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Methanol extract of *Elsholtzia fruticosa* promotes 3T3-L1 preadipocyte differentiation

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Abstract

Elsholtzia fruticosa (EF) is present in tropical regions throughout South Asian countries as well as the Himalayas. Although it has been used as a traditional medicine to treat digestive, respiratory, and inflammatory issues, its effect on preadipocyte differentiation is unknown. In this study, we examined the effects of a methanol extract prepared from EF on the differentiation of 3T3-L1 preadipocytes. Cell differentiation was assessed by microscopic observation and oil-red O staining. The expression of adipogenic and lipogenic genes, including *PPARγ* and *C/EBPα*, was measured by western blot analysis and quantitative real-time polymerase chain reaction (qRT–PCR), to provide insight into adipogenesis and lipogenesis mechanisms. The results indicated that EF promotes the differentiation of 3T3-L1 preadipocytes, with elevated lipid accumulation occurring in a concentration-dependent manner without apparent cytotoxicity. EF enhances the expression of adipogenic and lipogenic genes, including *PPARγ*, *FABP4*, *adiponectin*, and *FAS*, at the mRNA and protein levels. The effect of EF was more pronounced during the early and middle stages of 3T3-L1 cell differentiation. Treatment with EF decreased C/EBP homologous protein (CHOP) mRNA and protein levels, while increasing C/EBPα and PPARγ expression. Treatment with EF resulted in the upregulation of *cyclin E* and *CDK2* gene expression within 24 h, followed by a decrease at 48 h, demonstrating the early-stage impact of EF. A concomitant increase in cyclin-D1 levels was observed compared with untreated cells, indicating that EF modulates lipogenic and adipogenic genes through intricate mechanisms involving CHOP and cell cycle pathways. In summary, EF induces the differentiation of 3T3-L1 preadipocytes by increasing the expression of adipogenic and lipogenic genes, possibly through CHOP and cell cycle-dependent mechanisms.

Keywords: Adipocyte, Differentiation, Adipogenesis, 3T3-L1, *Elsholtzia fruticosa*, Lipogenesis

INTRODUCTION

Obesity, which is a multifaceted metabolic disorder characterized by a high body mass index (BMI) and the excessive accumulation of fat, has escalated into a global public health crisis. According to the

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Shrestha D, Suh SS, Kim SH, Seo JB.
Data curation: Shrestha D, Kim E.
Formal analysis: Shrestha D, Kim E.
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Writing - original draft: Shrestha D, Seo JB.
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Ethics approval and consent to participate
This article does not require IRB/IACUC approval because there are no human and animal participants.

World Obesity Federation Atlas 2023, >v2.6 billion people were overweight (BMI ≥ 25kg/m^2), and 1 billion people were obese (BMI ≥30kg/m^2) in 2022. This number is expected to be approximately 4 billion people by 2035 [1]. The prevalence of obesity is projected to increase by 18% in men and 21% in women by 2025 [2] and a total of 24% by 2035 [1]. This worldwide epidemic has resulted in several epidemiological studies, which have revealed a complex relationship between genetic, environmental, and behavioral risk factors that propel the onset and progression of obesity [3–6]. There are several health repercussions associated with obesity, including cardiovascular disease, type 2 diabetes, and the development of certain cancers, which place considerable strain on public health systems [7–10]. Current therapeutic strategies have failed to deliver sustained weight loss or address the physiological nuances of obesity, which highlights the need for novel treatment approaches. Adipose tissue is the central hub for lipid storage and is important in determining whole-body insulin sensitivity. The development and progression of obesity involve the white and brown adipose tissues, which are the predominant focus of obesity research [11]. The mouse-derived preadipocyte 3T3-L1 cell line is a widely used model for obesity research because of its robust and consistent differentiation into mature adipocytes. This makes it a suitable *in vitro* model for studying adipogenesis, insulin response, lipid metabolism, and therapeutic development[12,13]. The differentiation of preadipocytes into mature fat-storing cells is important to adipose tissue dynamics and function. Therapeutic targeting of this pathway offers significant potential in obesity management. Adipogenesis and lipogenesis are key processes that are important for managing fat storage and the conversion of excess energy into lipids within the liver and adipose tissues. This intricate process is associated with the expression of a myriad of genes that contribute to adipogenesis and lipogenesis [14]. The regulation of these genes and their ability to up- or downregulate adipogenesis and lipogenesis in various organs represent a significant area of study. Understanding the mechanisms underlying their regulation may reveal novel targets and strategies for treating obesity and its metabolic complications [15].

Natural products are major repositories for bioactive compounds that may be exploited for allopathic drug development [16]. Numerous well-established drugs, either isolated from or semisynthesized based on natural structures, are an important resource of potential therapeutic agents [17,18]. For example, metformin, a first-line drug for type 2 diabetes, was derived from a natural compound guanidine found in the French lilac [19]. Similarly, the weight loss drug orlistat is related to lipstatin, a natural product derived from the bacterium *Streptomyces toxytricini* [20]. With respect to obesity, several natural products have demonstrated promise, with certain compounds exhibiting a significant impact on the maturation of 3T3-L1preadipocytes by regulating various adipogenic and lipogenic genes [12,21,22].

Elsholtzia fruticosa (EF) is an aromatic plant belonging to the *Lamiaceae* family, which is the sixth-largest family of flowering plants [23]. This plant grows in elevated regions of the Himalayas, spanning Pakistan, Nepal, and China, at altitudes ranging from 1,800 m to 3,300 m [24, 25]. EF and its congeneric species have been used in traditional medicinal practices throughout the region. The essential oil of EF contains several bioactive compounds, such as eucalyptol, perillen, γ-terpinene, caryophyllene, limonene, p-cymene, 1,8-cineole, and carbofuran . These molecules contribute to its complex chemical profile and therapeutic activities. EF oil exhibits antiparasitic effects on Ditylenchus destructor and antibacterial properties [24–30]. The anti-inflammatory and antimicrobial effects have already been demonstrated [29,30]; however, their role in obesity management remains unclear.

In this study, EF promoted preadipocyte maturation in vitro and increased the expression of adipogenic genes, such as peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT enhancer-binding proteins (C/EBPα and C/EBPβ), fatty acid binding protein 4 (FABP4),

adiponectin, as well as lipogenic genes, such as fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), SCD2, and acetyl- CoA carboxylase (ACC), which play a significant role in preadipocyte maturation. Thus, EF has the potential as a natural therapeutic for the treatment of obesity.

MATERIALS AND METHODS

Methanol extract of *Elsholtzia fruticosa* (EF)

The methanol extract of EF was procured from the International Biological Material Research Center at Korea Research Institutes of Bioscience and Biotechnology, Daejeon, Korea. The deposit number is FBM198-050.

Cell culture and adipocyte differentiation

Mouse pre-adipocyte 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Gyeongsan, Korea) supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ in 5% CO_2. Cell differentiation began 2 days after seeding at 100% confluence (Day 0). Cell differentiation as performed as described before [31]. Initially, they were stimulated with a hormone cocktail (0.1 × MDI) consisting of 0.05 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1 μM dexamethasone, and 0.1 μg/ml insulin in 10% fetal bovine serum (FBS)-containing DMEM medium for two days in the presence or absence of EF. The differentiation medium was replaced with DMEM supplemented with 10% FBS and 1 μg/mL insulin every 2 days until day 6. The preadipocytes were transformed into mature adipocytes that were rounded and filled with oil droplets as determined by microscopy.

