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Anti-obesity effects of isoacteoside on 3T3-L1 adipocytes

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Abstract

Isoacteoside is a caffeoyl phenylethanoide glycoside found in various plant parts, such as the flower of *Magnolia denudata*. In particular, magnolia has been studied for its anti-obesity, anticancer, and anti-inflammatory effects. However, isoacteoside has not been extensively studied, except for its anti-inflammatory effects. In this study, the anti-obesity effects of isoacteoside were investigated in 3T3-L1 mouse pre-adipocytes. Isoacteoside treatment did not induce cytotoxicity in 3T3-L1 cells up to a concentration of 1000 μM. The anti-obesity effects on 3T3-L1 cells were confirmed using oil red O staining. In addition, the expression of obesity-related proteins and genes, such as peroxisome proliferator-activated acceptor gamma (*PPARγ*), CCAAT/enhancer-binding protein alpha (*C/EBPα*), and perilipin (*PLIN1*), was determined by western blotting and qRT-PCR assays to confirm the anti-obesity effects of isoacteoside. The results of this study suggest that isoacteoside, a natural substance isolated from plant extracts, is not highly toxic to normal cells, such as pre-adipocytes, and displays anti-obesity effects in vitro.

Keywords: 3T3-L1 cells, Anti-obesity, Isoacteoside, Oil Red O assay, Phenylethanoid glycosides

Introduction

Obesity is a disease that is prevalent worldwide, and as of 2016, 39% of the world's population is overweight and 12% is obese. The extent of obesity increased by three times compared to that in 1975 [1]. Obesity is a dangerous disease because it can cause chronic inflammation and lead to cancer and cardiovascular disease [2]. Therefore, studies investigating obesity in many models are being conducted.

Our previous studies have shown that the extract of *Magnolia danudata* flower exerts anti-obesity effects. Consequently, the chemical composition of the magnolia flower extract has been investigated and isoacteoside was identified as one of the 16 main compounds [3, 4]. Other studies have also reported that several plant extracts that exhibit anti-obesity effects contain isoacteoside [5, 6]. However, there is a lack of scientific evidence establishing that isoacteoside is responsible for these anti-obesity effects. In this study, we hypothesized that isoacteoside exerts anti-obesity effects. We used 3T3-L1 mouse pre-adipocytes to observe the inhibitory effects of isoacteoside on lipid accumulation in vitro and to study the expression of anti-obesity-related proteins following isoacteoside treatment.

Materials and methods

Materials

The 3T3-L1 pre-adipocytes were obtained from the Korean Cell Line Bank (Seoul, Korea). Isoacteoside was purchased from MedChemExpress Co. (#HY-N0022, purity > 99.27%; NJ, USA). Differentiation reagents such as 3-isobutyl-1-methylxanthine (IBMX, #I5879), dexamethasone (DEX, #D4902), and insulin (INS, #I6634) were purchased from Sigma-Aldrich Chemicals (MO, USA).

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Cell culture and differentiation

The 3T3-L1 pre-adipocytes were incubated at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, #LM001-01, Welgene, Gyeongsan, Korea) containing 10% bovine calf serum (BCS, #S003-01, Welgene), 100U/mL penicillin, and 100 μg/mL streptomycin (#LS202-02, Welgene). The cell culture was passaged with 10^4 cells/mL every 72 h. To differentiate 3T3-L1 pre-adipocytes into adipocytes, 1×10^5 cells per well were added into a 6-well plate and incubated for a week to reach confluency. Then, the medium was replaced with DMEM containing 10% fetal bovine serum (FBS, #S001-01, Welgene) with 0.05 mM IBMX, 1 μM DEX, and 20 μg/μL INS to initiate differentiation. The differentiation medium was replaced every 2 days with DMEM containing 20 μg/μL INS.

