Colocynth (*Citrullus colocynthis*) Flesh Extract Suppresses Adipogenesis by Down-Regulating Adipogenic Transcription Factors and Their Target Genes in 3T3-L1 Preadipocytes

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**Abstract**

*Citrullus colocynthis*, a member of the Cucurbitaceae family, is widely distributed in North Africa. The fruits are recognized for their wide range of medicinal uses and promising pharmaceutical potential. The present study aimed to investigate the anti-obesity effect of the ethanol extract of colocynth flesh (FCEE) in 3T3-L1 cells following treatment at different doses. The viability of 3T3-L1 preadipocytes was analyzed via MTT assay and triglycerides were stained with Oil red O to assess lipid accumulation. Additionally, adipogenesis-related gene expression was quantified via qRT-PCR. FCEE (0 - 150 µg/mL) dose-dependently suppressed intracellular triglyceride accumulation during the adipogenesis by 23% and 66% at 100 and 150 µg/mL, respectively, but did not affect cell viability. Analysis of the time-dependence of the effect of FCEE demonstrated that the greatest anti-adipogenic activity was observed during the early stages of differentiation. FCEE also decreased GPDH activity in a dose-dependent manner, with 98% decrease observed at 150 µg/mL. In addition, at same range of FCEE concentrations, the main transcription factors, including CCAAT/enhancer binding protein α (C/EBPα), peroxisome proliferator activated receptor γ (PPARγ), and sterol regulatory element-binding protein 1c (SREBP-1c), were downregulated by 90%, 89%, and 89%, respectively at 150 µg/mL. As these are the master regulators of adipogenesis. The inhibition of their downstream target genes was also observed. Colocynth may be useful in the treatment of obesity owing to its powerful effects on fat, which result in changes to adipocyte differentiation and fat mobilization.

**Keywords**

Adipogenesis, Colocynth, *Citrullus colocynthis*
1. Introduction

In spite of the current public awareness of obesity, its prevalence continues to increase. Obesity is increasing in both developed and developing countries [1]. It is one of the greatest public health problems, caused when energy intake exceeds energy expenditure and is considered a major risk factor associated with the development of metabolic disorders, such as type 2 diabetes, hypertension, and cardiovascular disease [2].

To study new therapeutic approaches, cell lines are used to investigate novel compounds and their effects on adipogenesis cells [3]. Many studies have suggested that in vitro mouse 3T3-L1 cells are one of the most well-characterized and reliable cell lines for the study of the differentiation of preadipocytes into adipocytes [4]. Adipogenesis involves the conversion of preadipocytes to mature spherical adipocytes. This conversion leads to both hypertrophy and hyperplasia of adipocytes [5], and it is characterized by morphological and biochemical changes in which the cells become round and begin to accumulate triacylglycerol and lipid droplets [6].

At cellular level, adipogenesis is tightly regulated by external factors that initiate the process, such as insulin, insulin-like growth factor (IGF), glucose, and free fatty acids. Among the external signals that trigger the activation of several transcription factors that promote preadipocyte differentiation and influence adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ) is considered the “master regulator of adipogenesis”. Other adipogenic transcription factors, which include the CCAAT/enhancer binding proteins, C/EBPα, C/EBPβ, and C/EBPδ, and sterol regulatory element-binding protein 1c (SREBP-1c), promote adipogenesis [2].

In this regard, many natural extracts have been found to have beneficial effects on health, and these extracts have drawn attention because of their relative safeness, and low cost as compared to synthetic drugs and accumulated evidence of physiological properties such as anti-obesity effect [7]. Recently, much attention has been focused on the use of medicinal plants in the form of a beverage or tea for the treatment of obesity. Natural products have historically and continually been investigated for promising new leads in pharmaceutical development. Many of the plant substances are used in traditional medicine because they are readily available in rural areas and cheaper compared to modern therapeutic agents [3]. Several reports have shown that natural products, including Hibiscus tea, Green tea, Caralluma fimbriata, and Yerba mate, showed promising potential as anti-obesity agents that do not result in unfavorable side effects [8] [9] [10].