Cell viability assay

The WST-8 Cell Viability Assay Kit (BIOMAX, Guri, Korea) was used to assess cell viability as described previously [31]. The assay measures the capacity of live cells to convert the WST-8 tetrazolium salt into formazan by mitochondrial dehydrogenases. The 3T3-L1 preadipocytes were seeded at a density of 1×10^4 cells/well in 96-well plates and cultured for 24 h. The cells were exposed to varying concentrations of EF extract and incubated for 24, 48, and 72 hours. The WST-8 reagent was then added and incubated for 4 h at 37℃. The absorbance of the resulting formazan was measured at 450 nm using an iMarkTM microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was determined relative to untreated cells, which were considered 100% viable.

Oil-red O staining

Cells were seeded into 12-well plates and subject to various treatments. Oil red O staining was performed as described previously [32]. Briefly, cells were rinsed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 1 h at room temperature. After washing, 100% isopropanol was added to each well and incubated for 25 min. Subsequently, the cells were stained with Oil-Red O solution for 30 min and repeatedly washed with distilled water until clear. The stained lipid droplets were observed with a NIB410 microscope (Nexcope, Ningbo, China) at 100× magnification.

Quantitative real-time polymerase chain reaction (qRT–PCR)

The expression of various genes associated with lipogenesis and adipogenesis in 3T3-L1 cells was analyzed by qRT–PCR. Total RNA was extracted using the RiboExTM reagent (GeneAll

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Biotechnology, Seoul, Korea). The RNA was reverse-transcribed into cDNA using the ReverTra AceTM qPCR RT kit (Toyobo, Japan). qRT–PCR was carried out using the SYBR Green PCR Master Mix on the CFX Connect Real-Time PCR system (both from Bio-Rad Laboratories). The mRNA levels of 36B4 were used as an internal control. The specific primer sequences are listed in Table 1.

Western blot analysis

Western blot analysis was used to measure protein expression in 3T3-L1 cells following EF treatment. The cells were lysed using the Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing an Xpert protease inhibitor cocktail (GenDEPOT, Katy, TX, USA). After centrifugation at 12,000 rpm for 10 min at 4℃, the supernatants were electrophoresed on precast gels (Bio-Rad Laboratories) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking buffer (BIOMAX, Seoul, Korea) for 5 min and probed overnight at 4℃ with antibodies against PPARγ (Cat. No. 2443, Cell Signaling Technology, Danvers, MA, USA), FABP-4 (Cat. No. SC-271529, Santa Cruz Biotechnology, Dallas, TX, USA), Mouse anti-CHOP IgG2a (L63F7) (Cat. No. 2895, Cell Signaling Technology), and HSP90 (Cat. No. SC-13119, Santa Cruz Biotechnology). After washing, the membranes were incubated with mouse and rabbit HRP-conjugated secondary antibodies (1:4,000 dilution, Bio-Rad Laboratories), washed, and the protein bands were detected using an ECL system (Bio-Rad Laboratories).

Cell counting

Following treatment with EF in 3T3-L1 cells, the cells were washed twice with Dulbecco's PBS (DPBS) and fixed with 4% formaldehyde at room temperature for 30 min. The cells were rinsed twice with DPBS and stained with 1 g/mL Hoechst 33342 for 10 min. After DPBS washing, cell morphology was assessed using a microscope at a magnification of 100×. Images were captured and the nuclei were counted manually to assess cell proliferation. The number of cells was measured using ImageJ software.

Table 1. Primer sets used for qRT–PCR analysis

Gene	Forward (5'-3')	Reverse (5'-3')
36B4	GGC CCT GCA CTC TCG CTT TC	TGC CAG GAC GCG CTT GT
PPARγ	TCG CTG ATG CAC TGC CTA TG	GAG AGG TCC ACA GAG CTG ATT
C/EBPα	GCG GGC AAA GCC AAG AA	GCG TTC CCG CCG TAC C
C/EBPβ	CAA CCT GGA GAC GCA GCA CAA G	GCT TGA ACA AGT TCG GCA GGG T
Adiponectin	TGT TCC TCT TAA TCC TGC CCA	CCA ACC TGC ACA AGT TCC CTT
FABP4	GGA TTT GGT CAC CAT CCG GT	CCA GCT TGT CAC CAT CTC GT
Pref-1	CGT GAT CAA TGG TTC TCC C	AGG GGT ACA GCT GTT GGT TG
FAS	CCA GAC AGA GAA GAG CCA TGG AGG	CCA ATG AGG TTG GCC CAG AAC TCC
SCD-1	TTC TTG CGA TAC ACT CTG GTG C	CGG GAT TGA ATG TTC TTG TCG T
SCD-2	GCA TTT GGG AGC CTT GTA CG	AGC CGT GCC TTG TAT GTT CTG
ACC	CTG ACG TAT ACT GAA CTG GTG TTG GAT G	TTT CCA GGC TAC CAT GCC AAT CTC
CDK2	CCC TTC CCA AAG CCC TTT TC	GAA GAG GGG AAG AAG CTG GT
Cyclin E	GTG GCT CCG ACC TTT CAG TC	CAC AGT CTT GTC AAT CTT GGC A
Cyclin D1	TAG GCC CTC AGC CTC ACT C	CCA CCC CTG GGA TAA AGC AC

qRT-PCR, quantitative real-time polymerase chain reaction.

Statistical analysis

The results are shown as the mean ± SEM, and n denotes the number of wells analyzed. Statistical analyses were performed using Prism 9 software (GraphPad Software, Boston, MA, USA). Data were analyzed using a two-way ANOVA followed by Tukey's post hoc test at $\alpha = 0.05$. A p-value < 0.05 was considered statistically significant.

RESULTS

Elsholtzia fruticosa stimulates 3T3-L1 adipocyte differentiation without cytotoxicity

When searching for natural products for anti-obesity treatment, the primary challenge is to assess their potential cytotoxicity, as complete plant extracts may contain toxic compounds. Therefore, we performed a cell viability assay to assess the effect of EF treatment on the viability of 3T3-L1 cells. As shown in Fig. 1, EF did not exhibit cytotoxic effects even up to concentrations of 100 μg/mL.

Next, we performed Oil-Red O staining following treatment of the cells with EF in differentiation media to determine its effect on the differentiation of 3T3-L1 preadipocytes into mature adipocytes (Fig. 2). EF treatment significantly increased lipid accumulation in 3T3-L1 cells and resulted in robust differentiation in a dose-dependent manner compared with the untreated control cells (Fig. 2A). These findings suggest that EF effectively promotes the maturation and differentiation of 3T3-L1 preadipocytes into mature adipocytes without causing cytotoxicity.

