Inhibition of Adipocyte Differentiation and Adipogenesis by the Extract from Sophora japonica Fruit

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Abstract

The world-wide rate of obesity is increasing continuously, representing a serious medical threat since it is associated with a variety of diseases including type 2 diabetes, cardiovascular disease, and numerous cancers. DFFXPXODWLRQRIFHOOXODUWULJ WUDQVFULSWLRQ IDFWRUV LQFOXGLQJ 33$ &% DQG &%,Q DGGLWLRQ ((6) SRWHQWLDOO\GZQ UHJXODWHG WKH HŢUHVVLRQ OHYHOV RI DGLSRFWHVSHFLĘ SURWHLQV LQFOXGLQJ D3 DQG OHSWLQ ,Q SDUWLFXODU ((6) WUHDWPHQW H#IFW enhanced the activation of the AMPK signaling pathway; however, the co-treatment with compound C, an inhibitor of AMPK, signifcantly restored the EESF-induced inhibition of pro-adipogenic transcription factors and adipocyte-specifc genes. These results indicate that EESF may exert an anti-obesity efect by controlling the AMPK signaling pathway, suggesting that the fruit extract of S. japonica may be a potential anti-obesity agent.

only cardiovascular and gastrointestinal disorders but also headache, severe thirst, constipation, insomnia, and palpitations. erefore, the development of a therapeutic agent with proven safety and e ectiveness is urgently needed [8,9]. One possible approach to this problem is through the identi cation of natural products with anti-obesity e ects.

In general, obesity is known to be caused by two critical factors including adipocyte hypertrophy as a result of the triglyceride accumulation induced by adipogenesis, and adipocyte hyperplasia caused by the proliferation and di erentiation of adipocytes [10]. Adipogenesis is the di erentiation process by which adipocytes are generated from broblastic pre-adipocytes, and is accompanied by changes in cell morphology, gene expression, and hormone sensitivity. Adipogenesis is known to be sequentially in uenced by adipogenic transcription factors including sterol response element binding protein 1c (SREBP1c), peroxisome proliferator-activated receptor γ (PPARγ), and cytidine-cytidine-adenosine-adenosine-thymidine (CCAAT)/enhancer binding proteins (C/EBPs) [11-14]. In addition, once adipocytes have been formed through adipogenesis, not only morphological characteristics, such as the triglyceride accumulation

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seen in white adipocytes, but also the expression of adipocyte-speci c genes including adipocyte-speci c lipid binding protein (aP2) and leptin is known to occur [15]. AMP-activated protein kinase (AMPK), in particular, is a key regulator of energy homeostasis that is known to control adipogenesis, and consequently has been studied as a drug target for obesity prevention and treatment [16]. e regulation of adipogenesis could be used as an important process to inhibit the

3T3-L1 Pre-adipocyte Di erentiation Induction and Morphological Observation

Prior to di erentiation, 3T3-L1 pre-adipocytes were maintained in a growth medium containing 10% BCS and 1% penicillin and streptomycin until they reached con uency. Con uent cells were then cultured using di erentiation medium containing 10% fetal bovine serum (FBS, Gibco BRL) and 1% penicillin and streptomycin for a further two days, a er which the medium was replaced with the one containing 10 µg/mL insulin, 1 µM dexamethasone, and 0.5 µM IBMX (MDI) for a further two days. A erwards, the medium was replaced with one containing 10 µg/mL insulin every two days. In addition, to assess inhibition of 3T3-L1 pre-adipocyte di erentiation, cells were treated with EESF by replacing the medium with one containing insulin and MDI. e 3T3-L1 pre-adipocytes whose di erentiation had been induced as described, were subsequently used in various experimental analyses. To examine the e ects of EESF on the morphological changes caused by di erentiation of 3T3-L1 pre-adipocytes into adipocytes, the same methods described above were used for di erentiation and EESF treatment, a er which the cells were observed under ×200 magni cation using an inverted microscope (Carl Zeiss, Gottingen, Germany). Images were taken using the Axio Vision program.

Cell Viability Measurement

To examine the e ects of EESF on the cell viability of 3T3-L1 pre-adipocytes, the cells were plated into a 6-well culture plate and cultured until con uent. e con uent cells were then treated with EESF. A er 72hrs, the supernatant was removed, and the cells were treated with 0.05% trypsin-EDTA to allow for their detachment from the plate. Next, phosphate-bu ered saline (PBS) and a 0.5% trypan blue solution (Gibco BRL) were added to each well. A er approximately two minutes, a hemocytometer was used to count the live cells in the sample under an inverted microscope and the relative cell counts were compared. As an alternative way to measure cell viability, the cells were prepared as described above, then a er removing the medium, 0.5 mg/mL tetrazolium bromide salt (MTT, Amresco, Solon, OH, USA) was added and the cells were cultured in complete darkness in a CO_2 incubator. A er 3 h, the MTT reagent was removed and DMSO was added to the wells to completely dissolve the formazan produced. e dissolved formazan (200 µL) was then transferred to a 96-well plate and the OD at 540 nm measured using ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Oil Red O Staining and Triglyceride Quanti cation