Researchers are highly interested in studying plants with the aim of isolating novel active drugs to replace synthetic drugs present in the market. Herbal medicines are one of the most important components of traditional medicines found worldwide. Citrullus colocynthis (CC), commonly known as “bitter apple”, belongs to the family of Cucurbitaceae and is a perennial herb used fre-
quent in herbal medicines [11]. This desert plant, originally from tropical Asia and Africa, is adapted to arid environments. Each plant produces between 15 and 30 round fruits approximately 5 - 7.5 cm in diameter. The fruits are globose or ellipsoid, smooth, indehiscent, mottled yellow-green, spongy, and extremely bitter [12] [13].

In traditional medicine, CC has been used to treat stomach pains owing to its high content of glucosides, including colocynthin, which is an effective cathartic and laxative [14]. Several plant secondary metabolites, including cucurbitacins, flavonoids, caffeic acid derivatives, and terpenoid phenolic compounds, have previously been reported from this plant [15]. Furthermore, the dried pulp of CC has been used to treat constipation, edema, bacterial infections, cancer, diabetes, fever, and leukemia [11] [13].

Presently, the effect of CC on adipogenesis and its regulatory mechanism in adipocytes remain unclear. Therefore, we have hypothesized that the ethanol extract of the flesh of colocynth (FCEE) mediates the inhibition of adipocyte differentiation. To the best of our knowledge, the antiobesity activity of CC is being reported herein for the first time. The aim of this study was to assess the effect of FCEE on obesity by using an in vitro model (the 3T3-L1 preadipocyte cell line) and to elucidate the underlying molecular mechanism.

2. Materials and Methods

2.1. Materials

3T3-L1 cells were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). High glucose Dulbecco’s modified Eagle’s medium (DMEM), Oil red O, isobutyl-3-methylxanthine (IBMX), dexamethasone (DEX), and streptomycin were purchased from Sigma-Aldrich, Missouri, USA. Fetal bovine serum (FBS) was purchased from HyClone, US and penicillin and insulin were purchased from Wako, Osaka, Japan.

2.2. Plant Extract Preparation

The plant material CC was collected from Tataouine province in the south of Tunisia. The flesh of colocynth was allowed to dry in the shade (at 25˚C), and then ground into a fine powder. The extraction mixture, which contained 6 g of powdered flesh in 20 mL 70% ethanol, was maintained for 1 week at 25˚C in the dark, with occasional stirring. The liquid fraction was centrifuged, filtered through a 0.22-µm filter, evaporated to dryness using a speed vacuum (Spin-DryerLite VC-36R, TAITEC), and stored at −20˚C until use.

2.3. Cell Culture and Differentiation

3T3-L1 cells were cultured in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37˚C. The cells were plated in 24-well plates at a sufficient density in order to reach confluence in 2 days (5 × 10⁴ cells/well).

After reaching confluence, the differentiation of 3T3-L1 preadipocytes was
induced using a hormone cocktail, which contained 1 μmol/L DEX, 0.5 mmol/L IBMX, and 10 mg/L insulin, for 2 days. The confluent cells were treated with various concentrations of FCEE in DMEM; the final quantity of the solvent was 0.1%. On day 4, DEX and IBMX were removed, leaving half insulin and extracts in the cell medium for an additional 2 days. Thereafter, the cells were maintained in DMEM and FCEE extract, and the medium was changed every 2 days until day 8.

2.4. Oil Red O Staining and Quantification

Oil red O staining was determined using the method of Drira et al., 2011 [16], it was used to measure intracellular lipid accumulation in differentiated adipocytes. Briefly, cell monolayers were incubated with FCEE extract (25, 50, 100, and 150 μg/mL) during the differentiation period. After differentiation, 3T3-L1 cells were washed twice with phosphate buffered saline (PBS(-), pH 7.4) and fixed with 4% paraformaldehyde (Wako, Tokyo, Japan) at 4°C overnight. The cells were washed twice with sterile water and images were taken using a microscope (BioZero BZ-8000; Keyence, Osaka, Japan). The differentiated adipocytes were stained with 0.3 g/L Oil red O (in 60% isopropanol), the dye was extracted with isopropanol, and the absorbance (OD = 420 nm) was measured by using a Spectra Max microplate reader (Spectra Max 190; Molecular Devices Corporation, CA, USA). The results were presented as the relative lipid content of each experimental group and all experiments were performed in triplicate.