Adipogenesis and lipogenesis are fundamental mechanisms in preadipocytes that are essential for their differentiation into mature adipocytes [33]. This process is mediated by various proteins and hormones, each playing a vital role in driving this transformation. Because increased lipid accumulation was observed in adipocytes differentiated with EF (Fig. 2A), we determined whether this was associated with the modulation of adipogenesis and lipogenesis-related genes in 3T3-L1 cells. A qRT–PCR analysis revealed a dose-dependent increase in the expression of several adipogenic genes, including *PPARγ, C/EBPα, C/EBPβ, adiponectin,* and *FABP4*, in EF-treated cells relative to the controls (Fig. 2B). Conversely, the expression of preadipocyte factor-1 (*Pref-1*; a marker gene for preadipocytes) was significantly decreased (Fig. 2B). To determine the relationship between these mRNA and protein expression, we examined the levels of PPARγ and FABP4

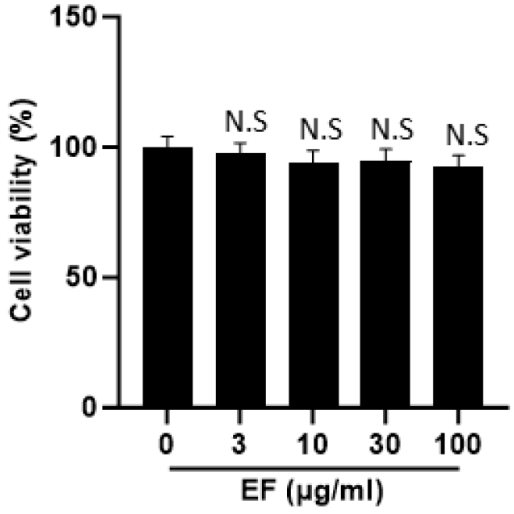

Fig. 1. Cytotoxicity of EF in 3T3-L1 preadipocytes. Viability of 3T3-L1 cells was assessed following treatment with 0, 3, 10, 30, and 100 μg/mL of EF using a WST-8 assay kit. N.S, not significant; EF, *Elsholtzia fruticosa*.

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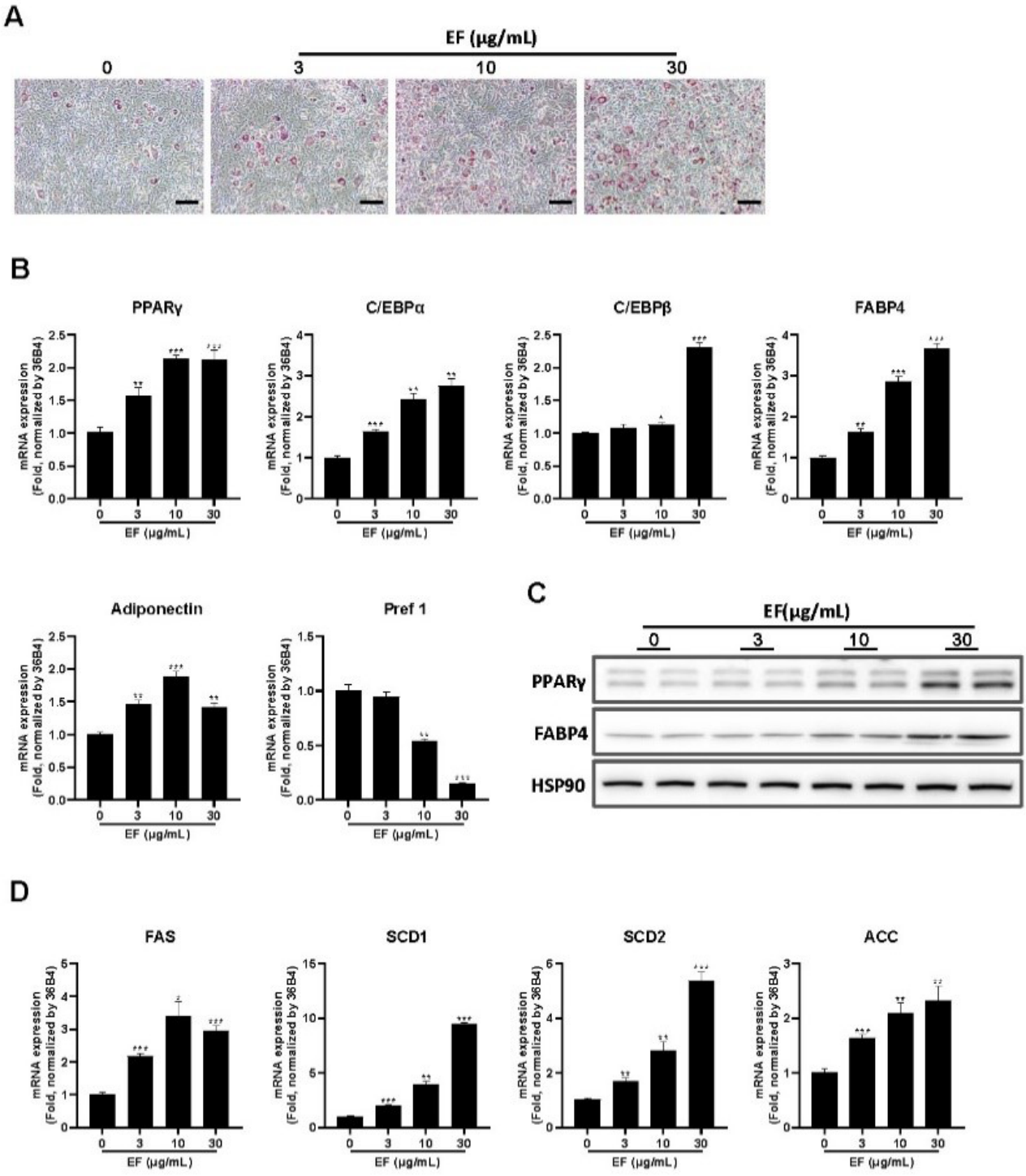

Fig. 2. The effect of EF on adipocyte differentiation in 3T3-L1 cells. (A) Differentiation of 3T3-L1 preadipocytes into adipocytes was initiated with IBMX, dexamethasone, and insulin in the differentiation media, followed by the addition of 3, 10, and 30 μg/mL of EF. After 6 days, adipocytes were stained with oil-red O, and images were captured. Scale bar: 100 μm. (B) At day 6, the cells were harvested for total RNA extraction, followed by qRT–PCR analysis of adipogenesis-related genes, *PPARγ*, *C/EBPα*, *C/EBPβ*, *FABP4*, *adiponectin*, and *Pref1*. (C) Analysis of the PPARγ and FABP4 proteins by western blot analysis was performed on total protein extracts after 6 days using HSP90 as a loading control. (D) Quantitation of *SCD1*, *SCD2*, *FAS*, and *ACC* mRNA levels was performed by qRT–PCR. Each bar represents the mean ± SEM. The findings were consistent across two independent experiments. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with the control group. EF, *Elsholtzia fruticosa*; IBMX, 3-Isobutyl-1-methylxanthine; qRT-PCR, quantitative real-time polymerase chain reaction.

proteins by western blot analysis. Interestingly, EF treatment also upregulated the expression of PPARγ and FABP4 in a dose-dependent manner (Fig. 2C).

Next, we measured the expression of lipogenesis-related genes in 3T3-L1 cells following EF treatment. As shown in Fig. 2D, EF enhanced the expression of genes, including *SCD-1, SCD-2, ACC,* and *FAS* in a dose-dependent manner compared with the control group. These results indicate that EF promotes 3T3-L1 preadipocyte differentiation by up-regulating the expression of adipogenesis- and lipogenesis-related genes.

