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Original Article

Effects of Persian Shallot Extract on Inhibition of Adipogenesis in 3T3-L1 Cells

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ABSTRACT

Today, the industrial world has encountered the problem of obesity which can be a risk factor for diseases such as cancer, cardiovascular diseases, and type 2 diabetes. Various strategies have been proposed for the management of obesity, one of which includes the utilization of medicinal plants. This study examines the impact of hydroalcoholic extract from Persian shallots on the adipogenesis of the 3T3-L1 cell line. The cell viability of Persian shallot extract was evaluated using the MTT assay following treatment with various concentrations of the extract, with assessments conducted at 24-hour and 48-hour intervals. The lipid content of the cells was evaluated through Oil-Red O staining, in conjunction with quantitative real-time PCR analysis to assess the expression of the PPARγ gene. The results from the MTT test indicated that concentrations below 250 µg/ml maintain cell viability levels exceeding 70%. However, the concentration of 125 µg/ml was also employed in this context. Upon reaching 80% confluency, the cells are treated with a differentiation medium for two days. The subsequent step involved transitioning to an expansion medium for six days. The expansion medium was refreshed every 48 hours during this period. During the differentiation process on Day 8, we observed the presence of lipid droplets that had accumulated. In the Oil-Red O staining analysis, our observations indicated a significant reduction in the lipid content of mature adipocytes treated with 125 µg/ml compared to the control group of mature adipocytes. The gene expression analysis revealed a significant decrease in PPARγ expression on the eighth day of differentiation, with a reduction of 77% compared to the control group (*P* value = 0.028). These findings indicate that Persian shallot may represent a viable treatment option for managing obesity.

Keywords: Obesity, Medicinal Plants, PPARγ, Adipogenesis

INTRODUCTION

Obesity as a multifactorial disease is affected by genetics, lifestyle, environment, or their combination [1]. Obesity has nearly tripled worldwide since 1975 [2]. Recent data indicates that more than 1.9 billion adults aged 18 and older are classified as overweight, with nearly 650 million of these individuals identified as obese. Moreover, the world's population mostly lives in countries where more people die from obesity and overweight than underweight. Yet, obesity is manageable and preventable. Obesity is both manageable and preventable. According to the World Obesity Federation's 2017 report, Iran ranks ninth among the ten countries with the highest obesity rates, with over 21 million individuals affected [3].

Among the common strategies for treating obesity are changing eating habits, exercising, decreasing calorie intake, and even altering living environments where nutritious food is more available than unhealthy foods [4].

Since genetics is considered to have a substantial role in the expansion and differentiation of stem cells into White Adipose Tissue (WAT), many studies have focused on genetics and its role in adipogenesis and obesity as a whole. Adipogenesis is a type of differentiation in which preadipocyte stem cells change irreversibly in function and morphology and mature to form adipocytes and white fat tissue [5]. Recent studies have identified several genes associated with the differentiation of undifferentiated cells and preadipocytes into adipocytes. This research highlights the critical roles these genes play in the adipogenic process. Several transcription factors contribute to adipogenesis, such as the CCAAT/enhancer-binding proteins (C/EBP) and peroxisome proliferator-activated receptor γ (PPARγ) coordinating the expression of several genes to develop the mature

adipocyte phenotype [6,7]. Overall, adipogenesis is a crucial process in the development and maintenance of white fat tissue in the body, and understanding the factors that regulate this process can provide valuable insights into the development and treatment of metabolic disorders such as obesity and type 2 diabetes.

According to the literature, substantial progress has been made concerning bioactive components in plant foods and their relation to obesity. PPARγ can be directly or/and indirectly regulated through the anti-adipogenic activities of different natural products. Two specific targets have been identified for regulation, which involves the activity and expression of PPARγ. To date, numerous natural products have been reported as having the ability to suppress adipogenesis by downregulating PPARγ [8].

Allium hirtifolium, belonging to the Allium L. genus, is known as Persian shallot (Mooseer) and is propagated through bulbs. [9]. Persian shallot is a perennial and medicinal plant that grows naturally in elevated lands. It can be found wild across the Zagros Mountains and is frequently used in Persian cuisine. Furthermore, it has demonstrated potential efficacy in the treatment of lipoma in southern Thailand and onchocerciasis, a neglected tropical disease recognized by the World Health Organization, in Nigeria [10-12].