2.5. MTT Assay

Cytotoxicity was assayed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction method. MTT assay estimated by the method of Drira et al., 2013 [17]. Briefly, 3T3-L1 cells were seeded in a 24-well plate (5 × 10^4 cells/well). After reaching confluence, the culture medium was replaced by 500 μL medium that contained various concentrations of FCEE (0 - 300 μg/mL) and the cells were incubated for a further 48 h. Subsequently, the culture medium was removed and replaced by 500 μL of fresh culture medium that contained 10% sterile filtered MTT (Sigma-Aldrich). After 3 h, 500 μL isopropanol was added to each well to dissolve the formazan crystals and the absorbance was measured at 570 nm and 630 nm. The inhibition (%) was expressed as the percentage of viable cells compared with that of the control cells. The absorbance of the untreated (control) cells was considered as 100% cell viability.

2.6. Measurement of GPDH Activity

GPDH activity was measured according to Drira et al., 2011 [16]. 3T3-L1 cells were differentiated in a 40-mm culture dish for 8 days in the presence of FCEE (0, 100, and 150 μg/mL). After treatment, the cells were rinsed twice with PBS, and then scraped into 200 μL enzyme extraction buffer (sucrose, 280 mmol/L; Tris-HCl [pH 8.0], 5 mmol/L; EDTA, 1 mmol/L; and β-mercaptoethanol, 0.2%).
The cell solutions were sonicated and centrifuged at 15,000×g at 4°C for 10 min. The supernatant was collected and GPDH activity was measured. The total protein quantity was quantified using a protein assay kit (Bio-Rad laboratories, Inc., Tokyo, Japan).

2.7. RNA Isolation and Gene Expression Analysis

Confluent cultures of 3T3-L1 cells in 4-well plates were induced as previously described. Total RNA was extracted from 3T3-L1 adipocytes by using Isoplus reagent (Wako Pure Chemical Industries) and quantified spectrophotometrically. After the extraction, cDNA was synthesized from 100 µg RNA by using the Takara cDNA kit in accordance with the manufacturer’s recommendations.

The mRNA expression level was analyzed via quantitative real-time reverse transcription-PCR by using the fluorescent dye SYBR Green I and SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Shiga, Japan). Each cDNA was amplified for 40 cycles of the following conditions: 95°C for 5 s, 60°C for 30 s, 72°C for 30 s.

The gene-specific primers used are listed in Table 1 and the mRNA level for each gene was normalized to that of β-actin. All analyses were carried out in triplicate.

2.8. Statistical Analysis

All calculations were performed using GraphPad Prism 5 software (GraphPad Software, CA, USA). The results were expressed as the mean ± S.D. of a minimum of three replicate experiments. The significance of the differences between means was analyzed using Student’s t-test. P values < 0.05 were considered statistically significant.

3. Results

3.1. Inhibitory Effect of FCEE Extract on Triglyceride Accumulation in 3T3-L1 Adipocytes

To investigate the potential effect and the optimal dose range of FCEE, 3T3-L1 preadipocytes were incubated with several concentrations of the extract (25, 50, 100, and 150 µg/mL) during the process of differentiation. Adipocytes differentiated for 8 days were stained with Oil red O and the total lipid accumulation was quantified. As shown in Figure 1(a), FCEE was found to significantly inhibit adipocyte differentiation at 100 and 150 µg/mL. These results indicated that FCEE efficiently abolished adipocyte differentiation and demonstrated anti-obesity potential in 3T3-L1 cells. The quantification of Oil red O staining (Figure 1(b)) showed that FCEE could inhibit total lipid accumulation in a dose-dependent manner.