Elsholtzia fruticosa has a greater effect on adipocyte differentiation at early and intermediate stages

To determine the specific phase of adipocyte differentiation influenced by EF, preadipocytes were cultured to post-confluence followed by treatment with EF according to the schedule outlined in Fig. 3A. Treatments ranged from untreated cells (group 1) to cells treated from days 0 to 6 (group 7). Oil-Red O staining (Fig. 3B) indicated that early treatment (groups 2, 5, and 7) resulted in the most significant lipid accumulation within adipocytes, followed by treatment during the intermediate phases (groups 3 and 6), and was the least effective when administered late (group 4), compared with the untreated group.

Subsequently, a qRT–PCR analysis of adipogenesis-related gene expression (Fig. 3) revealed that the expression of *PPARγ, C/EBPα, C/EBPβ, FABP4,* and *adiponectin* was all significantly increased in groups 2, 3, 5, 6, and 7, with group 4 exhibiting minimal to no increase in some genes compared with the untreated group. The translation of these mRNAs into protein was subsequently verified by western blot analysis (Fig. 3D). The data confirmed that the expression of adipogenic markers, PPARγ and FABP4, was consistent with the mRNA expression patterns, with a marked elevation in groups 2, 5, and 7, followed by groups 3 and 6, and to a lesser extent, group 4.

The effect of EF on lipid metabolism was determined by a qRT–PCR analysis of genes associated with *de novo* lipid synthesis pathways, including *SCD1, SCD2, FAS,* and *ACC* (Fig. 3E). EF markedly upregulated the expression of these genes in the early and intermediate treatment phases, which was consistent with the expression patterns observed for the lipogenic genes. These results demonstrate that EF significantly promotes adipocyte differentiation, particularly when administered at the early and intermediate stages. The consistent findings between lipid staining, mRNA expression, and protein expression suggest that EF is an effective agent for preventative therapy of obesity and highlights its efficacy at enhancing preadipocyte maturation and lipid synthesis.

Elsholtzia fruticosa promotes adipocyte differentiation via the regulation of C/EBP homologous protein expression and mitotic clonal expansion

C/EBP homologous protein (CHOP) and mitotic clonal expansion (MCE) are critical factors in the maturation of preadipocytes and the development of obesity [34]. CHOP is typically associated with the inhibition of adipocyte differentiation, whereas MCE is necessary for cell cycle reentry that precedes the differentiation process [35]. With these roles in mind, we used the most effective concentration of EF identified from previous assays to conduct a time-dependent study on gene expression modulation during adipogenesis.

EF treatment suppressed *CHOP* mRNA expression and concomitantly elevated the mRNA levels of key adipogenic transcription factors and enzymes including PPARγ, C/EBPβ, and C/EBPα (Fig. 4A). A western blot analysis confirmed that while PPARγ protein levels were increased, CHOP protein expression was significantly attenuated in a time-dependent manner when exposed to 30 μg/mL of EF, with HSP90 serving as a loading control (Fig. 4B).

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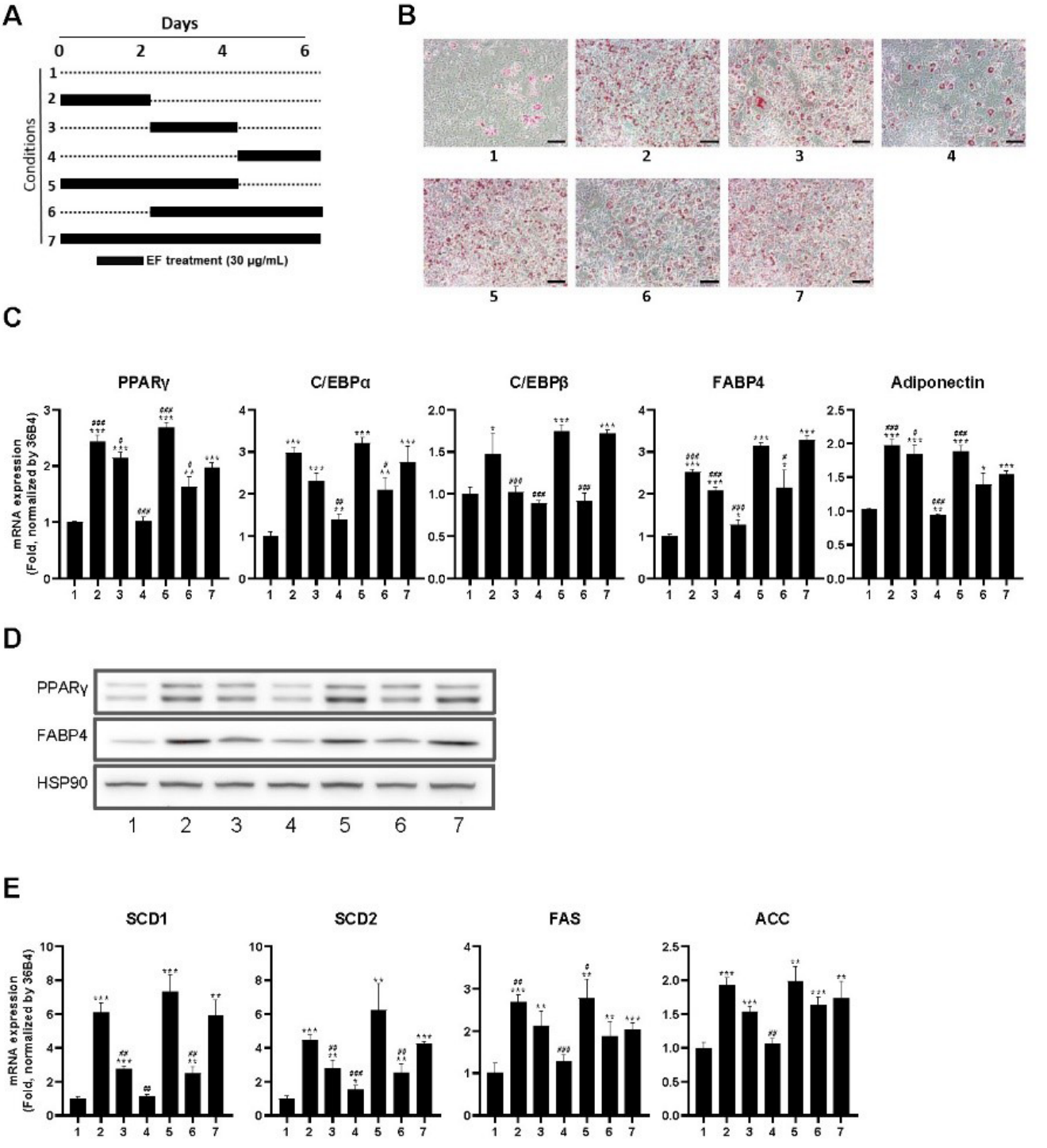

Fig. 3. Time-dependent effects of EF on adipocyte differentiation. (A) Schematic representation of the treatment regimen showing 3T3-L1 cells treated with 30 μg/mL EF for varying times (Groups 1 to 7) at 2 days post-confluence. (B) Cells were fixed with 4% formaldehyde and stained with oil-red O for visualization after 6 days of treatment. (C) On day 6, total RNA was extracted and the expression of adipogenesis-related genes (*PPARγ*, *C/EBPα*, *C/EBPβ*, *FABP4*, *Adiponectin*, and *Pref1*) were measured by qRT–PCR. (D) Total protein was extracted from the cells after 6 days and analyzed for PPARγ and FABP4 expression by western blotting, with HSP90 as a loading control. (E) qRT–PCR analysis of *SCD1*, *SCD2*, *FAS*, and *ACC* mRNA levels was done using total RNA extracted on day 6. Each bar represents the mean ± SEM with consistent results observed across two independent experiments. Significance is indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group. EF, *Elsholtzia fruticosa*; qRT-PCR, quantitative real-time polymerase chain reaction.

