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Alcea Rosea Çiçekleri Etanol Özütü *İn Vitro* Koşullarda Adipogenez Üzerine Baskılayıcı Etkiye Sahiptir
Ethanol Extract of *Alcea Rosea* Flowers Has a Suppressive Effect on Adipogenesis Under *In Vitro* Conditions

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ÖZET

Amaç: Obezite, ciddi sağlık komplikasyonlarına neden olan ve tüm dünyada görülen bir hastalıktır ve yeni, uzun vadeli ve etkili alternatif obezite karşıtı stratejiler geliştirme ihtiyacı vardır. Doğal ve bitkisel ürünler, anti-inflamatuar, antioksidan ve obezite karşıtı etkiler de dahil olmak üzere çeşitli biyolojik aktiviteleri nedeniyle obeziteyi tedavi etmede yaygın olarak kullanılmaktadır. Bu çalışmada, *Alcea rosea* çiçeğinin lipid birikimi ve adipogenezde önemli bir rol oynayan PPARγ ve C/EBPβ biyobelirteçleri üzerindeki etkilerini araştırmak amaçlanmıştır.

Materyal ve Metot: Adipogenezi in vitro ortamda mimiklemek için insan öncül adiposit hücreleri kullanıldı ve 37°C' de %5 CO_2 inkübatöründe bir preadiposit büyüme besiyerinde büyütüldü. Büyüyen hücreler 10^5 hücre/kuyu olacak şekilde ekildi, ardından 4 gruba ayrıldı ve her gruba farklı dozlarda (50 µg 100 µg 150 µg ve 200 µg) *Alcea rosea* etanol özütü (ARE) ve farklılaşma besiyeri uygulandı. 15. günün sonunda, lipid birikimini ölçmek için Oil O Red boyama yapıldı. PPARγ ve C/EBPβ gen ifadeleri RT-PCR yöntemi ile belirlendi.

Bulgular: Lipid birikiminin tüm ARE dozlarında önemli ölçüde ($p<0.05$) baskılandığı belirlendi. Ayrıca, ARE tüm dozlarda PPARγ gen ekspresyonunu baskıladı, ancak baskılama yalnızca 100 µg uygulanan seviyede istatistiksel olarak anlamlı bulundu ($p=0.05$). C/EBPβ geninin analizinde, baskılanma tüm dozlarda gözlemlendi, ancak baskılanma yalnızca 50 µg ($p=0.04$) ve 100 µg ($p=0.04$) dozlarında istatistiksel olarak anlamlı olarak bulundu.

Tartışma ve Sonuç: Sonuç olarak uygulanan ARE konsantrasyonlarının yağ oluşumunu baskıladığı ve obezite karşıtı özelliklere sahip olduğu tespit edilmiş olup, ancak çalışma bulgularının daha ileri çalışmalarla desteklenmesi önerilmektedir.

Anahtar Kelimeler: Adipogenez, Alcea Rosea, Obezite.

ABSTRACT

Objective: Obesity is a disease that causes serious health complications and is seen all over the world and there is a need to develop new long-term and effective alternative anti-obesity strategies. Natural and herbal products are widely used in treating obesity due to their various biological activities, including anti-inflammatory, antioxidant, and anti-obesity effects. It was aimed to investigate the effects of *Alcea rosea* flower on lipid accumulation and PPARγ and C/EBPβ biomarkers that have a key role in adipogenesis in this study.

Material and Methods: To mimic adipogenesis in vitro, human precursor adipocyte cells were used and grown in a preadipocyte growth medium at 37°C in a 5% CO_2 incubator. The grown cells were seeded at 10^5 cells/well, then divided into 4 groups, and different doses (50 µg 100 µg 150 µg and 200 µg) of *Alcea rosea* ethanol extract (ARE) and differentiation medium were applied to each group. At the end of the 15th day, Oil O Red staining was performed to measure lipid accumulation. PPARγ and C/EBPβ gene expressions were determined by the RT-PCR method.

Results: Lipid accumulation was significantly suppressed ($p<0.05$) at all ARE doses. In addition, ARE suppressed PPARγ gene expression at all doses, but the suppression was found to be statistically significant only at the 100 µg applied level ($p=0.05$). In the analysis of C/EBPβ gene, suppression was observed at all doses, but the suppression was found to be statistically significant only at the 50 µg ($p=0.04$) and 100 µg ($p=0.04$) doses.