3.2. Effect of FCEE on Viability of 3T3-L1 Preadipocytes

The cytotoxicity of 25, 50, 100, 150, 200, 250, and 300 µg/mL FCEE was evaluated in the 3T3-L1 cell line. The appropriate concentrations of FCEE for the
toxicity of 3T3-L1 preadipocytes were determined using the MTT assay. 3T3-L1 preadipocytes were incubated in the presence of the indicated concentrations of FCEE for 72 h. As shown in Figure 2, treatment with FCEE at these concentrations did not result in any significant changes in cell viability ($P < 0.05$). Hence, we considered that the concentrations equal to, or lower than, 300 µg/mL FCEE were suitable for use in further experiments.

3.3. Effect of Time-Dependent Treatment of FCEE on 3T3-L1 Differentiation

The activity of the extract was tested at different stages of the differentiation process. 3T3-L1 cells were differentiated in the presence of FCEE and the analysis was separated into the following periods: 0 - 2 days (early stage), 2 - 4 days (middle stage), and 4 - 6 days (late stage).

As shown in Figure 3, treatment with FCEE during the early stage of differentiation resulted in a quantitatively similar level of inhibition of adipogenesis to that produced by treatment with FCEE for 8 days. In contrast, treatment during the middle stage produced partial inhibition; whereas, no inhibition was observed in cells treated only during the late stage.

3.4. Effect of FCEE on GPDH Activity in 3T3-L1 Adipocytes

The GPDH enzyme occupies a central position in triglyceride synthesis; therefore, we measured GPDH activity to determine the effect of FCEE on the differentiation of 3T3-L1 preadipocytes to adipocytes. As shown in Figure 4, FCEE (100 or 150 µg/mL) significantly inhibited GPDH activity in a dose-dependent manner. These results indicated that FCEE suppressed lipid accumulation by the inhibition of GPDH activity in adipogenesis.

3.5. Effect of FCEE on the Gene Expression of PPARγ, C/EBPα, and SREBP-1c and Their Downstream Target Genes

As FCEE reduced adipocyte differentiation, we were prompted to investigate the effect of FCEE on the mRNA expression of PPARγ, C/EBPα, and SREBP-1c and their target genes Glut4, CD36, leptin, adiponectin, and FAS. To investigate whether FCEE downregulated the adipogenic gene expression, total RNA from 3T3-L1 cells differentiated for 8 days was purified and then analyzed via qRT-PCR. As shown in Figure 5(a), treatment of preadipocytes with FCEE at 100 or 150 µg/mL significantly and dose-dependently decreased mRNA levels of PPARγ, C/EBPα, and SREBP-1c. We further determined whether FCEE could downregulate their target genes. Similarly, a significant ($P < 0.05$) dose-dependent downregulation of the expression of CD36, Glut4, leptin, adiponectin, and FAS was observed (Figure 5(b)).

4. Discussion

Obesity is a growing epidemic worldwide and significantly increases the risk of
Table 1. Gene-specific primers used for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
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</thead>
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<tr>
<td>β-actin</td>
<td>Forward</td>
<td>AACGCAGCTCAGTAACAGTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGATGTGGATGACGCAAGCAGG</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Forward</td>
<td>AAACCTGGGAGATTCTCCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCATTGTGAATGGAATGTTCT</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Forward</td>
<td>GCCAAACTGAGACTCTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAAGCCTAAGTCTAGGC</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Forward</td>
<td>GCTTAGGCCTCTACACAAACTGGC</td>
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<tr>
<td></td>
<td>Reverse</td>
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</tr>
<tr>
<td>Glut 4</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
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</tr>
<tr>
<td>CD36</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAGATGCGCTCAATTGGGC</td>
</tr>
<tr>
<td>Leptin</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GAAACAAACTCCACAGAATTG</td>
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<tr>
<td>Adiponectin</td>
<td>Forward</td>
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<td></td>
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<tr>
<td>FAS</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACAGCCTGGGGTGATCTTTGCC</td>
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Figure 1. Inhibitory effects of FCEE on triglyceride accumulation in 3T3-L1 adipocytes. 3T3-L1 cells were incubated in the induction medium with 25, 50, 100, and 150 µg/mL of FCEE for 8 days. Cells were stained with Oil red O (a). Stained intracellular oil droplets were eluted with isopropanol and quantified spectrophotometrically at 420 nm, magnification of 100× (b). Each bar represents the mean ± SE of three determinations. ***P < 0.001, by Student’s t-test compared with the control group.
Figure 2. Effect of FCEE on viability and differentiation of 3T3-L1 preadipocytes. 3T3-L1 cells were treated with varying concentrations of FCEE and incubated for 72 h. Values represent the mean ± SE of three determinations.