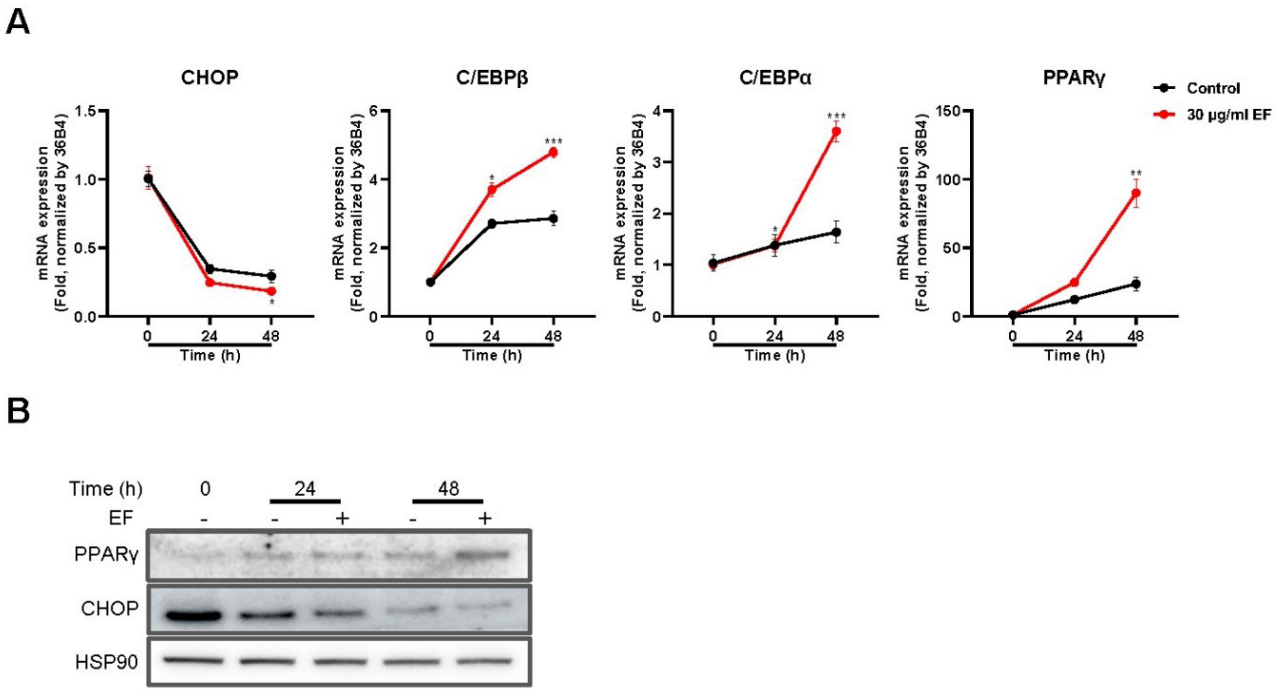

Fig. 4. Effect of EF on the expression of the major adipocyte transcription modular CHOP. (A) 3T3-L1 cells were cultured in 12-well plates and allowed to reach confluence over 48 h. The cells were then treated with differentiation media containing 30 μg/mL EF for 24 h and 48 h. Total RNA was extracted to assess the expression of *CHOP*, *PPARγ*, *C/EBPα*, and *C/EBPβ* using qRT–PCR analysis. (B) After 24 h and 48 h, total protein was extracted from the cells to analyze the expression of PPARγ and CHOP protein using western blotting, with HSP90 as a loading control. CHOP, C/EBP homologous protein; qRT-PCR, quantitative real-time polymerase chain reaction.

The cell cycle is an important process as it facilitates the MCE necessary for preadipocyte differentiation [36]. Upon examining the effect of EF on cell cycle-associated genes in 3T3-L1 preadipocytes, a distinct expression pattern was evident (Fig. 5A). *Cyclin E* mRNA levels increased at 24 h and decreased in the 48 h treatment group. A consistent biphasic pattern of mRNA expression was observed for cyclin-dependent kinase 2 (CDK2), whereas reduced cyclin D1 mRNA levels were observed compared with the control, followed by a slight increase at the 48-h time point compared with the 24-h mark. In addition, EF activated MCE during adipogenesis along with an increase in cell number (Figs. 5B and 5C). These results suggest that EF exerts a dual modulatory effect on adipocyte differentiation by downregulating CHOP, a negative regulator of adipogenesis, and inducing initial increases in cell cycle progression, which is necessary for preadipocyte maturation (Fig. 5D). These findings highlight the potential of EF as an adipogenesis modulator, with implications for the treatment and prevention of obesity.

DISCUSSION

Obesity is a significant global public health issue that is often linked to a sedentary lifestyle and a high-calorie Western diet [37]. It is a risk factor for various metabolic disorders, such as type 2 diabetes mellitus, cardiovascular disease, and certain cancers [38,39]. Adipose tissue is involved in the onset and progression of obesity and influences lipid storage, energy expenditure, and the development of insulin resistance [40,41]. 3T3-L1 cells are a cornerstone for obesity research because of their ability to differentiate into adipocytes. Thus, they are an invaluable model for