Cell viability

The 3T3-L1 pre-adipocytes (density: 5×10^4 cells per well) were cultured in 96-well cell culture plates (#30096, SPL Life Science, Pocheon, Korea) and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Isoacteoside was added to the cells after incubation for 24 h. After 24 and 48 h of incubation, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS, #LB001-02, Welgene), and 10 μL of water-soluble tetrazolium-1 (WST-1, #MK-400, Takara Bio, Shiga, Japan) solution was added to each well. Absorbance was measured at 450 nm using a microplate reader (EL800, Bio-Tek, MA, USA).

Oil red O staining

Nine days after differentiation, the adipocytes were stained with oil red O (#O0625, Sigma-Aldrich Chemicals) to measure the fat content of the adipocytes. The cells were washed twice with phosphate buffered saline (PBS) and fixed with 10% formalin (#252549, Sigma-Aldrich Chemical) for 1 h. Then, the cells were washed with distilled water and treated with oil red O dissolved in 60% isopropanol (#W292907, Sigma-Aldrich Chemical) for 30 min. The absorbance of the stained fat was measured at a wavelength of 490 nm after extraction of oil red O from the cell using 100% isopropanol.

Quantitative RT-PCR (qRT-PCR)

The cells were harvested 9 days after isoacteoside treatment, i.e., when the cells differentiated. TRIzol reagent (#15596026, Invitrogen, CA, USA) was used to isolate total RNA from the 3T3-L1 cells. The first-strand synthesis kit (GeneAll, Seoul, Korea) was used to synthesize cDNA, and the synthesized cDNA was quantified at 280 nm using a Nanodrop spectrophotometer (Coliri LB 915, JC Bio, Seoul, Korea). BrightGreen 2 × qPCR MasterMix (#MasterMix-S-XL, ABM, Vancouver, Canada) was used for 45 cycles of qPCR performed in a CFX Connect Real-Time PCR machine (Bio-Rad, CA, USA). Primer sequences are listed in Table 1. β-Actin gene was used as the housekeeping gene. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Protein was extracted from the cells 9 days, at which protein expression was the highest, after differentiation using radioimmunoprecipitation assay buffer (RIPA, #RC2002-050-00, Biosesang, Sungnam, Korea) containing a halt protease and phosphatase inhibitor cocktail (#78429, Thermo Fisher Scientific, MA, USA). The extracted proteins were quantified using a BCA protein assay kit (#23225, Thermo Fisher Scientific). The protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 μm, EMD Millipore, MA, USA). The membranes were blocked for 1 h with 5% skim milk or 5% BSA solution. The membranes were then incubated overnight at 4 °C with primary antibodies diluted to 1/1000. Primary antibodies against PPARγ (#2435), C/EBPα (#8178), and PLIN1 (perilipin, #9349) were purchased from Cell Signaling Technology (MA, USA). The membranes were washed twice with Tris-buffered saline (TBS) containing 0.1% Tween-20, and incubated for 2 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (#7074, Cell Signaling Technology) at room temperature. Finally, the membranes were washed twice with 0.1% Tween-20 in TBS and visualized using ECL western blotting detection reagents (Thermo Fisher Scientific). Luminescence was analyzed using ChemiDoc XRS imager (Bio-Rad) with the Image Lab Software (version 4.1, Bio-Rad).

Data analysis

Data analysis was performed using SPSS software (IBM, NY, USA) with Student's *t*-test. Data are presented as

Table 1 Primer sequences used in qRT-PCR analysis

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
C/EBPα	TGCTGGAGTTGACCAGTGAC	AAACCATCCTCTGGGTCTCC
PPARγ	TTTTCAAGGGTGCCAGTTTCA ATCC	AATCCTTGGCCCTCTGAGAT
PLIN1	CTCTGGGAAGCATCGAGAAG	GCATGGTGTGTCGAGAAAGA
β-actin	AGCCATGTACGTAGCCATCC	TCCCTCTCAGCTGTGGTGGTGAA

Primer sequences used in this study are shown in the table. The genes analyzed were as follows: *PPARγ*, *CEBPα*, *PLIN1* (perilipin), and *β-actin*

mean ± standard deviation (SD). Values of $p < 0.05$, < 0.01, or < 0.001 were considered statistically significant.