Figure 3. Effect of time-dependent treatment of FCEE on 3T3-L1 differentiation. 3T3-L1 cells were differentiated in the presence of FCEE at 0 - 2 days (early stage), 2 - 4 days (middle stage), 4 - 6 days (late stage), and 0 - 8 days. Total lipid quantity was quantified via Oil red O. The final concentration of ethanol was 0.1%. The error bars represent the standard error (±SE) of three independent trials.

Figure 4. Effect of FCEE on GPDH activity in 3T3-L1 adipocytes. GPDH activity was analyzed during the differentiation of 3T3-L1 in the presence of different concentrations of FCEE. Values represent the mean ± SE of three determinations. **P < 0.05 compared with the control group.
developing many chronic diseases, such as insulin resistance, diabetes mellitus, coronary heart disease, and hypertension [18]. White adipose tissue performs an important function in energy storage and organismal homeostasis through the secretion of molecules with endocrinial activities [19]. Adipocyte proliferation and hypertrophy are strongly associated with the development of obesity. As obesity is due to excessive adipogenesis, inhibitors of the differentiation of adipocytes may be effective in preventing obesity [2]. The study on adipocyte differentiation has been highly facilitated by cell culture models, such as fibroblastic 3T3-L1 cells [19]. The 3T3-L1 cell line is one of the most well-characterized and reliable models for studying the conversion of preadipocytes into adipocytes [20].

Plants have been employed for therapeutic purposes since ancient time. Many
phytochemicals served as an anti-obesity agent. The understanding of the activities of these compounds during adipogenesis is essential for the development of new treatments for obesity [10]. To the best of our knowledge, this is the first study to elucidate the effects of FCEE on 3T3-L1 adipocyte differentiation and analyze adipogenic gene expression.

In the present study, we demonstrated that FCEE suppressed lipid accumulation and GPDH enzyme activity in a dose-dependent manner. In fact, FCEE inhibited triglyceride accumulation by approximately 77% and 34% at 100 and 150 µg/mL, respectively (Figure 1). It is essential that the potential anti-obesity agents inhibit adipogenesis without cytotoxicity. Cell viability was measured using the MTT assay, in which cell viability is assessed by measuring mitochondrial activity in 3T3-L1 cells treated with different concentrations of crude extract [21]. We demonstrated that the plant extract was not cytotoxic at concentrations between 0 and 300 µg/mL (Figure 2).

Overall, our findings demonstrated that FCEE decreased adipogenesis in a dose-dependent manner without any effect on cell viability. Furthermore, GPDH activity was significantly inhibited by FCEE treatment in a dose-dependent manner. GPDH is important in the synthesis of fatty acids and triacylglycerols in adipocytes, which is increased during differentiation of preadipocytes into adipocytes. Therefore, the inhibition of GPDH activity may decrease differentiation in early-differentiating preadipocytes and lipid accumulation in mature adipocytes [2].

The analysis of the time-dependent effect of FCEE showed that inhibition was extract strongest during the early stage of differentiation, which runs parallel to clonal expansion. These results suggested that the inhibition of adipocyte differentiation by FCEE may start at the early stage of adipocyte differentiation. Our results agree with those of several previous studies, which showed that arresting or delaying the cell cycle of 3T3-L1 cells during the first 2 days of differentiation decreased the cell number and inhibited the differentiation rate of adipocytes [22] [23]. The differentiation of preadipocytes results in morphological and biochemical transition from the growth arrest of confluent preadipocytes, re-entry into the cell cycle for an additional several rounds of post-confluent mitosis, and terminal differentiation into mature adipocytes followed by changes in the genetic programs of lipid synthesis and storage [24]. Kuri-Harcuch et al. demonstrated that inhibition of DNA synthesis in 3T3-F442A cells blocked the formation of fat cells [25]. Tang et al. showed that blocking the entry of 3T3 cells into the S phase at the time of post-confluent mitosis prevented the differentiation of preadipocytes [26].