The potential benefits of this compound in cancer prevention, cardiovascular health, and diabetes management are noteworthy, attributed to its various organosulfur compounds and antioxidant properties. Furthermore, it can be used for other non-communicable diseases related to inflammatory and oxidative pathways most likely (indirect) owing to overweight or obesity [13-16]. Organosulfur compounds as a class of sulfur-containing compounds are distributed widely in Allium species, such as shallots. Allyl methyl trisulfide (AMTS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and allicin are among the most important organosulfur compounds existing in Persian shallot (and generally in the Allium family) [16]. It is worth noting that the extraction method can also affect the results. Hexane, ethanol, and methanol are commonly used for extraction representing more organosulfur content.

A novel approach is currently being pursued to explore the potential therapeutic properties of the traditional medicinal plant, Persian shallot. This natural remedy holds promise as an alternative intervention in the battle against obesity. By delving into the genetic and physiological aspects of adipogenesis and harnessing the medicinal properties of the Persian shallot, this research aims to offer a fresh perspective on managing obesity and improving public health outcomes by considering the many properties of the Persian shallot mentioned above that there is still no scientific evidence about its adipogenesis inhibition feature. In the present research, we aimed to investigate the effects of Persian Shallot extract on the prevention of adipogenesis in the 3T3-L1 cell line for the first time. The murine 3T3-L1 cell line serves as a widely recognized model in the study of adipose cell biology.

MATERIALS AND METHODS

Preparation of Persian Shallot Extraction

Persian Shallot was bought (1kg) from a local farmer in Hamedan province. A pharmacognosy expert from the School of Pharmacy, Alborz University of Medical Sciences, Karaj had approved the authenticity of the product, using visual characteristics of the herb. Persian shallots usually have 80 cm to 100 cm of erect scape and two or three leaves at the base and with a flower which is usually a violet or red color. After cleaning, 800g of shallots were diced and homogenized for extraction. Using 80% ethanol (1 liter), the mixture was passed through a strainer after incubation for 48 hours at room temperature. The cloudy liquid obtained was processed using a rotary extractor for further extraction purposes. The temperature was meticulously controlled and maintained at or below 37°C to prevent any alterations or deactivation of the active compounds due to elevated temperatures. The dark brown extract had a final weight of approximately 170g, a notable sulfur smell, and high viscosity. Through evaporation, the total extract was concentrated for 48 h at 37°C, and the dry mass was dissolved in PBS. The overall yield attained was around 60%, comprising 3% phenolic compounds.

Cell Culture

Iranian Biological Resource Center (IBRC Cell ID: C10152) provided the 3T3-L1 cell line. The cells were Briefly grown in DMEM complemented with 18mM Sodium bicarbonate, 10% FBS, and 100 U/mL penicillin-streptomycin in a humidified 5%CO_2 atmosphere. The 3T3-L1 cells were to grow for another 24 h by reaching the confluence. Followed by the confluence of 70 to 80 %, the cells were planted into a six-well plate, each

with about 80000 cells. The cells were then incubated in DMEM+FBS 10% for 48 hours and an additional 48 hours was spent in the same media as after 80% pre-confluency (Day 0). Then, the cells are treated with a differentiation medium comprising 0.5 mM IBMX (Merk, #I5879), 1.0 µM Dexamethasone (Merk, #D4902), and 5.0 µg/mL Insulin (Merk, #I6634) for 48 hours (Day 2). The media was then replaced with an expansion medium supplemented with 5.0 µg/mL of insulin for a span of six days. The expansion medium was replenished every 48 hours to account for the changes in viscosity and color attributed to nutrient consumption and fluctuations in pH during adipogenesis. On the last day of differentiation (Day 8), droplets of accumulated lipids were found in about 90% of the cells in the control group. DMEM was used as a basic medium for differentiation, as well as the expansion medium comprising 10% FBS in the absence or presence of Persian Shallot at the presented concentration until the 8th day. This study was ethically approved by Islamic Azad University, Isfahan (Khorasgan) Branch, Isfahan, Iran (Ethical Code: IR.IAU.KHUISF.REC.1402.303).