Discussion and Conclusion: As a result, it was determined that the applied ARE concentrations suppressed adipogenesis and had anti-obesity properties, but it is recommended that further studies support the study findings.

Keywords: Adipogenesis, Alcea rosea, Obesity.

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INTRODUCTION

Obesity is a chronic disease associated with many metabolic complications resulting from the accumulation of white adipose tissue in the body. It is caused by environmental, genetic, and behavioral factors and contributes to high morbidity and mortality as well as reducing the estimated life span. Many studies are needed to explain the molecular pathology of obesity, which is spreading rapidly around the world (1).

The most important molecular change in obesity, which causes many metabolic problems following lipid accumulation, is the triggering of white adipose tissue formation. Many molecular pathways play a role in this process known as adipogenesis (2). There are two important transcription factors involved in the differentiation of preadipocyte cells, stem cells, into mature adipocytes, which have functions such as triglyceride storage and secretion of many cytokines and hormones: CCAAT/Enhancer Binding Protein β (C/EBPβ) and Peroxisome Proliferator Activator γ (PPARγ). PPARγ, the main regulator of adipogenesis, is induced during the differentiation of preadipocytes into mature adipocytes and plays a critical role in both white and brown adipose tissues. Another transcription factor, C/EBPβ, has a role in terminal adipocyte differentiation and induces PPARγ expression (3).

Alcea rosea L. (syn. Althaea rosea (L.) Cav.), also known as Hollyhock, is a herbaceous plant belonging to the Malvaceae family. *Alcea rosea* (AR) flower is a medicinal aromatic plant that has an important place in the treatment of diseases due to its phenolic and antioxidant content. However, it exhibits antimicrobial (4) and immunomodulatory (5) effects and helps regulate serum triglyceride and glucose levels (6). Shahabipour et al. reported that it has an apoptotic effect in colon cancer cell lines and is an epigenetic regulator with a suppressive function on EZH2 (7). It has also been reported by Choi et al. to suppress neoplastic cell transformation (8).

This study analyzed the effect of the ethanol extract of *A. rosea* flowers on the adipogenesis process. It primarily aimed to illuminate the effect of this plant, which is very common and has many beneficial properties, on obesity, today's disease. By looking at its effects on lipid accumulation and gene expression, it was found that the ethanol extract of AR flowers suppressed adipogenesis differentiation.

MATERIALS AND METHODS

Extraction and Preparation of Ethanol Extracts from A. rosea Flowers

The flowers of the dried AR plant were ground in a grinding mill and turned into powder. Powdered plant material was added to cover the ethanol solvent and macerated 3 times with the help of an ultrasonic bath. After maceration, the liquid parts were filtered with filter paper

and after filtration, the extracts containing the ethanol solvent were evaporated in the rotary evaporator to obtain crude ethanol extracts.

A stock solution was obtained by dissolving the extracts in DMSO (dimethyl sulfoxide) to be applied to the cells. The final DMSO (Sigma, Germany) concentration in the cell culture did not exceed 0.1%. The main stock was prepared at 1 mg/ml and applied to the cells by dilution to obtain final concentrations of 50, 100, 150, 200 µg/ml.

Culture of human preadipocyte cells

Human preadipocyte cells (HPAD) stored as stock at -196 °C were cultured in a human preadipocyte growth medium (Thermo Fisher, USA) under appropriate conditions (at 37°C with 5% CO_2). When the cells were fully confluent, they were passaged and multiplied until they reached the ideal number for the experiment.

Application of ethanol extract of A. rosea flowers (ARE) and differentiation of preadipocyte cells

The seeded cells were followed until sufficient density was reached in the culture dishes. To determine the effect of ARE on adipogenesis at the determined concentrations, the differentiation stage of human preadipocyte cells into mature adipocyte cells was chosen as a model. For this, HPAD cells were counted as 10^5 cells/well in 12-well culture dishes and inoculated with 1ml growth medium. Preadipocytes were incubated regularly until 90% confluent and checked. After the cells were 90% confluent, they were photographed at 10X. The medium on the cells was removed, and 1 ml of human preadipocyte differentiation medium (Thermo Fisher, USA) and 50µg, 100µg, 150µg, and 200µg/ml ARE dissolved in DMSO were added. Differentiation medium and ARE were added every 3 days for 15 days.