Results and discussion

Effect of isoacteoside on cell viability

The wide diversity of natural products found in plants enables the discovery of novel compounds to combat chronic diseases such as those caused by obesity. Isoacteoside, a phenylethanoid glycoside and an isomer of acteoside (Fig. 1), has been isolated from a number of plant species, including *Magnolia denudata*, where it is found in the flower [3]. A few studies have demonstrated the physiological activity of isoacteoside. Isoacteoside inhibits a-amylase and lipase [7] and inhibits the production of pro-inflammatory cytokines in the human mast cell line HMC-1, by blocking the caspase-1, MAPK, and NF-kB signaling pathways [8]. Isoacteoside attenuates acute kidney injury induced by severe acute pancreatitis and septic acute lung injury. In addition, it exerts antioxidant, anti-inflammatory, and anticancer effects [9–11]. However, the in vitro bioactivity of isoacteoside with regard to its anti-obesity effects has not been studied. In this study, the anti-obesity effects of isoacteoside were investigated in 3T3-L1 pre-adipocytes.

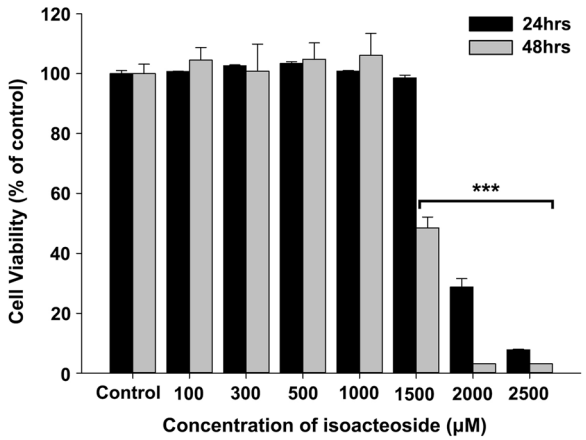

Fig. 2 Viability of 3T3-L1 cells after treatment with isoacteoside. 3T3-L1 cells were treated with isoacteoside (concentrations: 100 μM to 2500 μM). With increasing concentration, the cell viability was stable until 1000 μM. ***$p < 0.001$ compared to the control

The effects of isoacteoside on the viability of 3T3-L1 cells are shown in Fig. 2. Despite increasing the concentration of isoacteoside, cell viability remained unchanged until a certain concentration was reached. After 24 h of isoacteoside treatment, cell viability was approximately