Oil red O staining is a dye binding method used to quantify total lipids. e lipid-speci c binding allows for a quantitative analysis of the lipids produced by adipocytes. Oil red O staining was carried out to examine the e ects of EESF on the production of lipid droplets inside 3T3-L1 adipocytes following induction of di erentiation. For both control and 3T3-L1 adipocytes treated with varying concentrations of EESF, the medium was removed and the cells were washed with PBS then xed for 1 h using 3.7% formalin. A er xing, the cells were washed with 60% isopropanol and treated with the Oil Red O solution

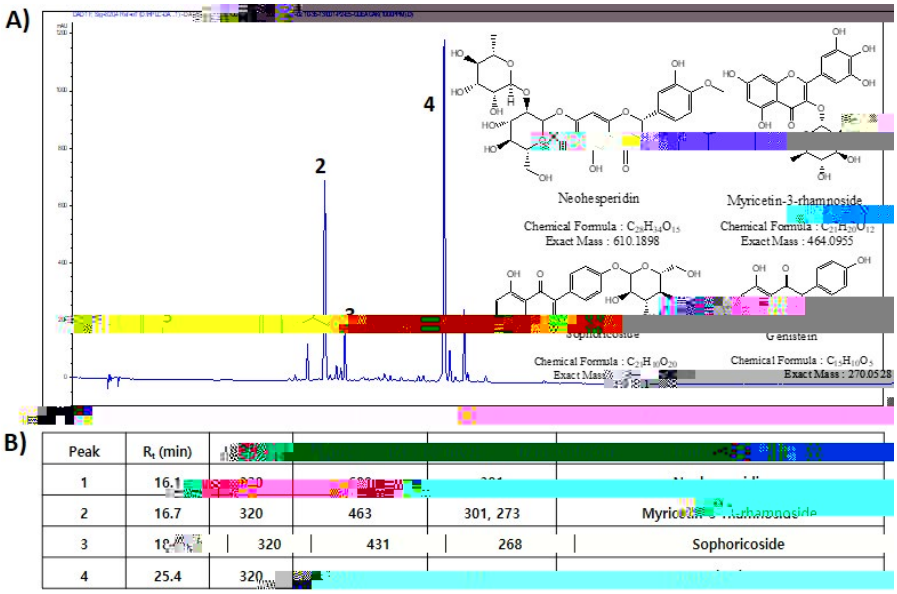

Figure 1: Fingerprint analysis of EESF. (**A**) HPLC analysis of the four reference compounds and EESF. (**B**) LC-MS/MS analysis of the major compounds in EESF.

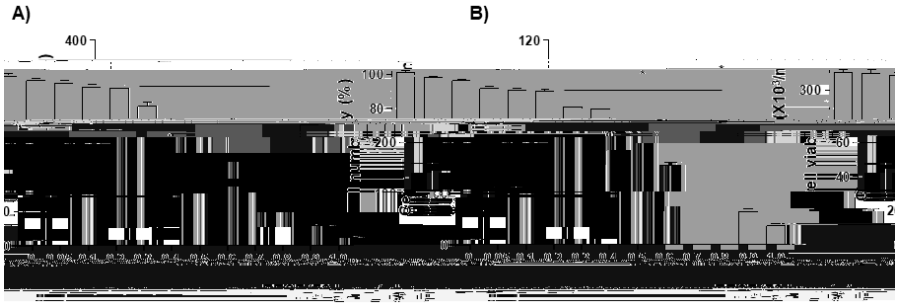

Figure 2: E fects of EESF on the growth of mouse 3T3-L1 pre-adipocytes**.** Cells were treated with the indicated concentrations of EESF for 72 h. Cell number (**A**) and viability (**B**) were determined by hemocytometer counts of trypan blue excluding cells and an MTT assay, respectively. The data are expressed as the mean ± SD of three independent experiments (ANOVA: ***** < 0.05 vs. untreated control).

E ects of EESF on the Production of Lipid Droplets

To examine the e ects of EESF on the production of lipid droplets during adipogenesis, 3T3-L1 pre-adipocytes which had been induced to di erentiate were treated with EESF and the extent of intracellular production of lipid droplets was observed under an inverted microscope before and a er Oil Red O staining. As shown in Figure 3, lipid droplets were not produced when di erentiation was not induced, but following MDI treatment to induce di erentiation, the intracellular production of lipid droplets was readily apparent. e number of lipid droplets was substantially inhibited in a dose-dependent manner upon EESF treatment. ese data indicate that EESF inhibits the di erentiation of 3T3-L1 pre-adipocytes into adipocytes (Figure 3).