Analysis of Gene Expression

After the application of A. rosea extract and the time required for differentiation, cells were harvested for total RNA (Ribonucleic Acid) isolation, cDNA (complementary DNA) synthesis, and PCR (Polymerase Chain Reaction) analysis at day 15. Total RNAs were obtained with the total RNA isolation kit (GeneJet RNA Purification Kit, Thermo Scientific, USA) by the manufacturer's recommendations. Purity and concentration analysis of the obtained RNAs were performed using a spectrophotometer (Thermo Scientific, USA). The concentrations of all RNAs were adjusted to 1000 ng/µl. To determine the gene expression level, cDNA synthesis using 1 µg of RNA was performed using a commercially available kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA). The PCR conditions for cDNA synthesis included an initial step of 10 min at 25°C, followed by 15 min at 42°C, and a final step of 5 min at 85°C. With the obtained cDNAs, the expression level of the genes whose primer

sequences are given in Table 1 was checked. PPARγ and C/EBPβ gene primer sequences and GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) gene primer sequence used as internal control are given in Table 1 and synthesized by Oligomer Biotechnology (Turkey). SyberGreen Master Mix kit (Real Q Plus 2X Master Mix Green, Amlpicon, Denmark) was prepared according to the manufacturer's recommendations, and 40 cycles in the RT-PCR (Real-Time Polymerase Chain Reaction) device; quantitative RT-PCR was performed using a temperature program of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C. The relative expression of each gene compared to control cells was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Gene primer sequences used for RT-PCR.

Target Gene Name	Forward (F) and Reverse (R) Primer Sequence
GAPDH	(F) 5'-CGAGATCCCTCCAAAATCAA-3'
	(R)5'-TTCACACCCATGACGAACAT-3'
PPARγ	(F) 5'TCGGTTTCAGAAATGCCTTG-3'
	(R) 5'AGGTCAGCGACTCTGGATT-3'
C/EBPβ	(F) 5'TTTGTCCAAACCAACCGCAC-3'
	(R) 5'GCATCAACTTCGAAACCGGC-3'

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, PPARγ: Peroxisome Proliferator-Activated Receptor Gamma C/EBPβ: CCAAT Enhancer Binding Protein Beta F: Forward R: Reserve

Determination of Lipid Accumulation

Determination of lipid accumulation in cells was done by the Oil Red O Staining method. For the analysis of the effect of *A. rosea* flower ethanol extracts on lipid accumulation during the adipocyte differentiation process, 10^5 cells/well of human preadipocyte cells were passaged into 12-well cell culture plates, and the extract was applied at determined concentrations every 3 days during the 15 days required for the preadipocytes to differentiate into mature adipocytes. On day 15, the procedure previously described by You et al. (9) was applied with slight modifications. Cells were washed 2 times with 1 ml of PBS after the medium was collected. Cells were fixed by incubation for 1 hour with 1 ml of 10% formalin. Following this, the cells were washed 2 times with 1,5 ml ddH_2O and incubated for 5 min. It was treated with 1ml of 60% isopropanol. The isopropanol was then withdrawn and left to dry. When drying was observed, it was kept in an Oil Red O (Merck, Germany) working solution for 30 min. Oil Red O dye was aspirated and cells were washed with 1,5 ml of ddH_2O. Cells were photographed under a 10X inverted microscope. After photographing, 1 ml of 60% isopropanol was added to each well and the absorbance of the dye solution at 570 nm was measured in a spectrophotometer.

Statistical Analysis of Data

Two-tailed distribution and two-sample t-test analysis were used for data analysis. The statistical significance of the results obtained was evaluated over a p-value of 0.05, and values with a p-value of ≤ 0.05 were considered reliable.

RESULTS

ARE Suppresses Adipogenesis Gene Expression Levels

It was observed that ARE applied to human preadipocyte cells at doses of 50, 100, 150, and 200 µg/ml during differentiation suppressed the PPARγ expression level up to 0.02, 0.002, 0.05, and 0.03, respectively, compared to control cells (cells without any substance). From these results, at a concentration of 100 µg/ml, the gene expression level was found to be 0.002, which was statistically significant (p=0.05) (Figure 1).