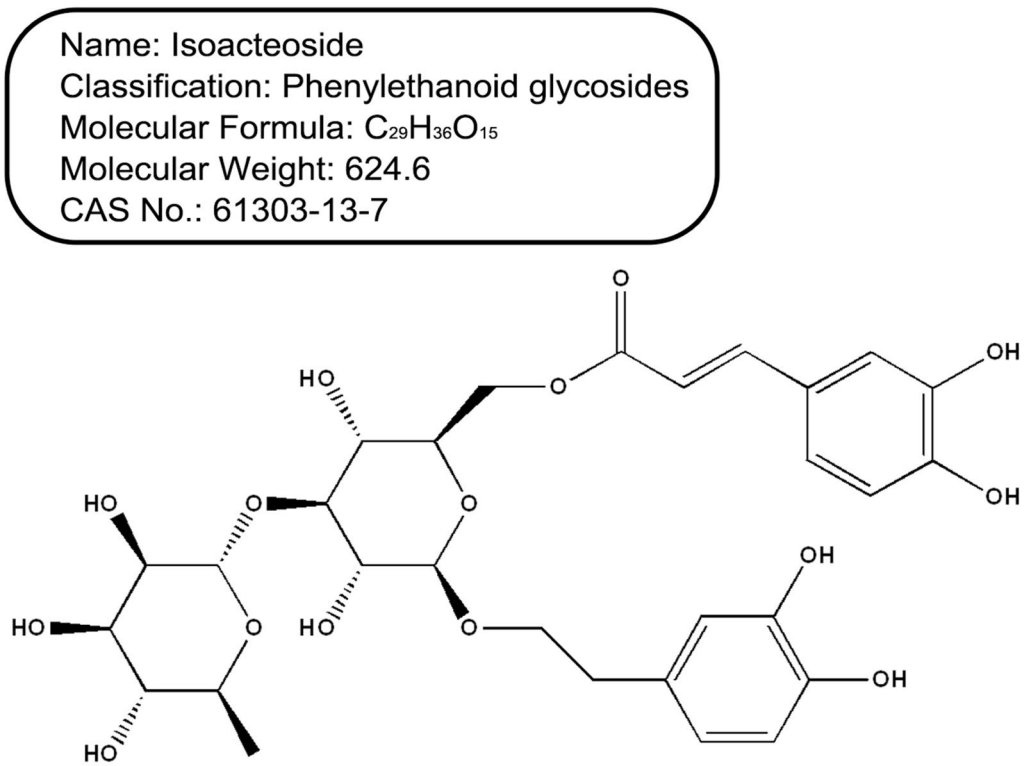

Fig. 1 Structure of isoacteoside. Information of isoacteoside

100.67 ± 0.15, 102.64 ± 0.31, 103.42 ± 0.58, 100.88 ± 0.17, 98.57 ± 0.93, 28.78 ± 2.77 and 7.82 ± 0.19% in comparison to the control group for 100, 300, 500, 1000, 1500, 2000, and 2500 μM isoacteoside-treatment groups, respectively. After 48 h of isoacteoside treatment, cell viability was approximately 104.51 ± 4.25, 100.80 ± 9.11, 104.80 ± 5.48, 106.10 ± 7.33, 48.41 ± 3.67, 3.10 ± 0.07, 3.09 ± 0.02% in comparison to the control group for 100, 300, 500, 1000, 1500, 2000, and 2500 μM isoacteoside-treatment groups, respectively. After 72 h of isoacteoside treatment, cell viability was similar to 48 h (data was not shown). These data suggest that isoacteoside does not significantly affect cell viability below a concentration of 1000 μM. Therefore, we inferred that while isoacteoside has almost no effect on cell viability, it might have other effects, such as on the adipogenic differentiation of 3T3-L1 cells.

Effect of isoacteoside on differentiation of 3T3-L1 cells

The pre-adipocytes were treated with differentiation medium, containing DMEM with IBMX, DEX, and INS in order to generate lipid droplets. These lipid droplets developed a red color upon staining with oil red O, as observed from the cell culture plates (Fig. 3A) and by optical microscopy (Fig. 3B). However, although the cells were treated with differentiation medium, lipid concentration decreased from 100% (control without treatment) to 68.69 ± 1.86% and 33.85 ± 0.22%, following treatment 75 and 150 mM isoacteoside, respectively. This result indicates that with an increase in the concentration of isoacteoside, the lipid content decreases (Fig. 3C). These results suggest that isoacteoside inhibits the differentiation of 3T3-L1 cells and thus the formation of lipid droplets.