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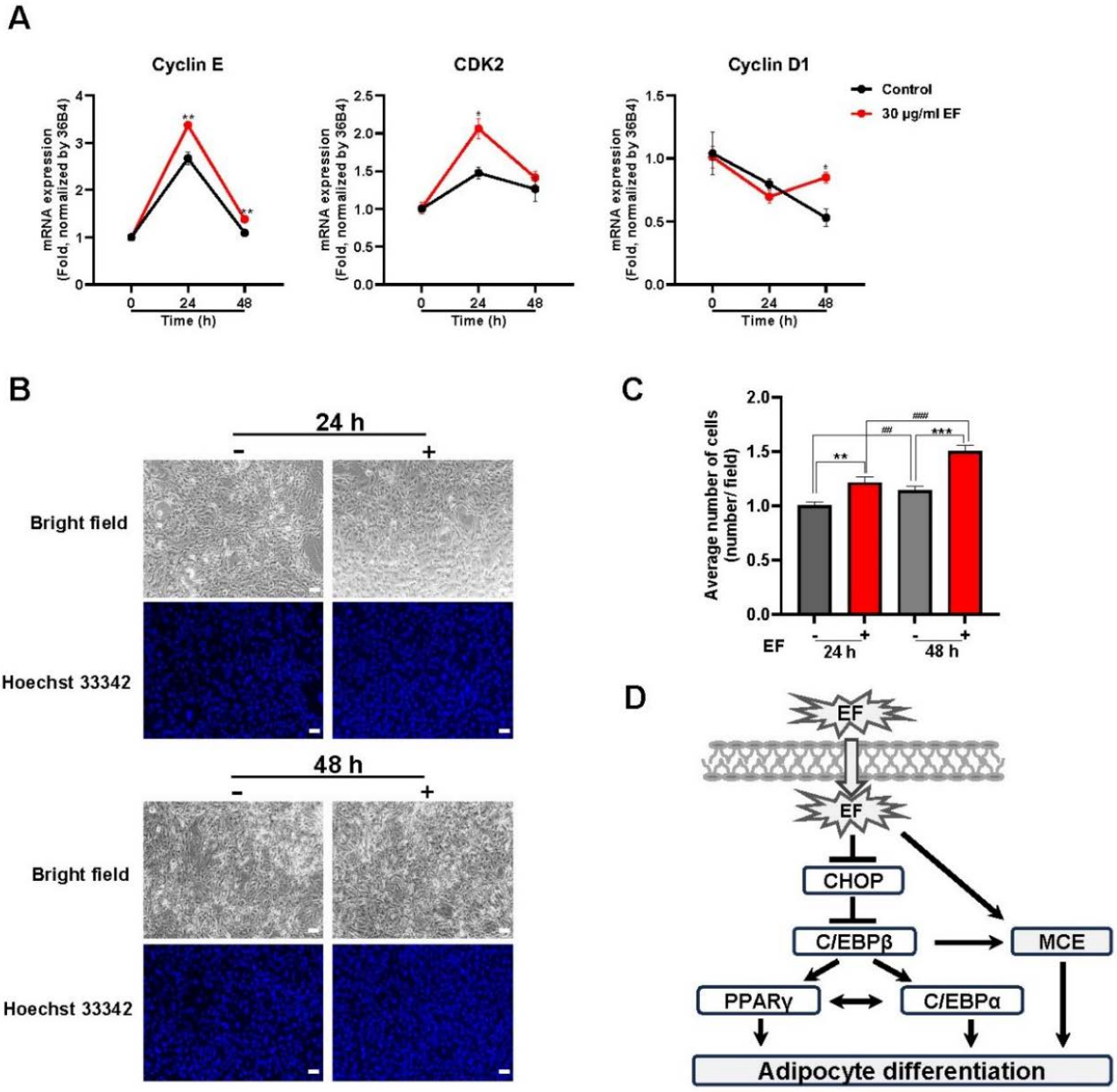

Fig. 5. Effect of EF on MCE during adipocyte differentiation. (A) On the same day, qRT-PCR was used to evaluate the expression of cell cycle-related genes (*Cyclin E*, *CDK2*, *Cyclin D1*). (B) After staining, the cells were counterstained with Hoechst 33342 and photographed using ImageJ software (N = 16). Scale bars = 100 μm. (C) The number of cells was measured using ImageJ software. (D) Working model for EF-induced adipocyte differentiation. Each bar represents the mean ± SEM, with consistent findings across two independent experiments. Statistical significance is indicated by *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$ compared with the control group. EF, *Elsholtzia fruticosa*; CHOP, C/EBP homologous protein; MCE, mitotic clonal expansion; qRT-PCR, quantitative real-time polymerase chain reaction.

understanding the intricacies of adipogenesis and its role in obesity and diabetes [42,43].

Our results indicate that EF potently induces the maturation of 3T3-L1 preadipocytes, as evidenced by the accumulation of lipids by oil-red O staining. An upregulation of adipogenic and lipogenic genes and a few at the protein level was observed. In contrast, numerous studies reported the inhibition of adipocyte differentiation by inhibiting transcription factors, such as PPARγ and C/EBPα in 3T3-L1 cells [44]. Previous studies in 3T3-L1 cells showed that resveratrol treatment

markedly reduced lipid accumulation and lowered the expression of C/EBPα, lipoprotein lipase (LPL), FAS, and SREBP 1c by activating AMPK [44]. The plant-derived alkaloid Berberine inhibits adipogenesis in 3T3-L1 cells by downregulating C/EBPα and PPARγ and up-regulating CHOP and basic helix-loop-helix family, member e41 (BHLHE41/DEC2), which is influenced by mitochondrial respiration [45]. The adipogenesis process involves a well-orchestrated transcriptional cascade, with key genes, such as *PPARγ, C/EBPα,* and *C/EBPβ* driving the differentiation of preadipocytes into mature fat cells, which is a process tightly linked to lipogenesis [46]. Adipogenesis involves elevated expression of adipogenic transcription factors, such as PPARγ and C/EBPα, which are the key adipogenic transcription factors that are activated during the differentiation of 3T3-L1 cells into adipocytes [47].

Natural products have long been studied for their therapeutic potential, and several, such as resveratrol, curcumin, and epigallocatechin gallate, have demonstrated significant effects on adipocyte differentiation and the regulation of adipogenic and lipogenic genes [48]. EF did not exhibit cytotoxic effects, which is an important consideration for its potential use as a therapeutic. Unlike some complete plant extracts that may contain toxic compounds, EF was well-tolerated by 3T3-L1 cells, even at high concentrations.

Furthermore, the upregulation of both adipogenic and lipogenic genes following EF treatment is noteworthy. The dose-dependent increase of PPARγ and FABP4 mRNA and protein underscores the role of EF in enhancing adipocyte differentiation. The effect of EF on 3T3-L1 preadipocyte differentiation is similar to that of well-established anti-diabetes drugs, such as pioglitazone and rosiglitazone from the thiazolidinediones group, particularly for promoting the expression of PPARγ and adiponectin [49]. Pioglitazone enhances peripheral insulin sensitivity. Pioglitazone up-regulates adiponectin receptor 2 in 3T3-L1 adipocytes, whereas rosiglitazone increases GLUT-4 expression in muscle cells [49]. Our results also highlight the unique properties of EF, including the inhibition of CHOP expression at the mRNA and protein level (Figs. 4A and 4B). CHOP, a negative regulator of adipocyte differentiation, inhibits the function of CEBP isoforms by forming dimers [50]. Its degradation enhances preadipocyte differentiation and the EF extract-mediated decrease in CHOP protein levels increases the function of C/EBPα, C/EBPβ, and PPARγ. These suggests that the role of EF in preadipocyte differentiation may mediated through CHOP protein degradation [35].

The specific molecule within EF that is responsible for enhancing preadipocyte maturation remains unknown. Eucalyptol, perillen, γ-terpinene, caryophyllene, limonene, p-cymene, 1,8-cineole, and carbofuran have been identified as the major compounds in EF essential oil. Of these, eucalyptol has a potential anti-obesity role as demonstrated by its inhibition of digestive enzymes, specifically α-amylase and pancreatic enzymes [51]. In addition, the antioxidant activity of eucalyptol has been reported [52]; however, the specific anti-diabetic effects of other compounds in the extract have not been evaluated. Further studies involving compound isolation and their activity are warranted.

The promotion of adipocyte differentiation by EF may play an important role in the development of new metabolic disease treatments. In the context of adipocyte biology, obesity is often associated with compromised insulin sensitivity and elevated blood lipid levels. This results in the ectopic accumulation of lipids in muscles, liver, and other organs, which can exacerbate metabolic complications [40]. By promoting the formation of healthy adipocytes, EF can maintain energy homeostasis and mitigate such complications. Enhancing healthy adipose tissue may provide a buffer for lipid storage to prevent lipid overflow into nonadipose tissues, which is a key factor in maintaining insulin sensitivity and metabolic health [53].

