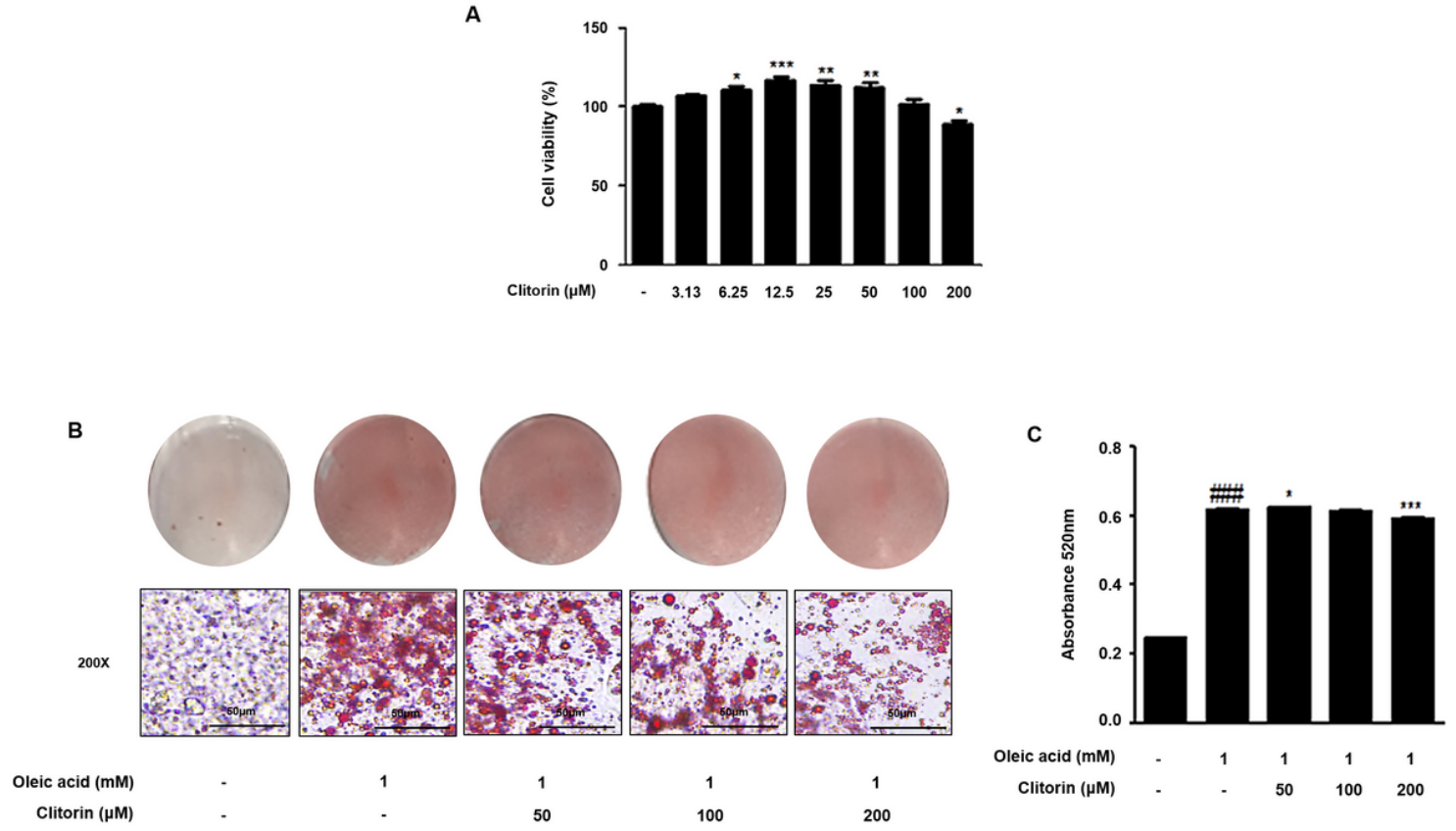

Figure 4

Effect of clitorin on oleic acid-induced steatosis in HepG2 Cells. (A) Cell viability was evaluated using the MTT assay in HepG2 cells. (B) The lipid accumulation rate was measured by Oil Red O staining. (C) The lipid content was quantified by measuring absorbance. The values are represented as mean ± S.D of three independent experiments. ###P < 0.001 vs. non-treated cells; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. oleic acid-treated cells; significances were determined using one-way ANOVA followed by Dunnett's post hoc test.