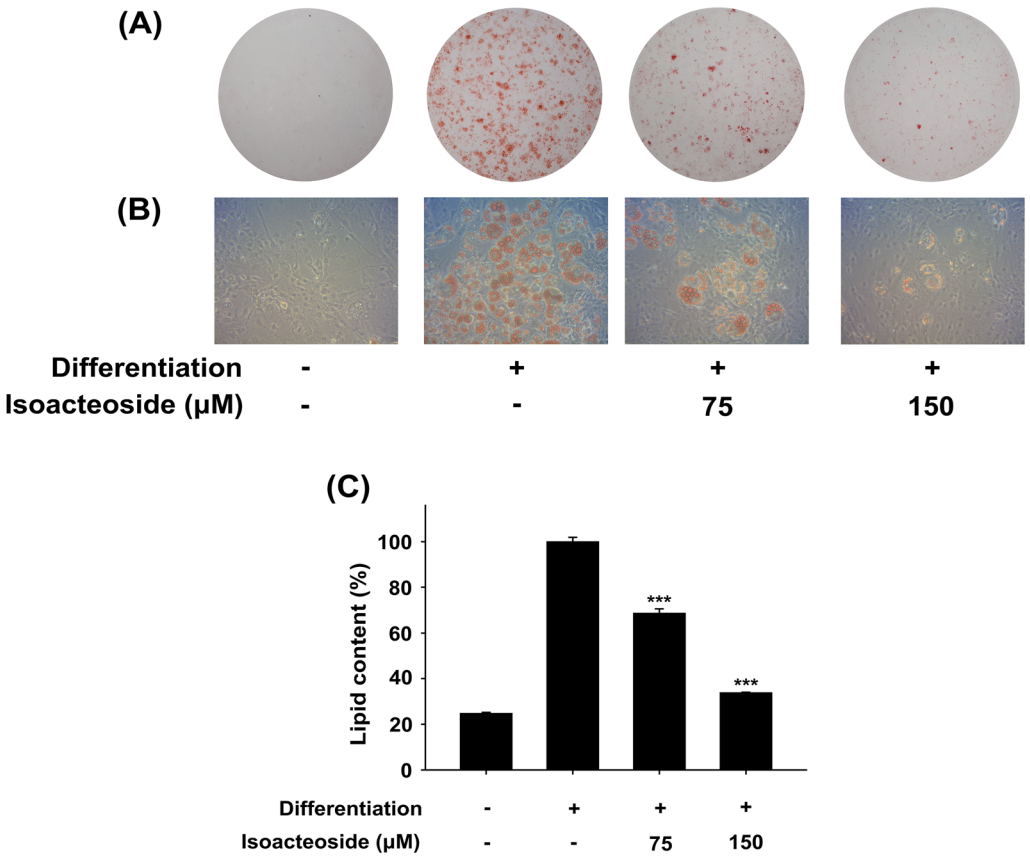

Fig. 3 Effects of isoacteoside on lipid accumulation in differentiating 3T3-L1 cells. **A** Images of cell culture plate of 3T3-L1 cells after staining lipid droplets with oil red O. **B** Images of 3T3-L1 cells obtained using a light microscope after staining lipid droplets with oil red O. **C** Lipid content of 3T3-L1 cells following isoacteoside treatment. The percentage was normalized based on the differentiated control group (+/−) as 100. ***$p <$ compared to the differentiated cell group without isoacteoside treatment