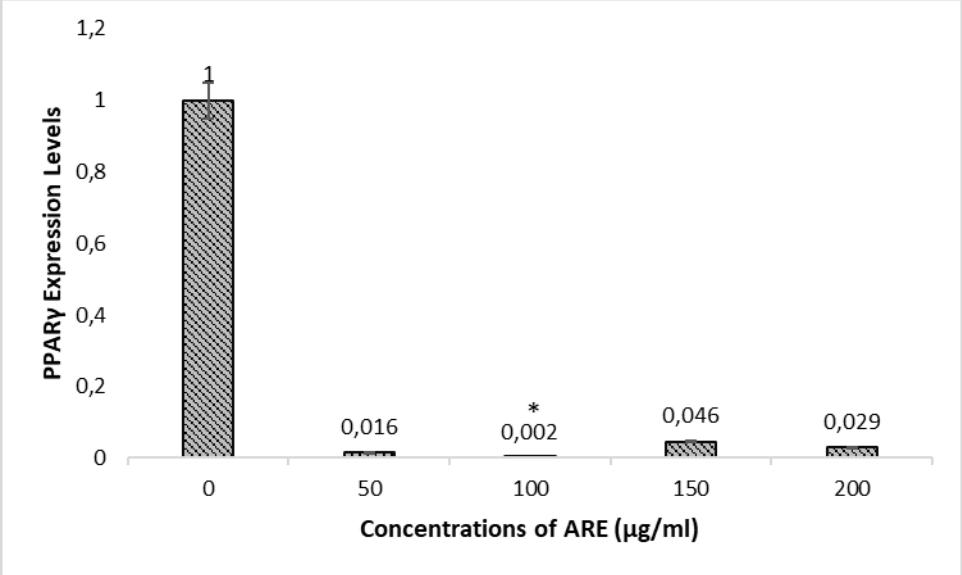

Figure 1: PPARγ gene expression level (*: statistically significant)

It was determined that ARE at different concentrations had a suppressive role on C/EBPβ expression during differentiation. A decrease in gene expression was detected at concentrations of 50, 100, 150, and 200 µg/ml, reaching levels of 0.001, 0.001, 0.065, and 0.028, respectively (Figure 2). Gene expression values at 50 and 100 µg/ml concentrations were found to be statistically significant (p= 0.04).

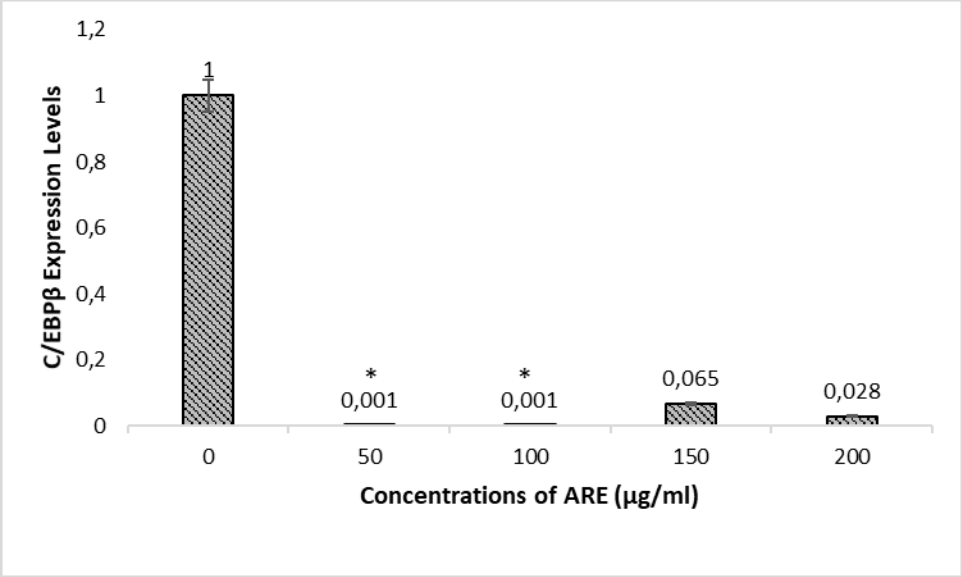

Figure 2: C/EBPβ gene expression level (*: statistically significant)

ARE Reduces Lipid Accumulation

As a result of examining the effect of ARE applied to preadipocytes at different concentrations on the adipogenesis process by Oil Red O staining, it was determined that lipid accumulation decreased at each concentration compared to the control. After staining, the absorbance measurements of the dye obtained from the cells were normalized with the absorbance value of the control cells. According to the result, absorbance measurements of 0.67, 0.57, 0.55, and 0.44 values were obtained at 50, 100, 150, and 200 µg/ml concentrations, respectively. It was determined that lipid accumulation decreased depending on concentration. All results were statistically significant for each concentration (p=0.04) (Figure 3).