E ects of EESF on the Production of Triglycerides

To determine the e ects of EESF on the production of triglycerides, the Oil Red O-stained lipid droplets were extracted using isopropanol and the triglyceride content was determined as shown in Figure 4. During the process of MDI-induced di erentiation into adipocytes, triglyceride production markedly increased; however, EESF treatment led to a gradual decrease in triglyceride production, with the maximum concentration 0.4 mg/mL EESF producing an approximately 80% inhibition of triglyceride production. e result indicates that there is an inhibitory e ect of EESF on the production of triglycerides, and this is correlated with the inhibition of lipid droplet formation (Figure 4).

E ects of EESF on the Expression of Adipogenic Transcription Factors and Adipocyte-Speci c Genes

We examined the e ects of EESF on the expression of adipogenic transcription factors and adipocyte-speci c proteins. As shown in Figure 5A, substantially increased expression levels of PPARγ, C/EBPα and C/EBPβ were observed when the di erentiation was induced without EESF treatment; however, upon EESF treatment, a dose-dependent decrease in expression levels was observed. EESF treatment also signi cantly decreased the expression levels of the adipocyte-speci c proteins aP2 and leptin, as shown in Figure 5B. Collectively, these results indicate that EESF leads to the inhibition of adipogenesis by inhibiting the expression of adipogenic transcription factors, leading to a reduction in the production of lipid droplets and triglycerides and the expression of adipocyte-speci c proteins (Figure 5).

E ects of EESF on the Inhibition of Adipogenesis through the AMPK Signaling Pathway

Finally, we examined whether the AMPK signaling pathway was involved in the EESF-induced inhibition of adipogenesis. First, the expression of ACC acting on AMPK and the step downstream was examined for changes, and as shown in Figure 6A and C, the

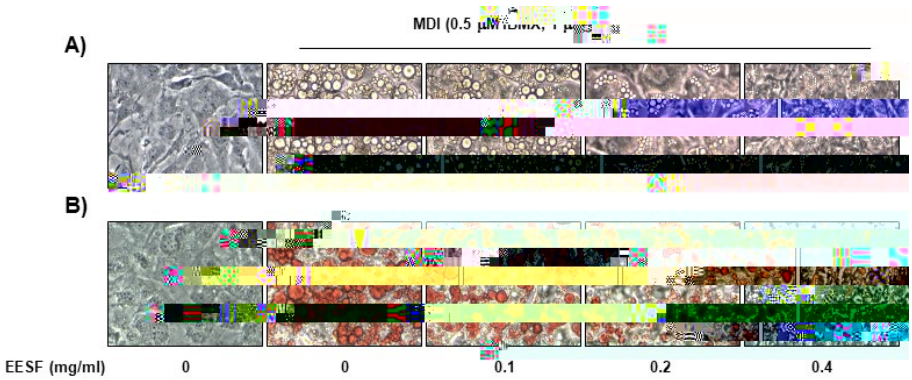

Figure 3: E fects of EESF on the microscopic morphological changes and lipid droplet accumulation in di erentiated mouse 3T3-L1 adipocytes. Di erentiation of con uent mouse 3T3-L1 pre-adipocytes was initiated with MDI (0.5 µM 3-isobytyl-1-methylxanthine, 1 µM dexamethasone, and 10 µg/mL insulin) and maintained in DMEM-5% FBS medium (maintenance di erentiation medium) in the absence or presence of EESF for 8 days. (**A**) Di erentiating 3T3-L1 cells were visualized by light microscopy. Magni cation×200 (**B**) Cells were fxed and stained with Oil Red O to visualize lipid droplets by light microscopy. Magni cation×200.

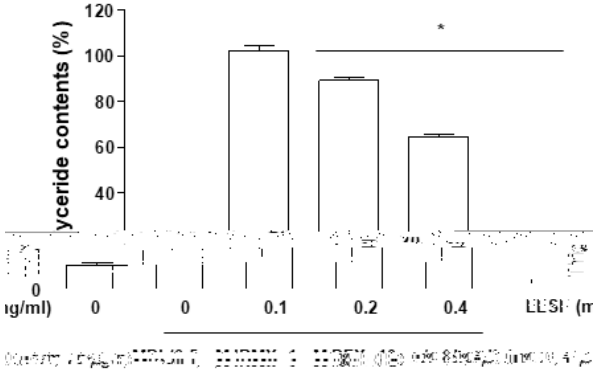

Figure 4: Inhibitory e fects of EESF on triglyceride accumulation in di erentiated mouse 3T3-L1 adipocytes. Triglyceride contents were determined by Oil Red O staining after treatment in the absence or presence of EESF. Quanti cation of triglyceride content was performed by measuring the absorbance at λ=500 nm using an ELISA reader. The data are expressed as the mean ± SD of three independent experiments. (ANOVA: ***** <0.05 vs untreated control).