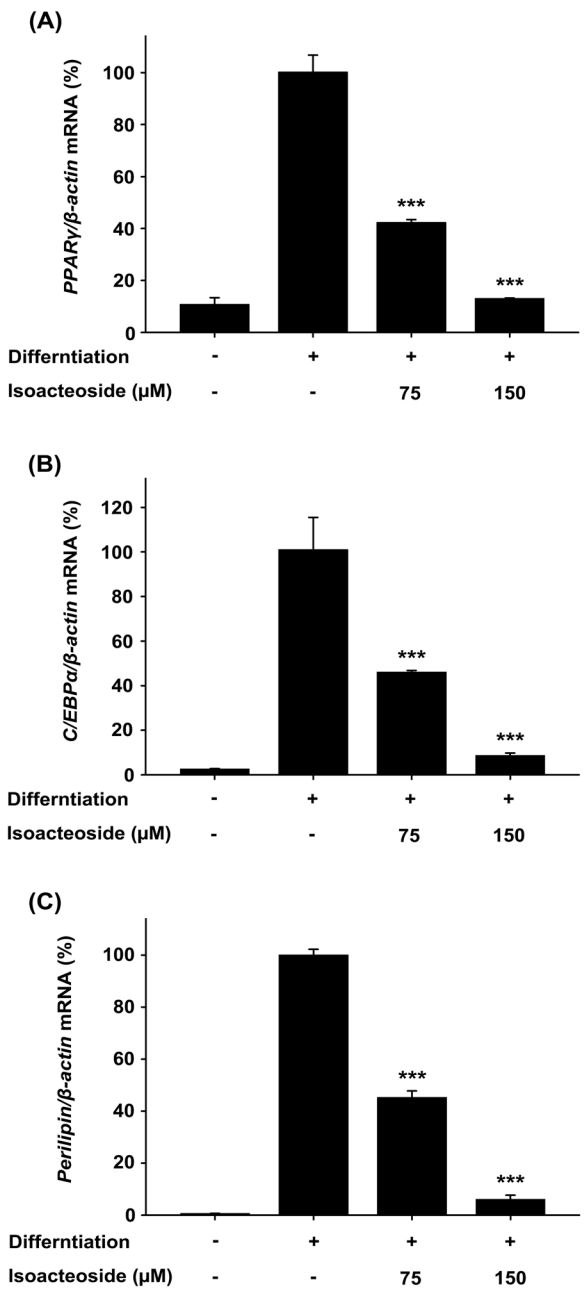

Fig. 4 Quantitative real-time PCR (qRT-PCR) analysis of adipogenesis- and lipogenesis-related genes after isoacteoside treatment of 3T3-L1 cells**. A** Relative mRNA level of *PPARγ* was normalized with that of β-actin gene of each group. **B** Relative mRNA level of *C/EBPα* was normalized with that of β-actin gene of each group. **C** Relative mRNA level of *PLIN1* (perilipin) was normalized with that of β-actin gene of each group. The levels of mRNA for each group were normalized based on the differentiated control group (+/−). Primer sequences used for qRT-PCR analysis are shown in Table 1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the differentiated cell group without treatment of isoacteoside

Effect of isoacteoside on the expression of mRNAs related to adipogenesis and lipogenesis

To observe the extent of inhibition of adipogenesis and lipogenesis by isoacteoside, qRT-PCR was performed to quantify the expression levels of the related genes (Fig. 4). The primer sequences used for this experiment are listed in Table 1. The mRNA expression of peroxisome proliferator-activated acceptor gamma (*PPARγ*) was 10.78 ± 2.62% in the undifferentiated control group (−/−), compared to that in the differentiated control group (+/−). The mRNA expression of *PPARγ* decreased to 42.34 ± 1.08% and 13.13 ± 0.17%, following treatment with 75 μM and 150 μM isoacteoside (Fig. 4A), respectively. The mRNA expression of CCAAT/enhancer-binding protein alpha (*C/EBPα*) was 2.65 ± 0.17% in the undifferentiated control group (−/−), compared to that in the differentiated control group (+/−). The mRNA expression of *C/EBPα* decreased to 46.15 ± 0.70% and 8.73 ± 1.09% upon treatment with 75 μM and 150 μM isoacteoside, respectively (Fig. 4B). The mRNA expression of perilipin (*PLIN1*) was 0.63 ± 0.06% in the undifferentiated control group (−/−), compared to that in the differentiated control group (+/−). The mRNA expression of *PLIN1* decreased to 45.25 ± 2.53% and 6.13 ± 1.58%, upon treatment with 75 μM and 150 μM isoacteoside, respectively (Fig. 4C). These results suggest that isoacteoside suppresses the expression of genes related to adipogenesis and lipogenesis.