In conclusion, the ability of EF to modulate adipocyte differentiation and potentially support the

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development of healthy adipose tissue indicates its potential as a treatment for metabolic diseases. Future studies are needed to elucidate the precise mechanisms by which EF influences adipogenesis and lipogenesis and to determine its efficacy and safety in vivo. With the rising demand for safe and effective treatments for metabolic syndromes, natural products, such as EF, represent potential therapies.

REFERENCES

1. World Obesity Federation. World obesity atlas 2023 [Internet]. 2023 [cited Nov 23]. https://data.worldobesity.org/publications/WOF-Obesity-Atlas-V5.pdf
2. NCD Risk Factor Collaboration(NCD-RisC). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet. 2016;387:1377-96. https://doi.org/10.1016/S0140-6736(16)30054-X
3. Albuquerque D, Stice E, Rodríguez-López R, Manco L, Nóbrega C. Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective. Mol Genet Genomics. 2015;290:1191-221. https://doi.org/10.1007/s00438-015-1015-9
4. Hruby A, Hu FB. The epidemiology of obesity: a big picture. PharmacoEconomics. 2015;33:673-89. https://doi.org/10.1007/s40273-014-0243-x
5. MacLean PS, Blundell JE, Mennella JA, Batterham RL. Biological control of appetite: a daunting complexity. Obesity. 2017;25:S8-16. https://doi.org/10.1002/oby.21771
6. Raciti GA, Spinelli R, Desiderio A, Longo M, Parrillo L, Nigro C, et al. Specific CpG hypermethylation leads to Ankrd26 gene down-regulation in white adipose tissue of a mouse model of diet-induced obesity. Sci Rep. 2017;7:43526. https://doi.org/10.1038/srep43526
7. Ortega FB, Lavie CJ, Blair SN. Obesity and cardiovascular disease. Circ Res. 2016;118:1752-70. https://doi.org/10.1161/CIRCRESAHA.115.306883
8. Gross B, Pawlak M, Lefebvre P, Staels B. PPARs in obesity-induced T2DM, dyslipidaemia and NAFLD. Nat Rev Endocrinol. 2017;13:36-49. https://doi.org/10.1038/nrendo.2016.135
9. Hecker J, Freijer K, Hiligsmann M, Evers SMAA. Burden of disease study of overweight and obesity; the societal impact in terms of cost-of-illness and health-related quality of life. BMC Public Health. 2022;22:46. https://doi.org/10.1186/s12889-021-12449-2
10. Pati S, Irfan W, Jameel A, Ahmed S, Shahid RK. Obesity and cancer: a current overview of epidemiology, pathogenesis, outcomes, and management. Cancers. 2023;15:485. https://doi.org/10.3390/cancers15020485
11. Ying T, Simmons RA. The role of adipocyte precursors in development and obesity. Front Endocrinol. 2021;11:613606. https://doi.org/10.3389/fendo.2020.613606
12. Guru A, Issac PK, Velayutham M, Saraswathi NT, Arshad A, Arockiaraj J. Molecular mechanism of down-regulating adipogenic transcription factors in 3T3-L1 adipocyte cells by bioactive anti-adipogenic compounds. Mol Biol Rep. 2021;48:743-61. https://doi.org/10.1007/s11033-020-06036-8
13. Molani-Gol R, Rafraf M. The anti-obesity effects of resveratrol on the 3T3-L1 adipocytes. Int J Vitam Nutr Res. Forthcoming 2023. https://doi.org/10.1024/0300-9831/a000784
14. Kusminski CM, Bickel PE, Scherer PE. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat Rev Drug Discov. 2016;15:639-60. https://doi.org/10.1038/nrd.2016.75
15. Wen X, Zhang B, Wu B, Xiao H, Li Z, Li R, et al. Signaling pathways in obesity: mechanisms and therapeutic interventions. Signal Transduct Target Ther. 2022;7:298. https://doi.org/

10.1038/s41392-022-01149-x
16. Aware CB, Patil DN, Suryawanshi SS, Mali PR, Rane MR, Gurav RG, et al. Natural bioactive products as promising therapeutics: a review of natural product-based drug development. S Afr J Bot. 2022;151:512-28. https://doi.org/10.1016/j.sajb.2022.05.028
17. Naik SR, Kokil GR. Development and discovery avenues in bioactive natural products for glycemic novel therapeutics. In: Atta-ur-Rahman FRS, editor. Studies in natural products chemistry: bioactive natural products. Amsterdam: Elsevier; 2013. p. 431-66.
18. Imran M, Insaf A, Hasan N, Sugandhi VV, Shrestha D, Paudel KR, et al. Exploring the remarkable chemotherapeutic potential of polyphenolic antioxidants in battling various forms of cancer. Molecules. 2023;28:3475. https://doi.org/10.3390/molecules28083475
19. Thomas I, Gregg B. Metformin; a review of its history and future: from lilac to longevity. Pediatr Diabetes. 2017;18:10-6. https://doi.org/10.1111/pedi.12473
20. Lunagariya NA, Patel NK, Jagtap SC, Bhutani KK. Inhibitors of pancreatic lipase: state of the art and clinical perspectives. EXCLI J. 2014;13:897-921.
21. Park KS. Raspberry ketone, a naturally occurring phenolic compound, inhibits adipogenic and lipogenic gene expression in 3T3-L1 adipocytes. Pharm Biol. 2015;53:870-5. https://doi.org/10.3109/13880209.2014.946059
22. Feng S, Reuss L, Wang Y. Potential of natural products in the inhibition of adipogenesis through regulation of PPARγ expression and/or its transcriptional activity. Molecules. 2016;21:1278. https://doi.org/10.3390/molecules21101278
23. Li B, Cantino PD, Olmstead RG, Bramley GLC, Xiang CL, Ma ZH, et al. A large-scale chloroplast phylogeny of the Lamiaceae sheds new light on its subfamilial classification. Sci Rep. 2016;6:34343. https://doi.org/10.1038/srep34343
24. Pradhan S, Paudel HR, Maharjan R, Sharma K. Essential oils from six aromatic plants of langtang national park: insights on their chemical constituents via GC-MS analysis. Separations. 2023;10:52. https://doi.org/10.3390/separations10010052
25. Yu X, Song YR, Zhao ZN. The complete chloroplast genome of Elsholtzia fruticosa (D. Don) Rehd. (Labiatae), an ornamental plant with high medicinal value. Mitochondrial DNA B Resour. 2023;8:336-41. https://doi.org/10.1080/23802359.2023.2183069
26. Taylor RS, Manandhar NP, Towers GHN. Screening of selected medicinal plants of Nepal for antimicrobial activities. J Ethnopharmacol. 1995;46:153-9. https://doi.org/10.1016/0378-8741(95)01242-6
27. Saini R, Guleria S, Kaul VK, Lal B, Babu GDK, Singh B. Comparison of the volatile constituents of Elsholtzia fruiticosa extracted by hydrodistillation, supercritical fluid extraction and head space analysis. Nat Prod Commun. 2010;5:641-4. https://doi.org/10.1177/1934578X1000500430
28. Kang SH. Research progress on essential oils and pharmacology of Elsholtzia in China. J Northwest Minzu Univ (Nat Sci). 2012;33:58-63.
29. Liang J, Ning A, Lu P, Shao Y, Xu J, Yang YY, et al. Chemical composition and biological activity of essential oil extracted from the aerial part of Elsholtzia fruticosa against Ditylenchus destructor. J Essent Oil Bear Plants. 2020;23:575-82. https://doi.org/10.1080/0972060X.2020.1778542
30. Fusani P, Ronga D, Carminati D, Mandrioli M, Manicardi GC, Giannì S, et al. Composition and biological activity of essential oils from Artemisia roxburghiana Besser and Elsholtzia fruticosa Rehder cultivated in Italy. Ind Crops Prod. 2022;187:115317. https://doi.org/10.1016/j.indcrop.2022.115317
31. Eom J, Choi J, Suh SS, Seo JB. SLC3A2 and SLC7A2 mediate the exogenous putrescine-