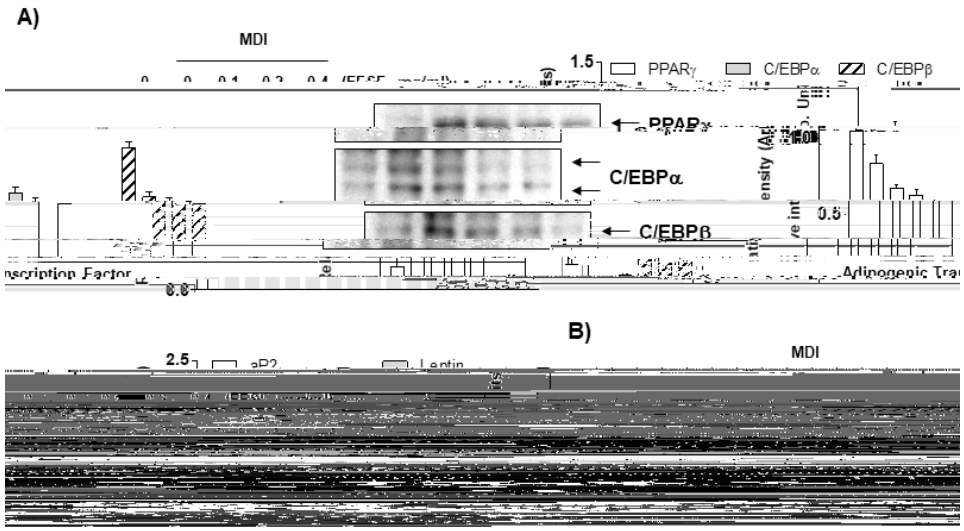

Figure 5: E fects of EESF on the levels of adipogenic transcription factors and adipocyte-speci c genes expression in di erentiated mouse 3T3-L1 adipocytes. Di erentiation of con uent 3T3-L1 mouse pre-adipocytes was carried out in absence or presence of EESF for 8 days after initiation with MDI. (**A**) Cells were lysed and proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (n=3 biologically independent samples). The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. (**B**) Protein band intensities were quanti fed by densitometry using the image J software and relative band intensities were normalized for each actin band. This experiment was repeated at least thrice with equivalent results.

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aP2 and leptin are also critically important for the di erentiation of pre adipocytes into mature adipocytes [36,37]. erefore, the inhibition of adipogenic transcription and adipocyte-speci c factors would decrease the adipocyte di erentiation associated biosynthesis of fatty acids and Triglycerides. In order to investigate the role of EESF in regulating the expression level of the adipogenic transcription factors during adipogenesis of 3T3-L1 cells, we compared their expression levels in the presence and absence of EESF. In addition, EESF also reduced the expression of adipocyte markers aP2 and leptin on the 3T3-L1 cells. aP2 is a carrier protein that can trigger the accumulation of lipid droplets in the cytoplasm of di erentiating adipocytes [38,39], and leptin up regulates the adipocyte genes involved in lipid oxidation, enhancing lipid accumulation in the adipocytes [40,41]; therefore, our results also suggest that EESF strongly suppress the de novo synthesis of Triglyceride and di erentiation of adipocytes.

Accumulating evidence suggests that the AMPK signaling pathway is a target for the energy balance and metabolic disorders involved in the maintenance of lipid and cholesterol homeostasis. During pre-adipocyte di erentiation and adipogenesis, AMPK is inactivated by lower phosphorylation levels. In addition, AMPK activation by phosphorylation can reduce the degree of obesity by inhibiting adipocyte di erentiation by changing the expression and activity of the enzymes and proteins involved in lipid metabolism [42-44]. Furthermore, ACC, a downstream substrate of AMPK, is a rate-limiting enzyme that limits the critical rates in fatty acid synthesis and oxidation, reducing the fatty acid and lipid synthesis to inhibit the onset and progression of obesity [45]. erefore, AMPK signaling has gained the attention of researchers as a molecular target for ghting obesity. e present study showed EESF markedly elevates the phosphorylation level of AMPK in a dose-dependent manner. e ACC phosphorylation was also increased, indicating that the AMPK signaling pathway was activated following EESF administration. In addition, the elevated phosphorylation of ACC and AMPK was inhibited simultaneously by treatment with compound C, as well as the EESF-induced reduction in the expression of transcription factors such as PPARγ, C/EBP members, and SREBP-1c was reversed by the suppression of AMPK activity by compound C.

e results collectively indicate that EESF stimulated AMPK activity, suppressing the adipocyte di erentiation regulators and consequently their target lipogenic enzymes and proteins, ultimately resulting in

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