Effect of isoacteoside on the expression of adipogenesis and lipogenesis-related proteins

The expression of three proteins involved in the adipogenesis of 3T3-L1 cells and lipid droplet accumulation is shown in Fig. 5A. The expression of PPARγ in the undifferentiated control group (−/−) was 17.16 ± 3.85% compared to that in the differentiated control group (+/−). PPARγ expression levels were 38.50 ± 3.14% and 30.02 ± 8.42% in the groups treated with 75 μM and 150 μM isoacteoside, respectively, compared to that in the differentiated control group (+/−) (Fig. 5B). The expression of C/EBPα in the undifferentiated control group (−/−) was 12.53 ± 1.72% compared to that in the differentiated control group (+/−). C/EBPα expression levels were 33.16 ± 3.42% and 18.81 ± 0.22% in the groups treated with 75 μM and 150 μM isoacteoside, respectively, compared to that in the differentiated control group (+/−) (Fig. 5C). The expression of PLIN1 in the undifferentiated control group (−/−) was 8.22 ± 1.55% compared to that in the differentiated control group (+/−). PLIN1 expression levels were 31.70 ± 1.79% and 6.35 ± 2.78% in the groups treated with 75 μM and

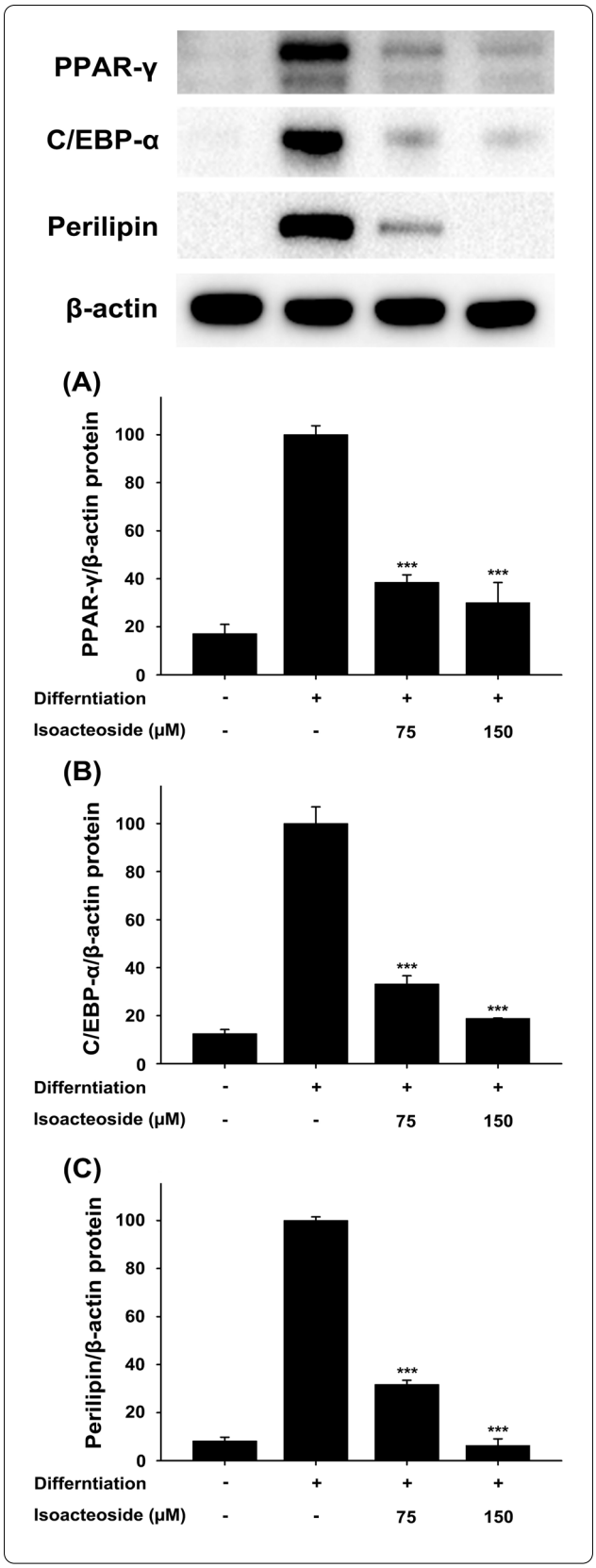

Fig. 5 Western blot analysis of adipogenesis- and lipogenesis-related proteins after isoacteoside treatment of 3T3-L1 cells. **A** Proteins related to adipogenesis and lipogenesis were quantified by western blotting. **B** Levels of PPARγ were normalized to that of β-actin of each group. **C** Levels of CEBPα were normalized to that of β-actin of each group. **D** Levels of PLIN1 (perilipin) were normalized to that of β-actin of each group. The percentage was normalized using the differentiated control group (+/−) as 100. ***$p < 0.001$ compared to the differentiated cell group without isoacteoside treatment

150 μM isoacteoside, respectively, compared to that in the differentiated control group (+/−) (Fig. 5D).

PPARγ and C/EBPα are two important proteins involved in adipogenesis and lipogenesis, respectively [12]. PPARγ is a nuclear receptor protein within the PPAR group and acts as a transcription factor involved in the differentiation of pre-adipocytes and lipid metabolism [13]. PPARγ targets fat-specific marker genes such as genes encoding adipocyte fat acid-binding protein (aP2) and sterol regulatory element-binding protein 1 (SREBP-1). PPARγ promotes adipose tissue production and reduces the expression of leptin, thereby inhibiting lipolysis and promoting lipid accumulation [14–16]. C/EBPα