https://doi.org/10.5187/jast.2024.e6

induced adipocyte differentiation. Mol Cells. 2022;45:963-75. https://doi.org/10.14348/molcells.2022.0123
32. Shrestha J, Baek DJ, Oh YS, Cho SS, Ki SH, Park EY. Protective effect of Cudrania tricuspidata extract against high-fat diet induced nonalcoholic fatty liver disease through Nrf-2/HO-1 pathway. Molecules. 2021;26:2434. https://doi.org/10.3390/molecules26092434
33. Moreno-Navarrete JM, Fernández-Real JM. Adipocyte differentiation. In: Symonds ME, editor. Adipose tissue biology. New York, NY: Springer; 2017. p. 69-90.
34. Chang E, Kim CY. Natural products and obesity: a focus on the regulation of mitotic clonal expansion during adipogenesis. Molecules. 2019;24:1157. https://doi.org/10.3390/molecules24061157
35. Shin MC, Jung YH, Jeong Y, Oh AR, Lee SB, Kim K. Kctd17-mediated Chop degradation promotes adipogenic differentiation. Biochem Biophys Res Commun. 2023;653:126-32. https://doi.org/10.1016/j.bbrc.2023.02.068
36. Audano M, Pedretti S, Caruso D, Crestani M, De Fabiani E, Mitro N. Regulatory mechanisms of the early phase of white adipocyte differentiation: an overview. Cell Mol Life Sci. 2022;79:139. https://doi.org/10.1007/s00018-022-04169-6
37. Kopp W. How western diet and lifestyle drive the pandemic of obesity and civilization diseases. Diabetes Metab Syndr Obes. 2019;12:2221-36. https://doi.org/10.2147/DMSO.S216791
38. Gallagher EJ, LeRoith D. Obesity and diabetes: the increased risk of cancer and cancer-related mortality. Physiol Rev. 2015;95:727-48. https://doi.org/10.1152/physrev.00030.2014
39. Karczewski J, Begier-Krasińska B, Staszewski R, Popławska E, Gulczynska-Elhadi K, Dobrowolska A. Obesity and the risk of gastrointestinal cancers. Dig Dis Sci. 2019;64:2740-9. https://doi.org/10.1007/s10620-019-05603-9
40. Longo M, Zatterale F, Naderi J, Parrillo L, Formisano P, Raciti GA, et al. Adipose tissue dysfunction as determinant of obesity-associated metabolic complications. Int J Mol Sci. 2019;20:2358. https://doi.org/10.3390/ijms20092358
41. Zatterale F, Longo M, Naderi J, Raciti GA, Desiderio A, Miele C, et al. Chronic adipose tissue inflammation linking obesity to insulin resistance and type 2 diabetes. Front Physiol. 2020;10:1607. https://doi.org/10.3389/fphys.2019.01607
42. Jang KO, Choi JS, Choi KH, Kim S, Kim H, Chung DK. Anti-obesity potential of heat-killed Lactiplantibacillus plantarum K8 in 3T3-L1 cells and high-fat diet mice. Heliyon. 2023;9:e12926. https://doi.org/10.1016/j.heliyon.2023.e12926
43. Molonia MS, Muscarà C, Speciale A, Salamone FL, Costa G, Vento G, et al. Low concentrations of antimony impair adipogenesis and endoplasmic reticulum homeostasis during 3T3-L1 cells differentiation. Food Chem Toxicol. 2023;181:114107. https://doi.org/10.1016/j.fct.2023.114107
44. Marcelin G, Silveira ALM, Martins LB, Ferreira AVM, Clément K. Deciphering the cellular interplays underlying obesity-induced adipose tissue fibrosis. J Clin Invest. 2019;129:4032-40. https://doi.org/10.1172/JCI129192
45. Pham TP, Kwon J, Shin J. Berberine exerts anti-adipogenic activity through up-regulation of C/EBP inhibitors, CHOP and DEC2. Biochem Biophys Res Commun. 2011;413:376-82. https://doi.org/10.1016/j.bbrc.2011.08.110
46. Ali AT, Hochfeld WE, Myburgh R, Pepper MS. Adipocyte and adipogenesis. Eur J Cell Biol. 2013;92:229-36. https://doi.org/10.1016/j.ejcb.2013.06.001
47. Smith A, Yu X, Yin L. Diazinon exposure activated transcriptional factors CCAAT-enhancer-binding proteins α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) and induced adipogenesis in 3T3-L1 preadipocytes. Pestic Biochem Physiol. 2018;150:48-58.

https://doi.org/10.1016/j.pestbp.2018.07.003
48. Jakab J, Miškić B, Mikšić Š, Juranić B, Ćosić V, Schwarz D, et al. Adipogenesis as a potential anti-obesity target: a review of pharmacological treatment and natural products. Diabetes Metab Syndr Obes. 2021;14:67-83. https://doi.org/10.2147/DMSO.S281186
49. Teixeira C, Sousa AP, Santos I, Rocha AC, Alencastre I, Pereira AC, et al. Enhanced 3T3-L1 differentiation into adipocytes by pioglitazone pharmacological activation of peroxisome proliferator activated receptor-gamma (PPAR-γ). Biology. 2022;11:806. https://doi.org/10.3390/biology11060806
50. Chen R, Harrod KS, Olson JW, Gillespie MN. Regulation of gadd153 mRNA expression by hypoxia in pulmonary artery smooth muscle cells. Res Commun Mol Pathol Pharmacol. 2000;108:3-14.
51. Hegazi NM, Tantawy MA, Emam M, Bakry SM, Hussein SAA. Headspace gas chromatography/mass spectrometry analysis endorses melaleuca species as abundant source of medicinal eucalyptol and its proposed anti-obesity activity. Egypt J Chem. 2022;65:607-18.
52. Liu H, Sun W, Zhu H, Guo J, Liu M, Xu S. Eucalyptol relieves the toxicity of diisobutyl phthalate in Ctenopharyngodon idellus kidney cells through Keap1/Nrf2/HO-1 pathway: apoptosis-autophagy crosstalk and immunoregulation. Fish Shellfish Immunol. 2022;130:490-500. https://doi.org/10.1016/j.fsi.2022.09.056
53. Ahmed B, Sultana R, Greene MW. Adipose tissue and insulin resistance in obese. Biomed Pharmacother. 2021;137:111315. https://doi.org/10.1016/j.biopha.2021.111315

https://doi.org/10.5187/jast.2024.e6