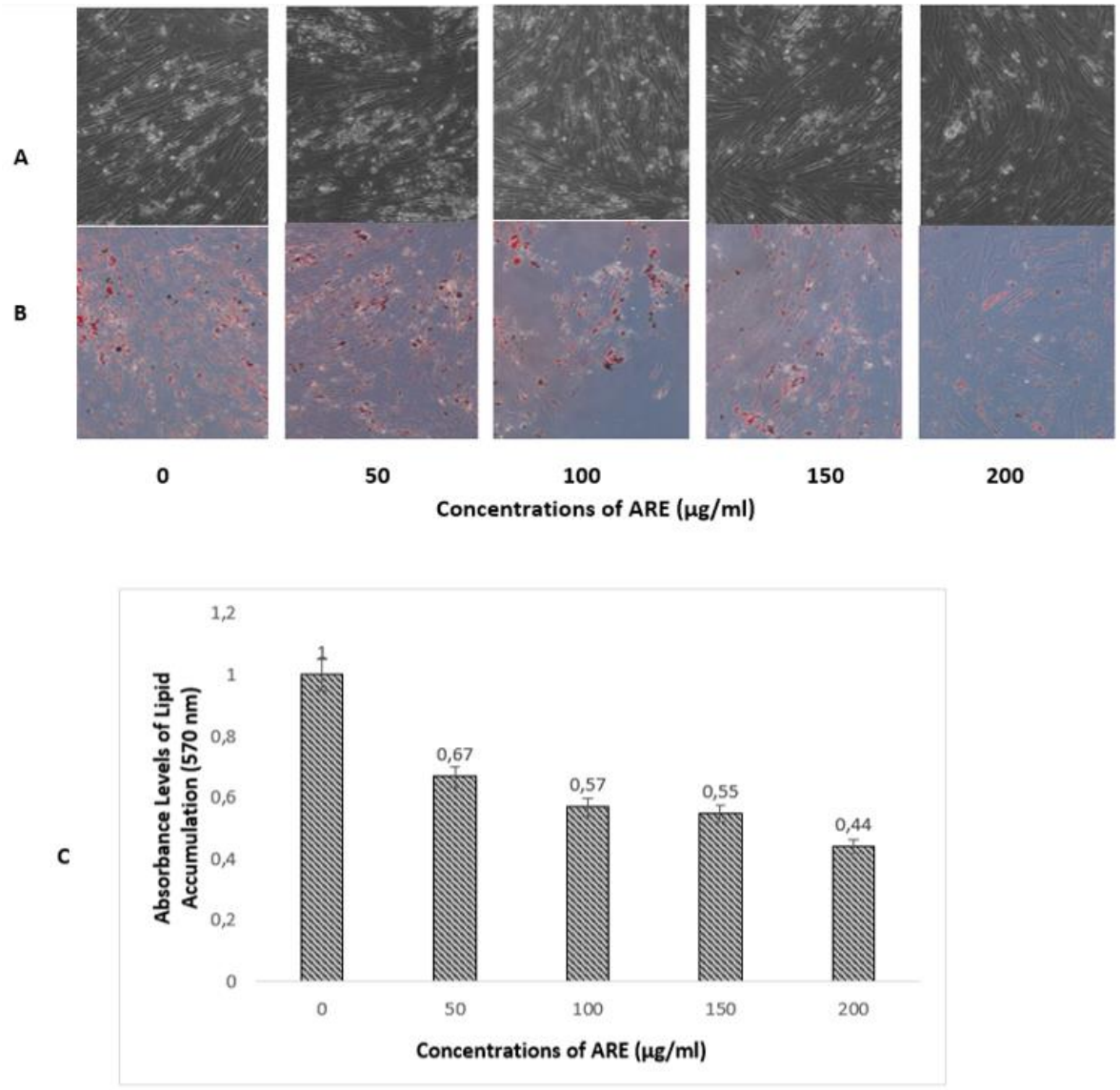

Figure 3: Effect of ARE on differentiation of human preadipocyte cells. Lipid accumulation was detected by Oil Red O staining. A: Microscopic images of differentiated adipocytes before oil red o staining. B: Microscopic images of differentiated adipocytes after oil red o staining (scale bar = 100 µm). C: Comparison of optical absorbance values at 570 nm of dye removed from adipocytes.