Adipogenesis involves several sequential events, such as cell cycle arrest, clonal expansion, and differentiation. These events involve a highly regulated cascade of transcription factors; it is believed that the process begins with the activation of C/EBPβ and C/EBPδ induced in response to isobutylmethylxanthine, dexamethasone, and insulin, which, in turn, activate PPARγ and C/EBPα. The final stages of differentiation require the expression of genes that contribute to the
maintenance of the adipocyte phenotype and function, such as the enzymes of lipid metabolism [27]. The expression of PPARγ and C/EBPα markedly decreased the expression of their downstream target genes involved in triacylglycerol metabolism including the fatty acid transporter CD36 and glucose transporter GLUT4. CD36, a target gene of PPAR-γ, plays a key role in adipogenesis [28]. In agreement with these results, CD36 mRNA expression was also dramatically decreased in FCEE-treated cells.

Next, we examined the expression of adiponectin and leptin. Adiponectin and leptin are two hormones secreted by white adipose tissue and regulate insulin sensitivity and energy balance [29]. Leptin is synthesized by adipocytes and is an important regulator of energy balance through its actions within the brain on appetite and energy expenditure. Adiponectin (also called Arcp30, GBP28, apM1, or AdipoQ) is produced by adipocytes, and plays an insulin-sensitizing role during glucose and lipid metabolisms of adipocytes, skeletal myocytes, and hepatocytes [30].

C/EBP and PPAR expression depends on other genes, which are also essential to adipogenesis, such as cAMP responsive element binding protein 1 (SREBP-1c). Our results showed that FCEE increased the expression of SREBP-1c and its downstream target gene (FASN) in a dose-dependent manner. SREBP-1c was identified as a pro-adipogenic basic helix-loop-helix transcription factor that induced PPARγ expression and possibly generated an as-yet-unknown PPARγ ligand [31]. SREBP-1c also mediated the induction of lipid biosynthesis by insulin in adipocytes that increased gene expression of the main lipogenic genes, such as fatty acid synthase and acetyl-CoA carboxylase [32]. SREBP-1c, a transcription factor that controls fatty acid synthesis, was found to be a crucial transcriptional regulator involved in adipogenesis, which was associated with the production of an endogenous PPARγ ligand that reinforces PPARγ activity [33]. SREBP-1c expression significantly increased in 3T3-L1 adipocytes in response to insulin [32], and its transcriptional activity increased in response to oxidative stress [34]. The SREBP family has been found to directly regulate a group of genes involved in TG and cholesterol synthesis [35]. In previous studies, dominant negative SREBP-1c expression was found to inhibit preadipocyte differentiation and HLH overexpression to enhance the adipogenic activity of PPARγ [36].

Moreover, it has been proven that FAS, an important fatty acid synthesis enzyme, facilitates the synthesis of triglycerides from free fatty acid [37]; FAS expression was significantly lower in FCEE-treated cells.

The data suggest that the anti-adipogenic effect of FCEE was mediated by downregulation of the expression of transcription factors, such as PPARγ, C/EBPα, and SREBP-1c, during adipocyte differentiation.

5. Conclusion

This study showed that FCEE effectively reduced lipid accumulation in 3T3-L1
preadipocytes through the inhibition of GPDH activity and downregulation of the expression of genes involved in the adipogenesis process. These results suggested that this extract could be useful in the prevention or treatment of obesity. However, in vivo studies should be conducted to confirm the anti-obesity effect of FCEE in animal models.

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**Author Disclosure Statement**

No competing financial interests exist.

**Conflicts of Interest**

The authors declare no conflicts of interest regarding the publication of this paper.

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**Abbreviations**

**FCEE**: Flesh Colocynth Ethanolic Extract  
**CC**: *Citrullus colocynthis*  
**OD**: Optical Density