is also a transcription factor that binds aP2 with PPARγ and regulates adipogenesis [17, 18]. C/EBPα is PPARγ-dependent, indicating that C/EBPα alone cannot induce adipogenesis in the absence of PPARγ [19]. Perilipin is a protein found in lipid droplets present on the surface of differentiated adipocytes, where it blocks access to lipases to regulate lipogenesis and promotes PPARγ expression [20]. Therefore, our results indicate that the expression of adipogenesis and lipogenesis-related proteins was suppressed by isoacteoside treatment (Fig. 5). In general, natural products have few side effects on the human body, except in a few cases [21].

The present study aimed to investigate whether isoacteoside inhibits adipogenesis and lipogenesis in 3T3-L1 pre-adipocytes. Although the viability of 3T3-L1 cells was not affected by isoacteoside (< 1000 μM), anti-obesity effects were observed at concentrations lower than 1000 μM. This was supported by the fact that lipid content decreased in the cell culture after treatment with 75 μM and 150 μM isoacteoside, as shown by oil red O staining and the expression levels of adipogenesis- and lipogenesis-related proteins and mRNAs. These results showed that isoacteoside exerted anti-obesity effects on 3T3-L1 cells.

Although, more studies are needed to support this promising mechanism, this study might provide implication in animal and human anti-obesity effect. However, isoacteoside will need to be compared for their efficacy with lipid-lowering medications, such as simvastatin,

mevinolin, or orlistat. In the future, in vivo studies analyzing other tissues involved in obesity are required to validate the anti-obesity effects of isoacteoside.

Abbreviations
DEX: Dexamethasone; ECL: Enhanced chemiluminescence; HRP: Horseradish peroxidase; IBMX: 3-Isobutyl-1-methylxanthine; INS: Insulin; PVDF: Polyvinylidene fluoride; SD: Standard deviation; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS: Tris-buffered saline; PPARγ: Peroxisome proliferator-activated acceptor gamma; CEBPα: CCAAT/enhancer-binding protein alpha; WST-1: Water-soluble tetrazolium-1.

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Author contributions
CGC wrote and revised the manuscript and performed the experiments. DJL performed the experiments. NC and YHJ supervised the study, interpreted the data, and edited the manuscript. All the authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare no conflict of interest.

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