DISCUSSION

In obesity, which occurs due to increased food intake (hyperphagia) and adipocyte proliferation (hyperplasia), numerous studies focus on both prevention and treatment methods for obesity. The suppression of the adipogenesis mechanism plays a critical role in studies aimed at treating and preventing obesity. For this purpose, many studies have been conducted on the effects of medicinal and aromatic plants on transcription factors such as PPARγ and C/EBPβ, which play a key role in the adipogenesis process at the molecular level. *A. rosea* has been studied for years due to its anti-inflammatory, antimicrobial, anticancer effects, and rich phenolic content. The effects of phenolic compounds similar to those found in *A. rosea* have been studied in

relation to obesity, but mostly by isolating these compounds from other plants. So this study is unique in this respect. In this study, the effects of ARE on the differentiation of human preadipocyte cells were investigated.

As a result of this study, it was observed that ARE applied in 50µg, 100 µg, 150 µg, and 200 µg doses suppressed lipid accumulation in human adipocyte cells. Swamy et al. isolated astragalin (3-0-glucoside of kaempferol) from *Moringa oleifera* leaves and investigated its effects on adipogenesis and adipokines in 3T3-L1 cells. In a dose-dependent study, it was concluded that astragalin significantly reduced triglyceride content and lipid accumulation (10). In our study, lipid accumulation was similarly reduced in cells where A. rosea extract was applied. Similarly, kaempferol flavonoid found in A. rosea flowers reduces lipid accumulation in 3T3-L1 cells (11); another flavonoid, isoquercetin, has been reported to reduce lipid accumulation in H4IIE cells (12) and 3T3-L1 cells (13). As a result of the study by Dudek et al., the presence of phenolic acids such as syringic acid, vanillic acid, ferulic acid, p-hydroxybenzoic acid, and pcoumaric acid was determined in *A. rosea* flowers (14). Among these phenolic acids, pcumaric acid (15), ferulic acid (16), and vanillic acid (17) have been reported to significantly reduce lipid accumulation.

It was determined that the gene expression level of PPARγ and C/EBPβ transcription factors, which play a role as the main regulator in adipogenesis, was suppressed at each dose following the application of ethanol extract of *A. rosea* flowers. Vanillic acid, a component of the phenolic content in A. rosea, reduces the expression of PPARγ and C/EBPα in 3T3-L1 cells (17). Similarly, myricetin has been reported to suppress the expression levels of PPARγ and C/EBPβ with statistical significance (18). Another flavonoid luteolin in *A. rosea* significantly reduces the expression of PPARγ and C/EBPα mRNA in 3T3-L1 cells (19) and inhibits adipose differentiation in bone marrow cells by suppressing PPARγ (20) has been revealed in studies. Additionally, other compounds such as delphinidin-3-O-β-glycoside (21), quercetin (22), and kaempferol (23) have also been observed to suppress PPARγ and C/EBPβ expression in 3T3-L1 cells.

CONCLUSION

As a result of this study, it was determined that ARE suppressed adipogenesis under in vitro conditions. Based on these results, it is recommended to conduct in vivo studies on ARE and to investigate the molecular mechanisms involved in adipogenesis using more comprehensive methodologies.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Ethics Approval and Consent

This study was conducted with in vitro experimental methods with cell lines. Therefore, any ethical approval needed for clinical experiments was not obtained.

Conflict of Interest

No conflict of interest was declared by the authors.

Author Contributions

Ebru Demir/ Ceyda Okudu/ Özlem Ağırel/ Hüseyin Servi; in vitro experiment and writing/hypothesis, in vitro experiment and writing/ in vitro experiment and writing/ extraction of ARE.

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