Cell Viability Assay

The MTT assay was used to determine the Cell viability. The tests were conducted in 96-well plates. Seeding the 3T3-L1 cells (2000 cells/well), they were grown to confluency and allowed to grow for an additional 24 hours. The culture medium was substituted with 200 µL serial dilutions of Persian Shallot extract (1mg/ml, 250µg/ml, 500µg/ml, 62.5µg/ml, 125µg/ml, 31.25µg/ml and 15.6µg/ml), after 24 h, with 5 repeats. The cells were then incubated for 24 and 48 h. Removing the culture solutions, they were replaced by a culture medium (90 µL). A final concentration of 0.5 mg MTT/mL was obtained by adding 10 µL of a sterile, filtered MTT solution (DNA biotech, #DMA300) (5 mg/mL) in phosphate-buffered saline to each. The unreacted dye was eliminated after 5 h. The insoluble formazan crystals were then dissolved in DMSO (200 µL/well) and spectrophotometrically measured in EPOCH spectrophotometer at 570 nm. To state the cell population growth percentage (%), the cell growth percentage was considered against the control using A_{570nm} [Persian Shallot]/A_{570nm}[control] X 100. To calculate IC_{50}, the Persian Shallot concentration was used under which cell population growth was inhibited 50% more than the untreated controls.

Oil-Red O Staining

Oil-Red O staining is extensively used for intracellular lipid staining, which was conducted on the 8th day (Mature adipocytes). Briefly, cell monolayers were rinsed twice with PBS, and it was fixed for 1 hour using 10% formaldehyde in PBS (pH= 7.4) and incubated at room temperature. Then, to stain the cells, 1.2 mg Oil-Red O C.I.26125 (Sigma-Aldrich, #1.05230) was used, which was dissolved into 60% isopropanol (1 ml). The dye solution-containing flasks were incubated at room temperature for 20 min. The staining process was monitored Every 5 minutes.

Three images were charged for each dish specifically for control cells treated with 10µl of PBS and 125 µg/ml behind the mounting process, an Optika IM-3FL microscope. Among three images, one was randomly selected to maintain the principle of neutrality.

Quantitative real time RT-PCR

To extract the total RNA, mature adipocytes (Day8) were used Trizol reagent (DNA biotech, #DB9683). The non-treated differentiated cells served as the negative control. To synthesize the first-strand cDNA, a total of 0.25 µg RNA was utilized. Reverse transcription was conducted in a programmable thermal cycler in 20 µL comprising 0.3% b-mercaptoethanol, 25 mM Tris–HCl, 1 mM of each deoxynucleotide triphosphate, 50 mM $(NH_4)_2SO_4$, 0.1 g/L bovine serum albumin, 2.5 units RNase inhibitor, 5 mM $MgCl_2$, and 0.5 mM oligo dT and Moloney murine leukemia virus reverse transcriptase.

Primer design for PPARγ2 and β-actin genes was done via the NCBI Primer design tool. The real-time primers were as follows and the primers for discovering PPARγ2 (PCR product size: 198 bp) were as follows:

Forward: 5'-TTTTCAAGGGTGCCAGTTTC-3'

Reverse: 5'-AATCCTTGGCCCTCTGAGAT-3'

For β-actin (PCR product size: 227 bp), the primers were used as a housekeeping gene:

Forward: 5'-CACCATTGGCAATGAGCGGTTC-3'

Reverse: 5'-AGGTCTTTGCGGATGTCCACGT-3'

Then, the Thermocycler (Applied biosystems Step-One plus) was programmed to 40 cycles, 5 minutes at 60˚C for annealing, 30 seconds at 95˚C for denaturation, and 1 minute for 72˚C for extension. To measure relative gene expression, the $2^{-\Delta\Delta C_T}$ method was used along with Average Fold Change measurement.

Statistical Analysis

All experiments were performed independently in Four repetitions (cell viability with 5 repeats) and stated as mean ± standard deviation (SD). To check the normality of the data, the Shapiro-Wilk test was used at different concentrations in the MTT assay. In addition, independent *t*-tests and one-way ANOVA were compared to detect differences between groups. Tukey's post hoc test was used to make pairwise comparisons between concentrations and control and $P < 0.05$ was considered statistically significant.

RESULTS

Persian Shallot Extract

The obtained Persian Shallot extract was dark brown with a dense texture, higher viscosity, and a very considerable unique smell. It was kept at -20˚C for further examinations. No freezing was performed for the extract during the storage, owing to very low water and additional content.

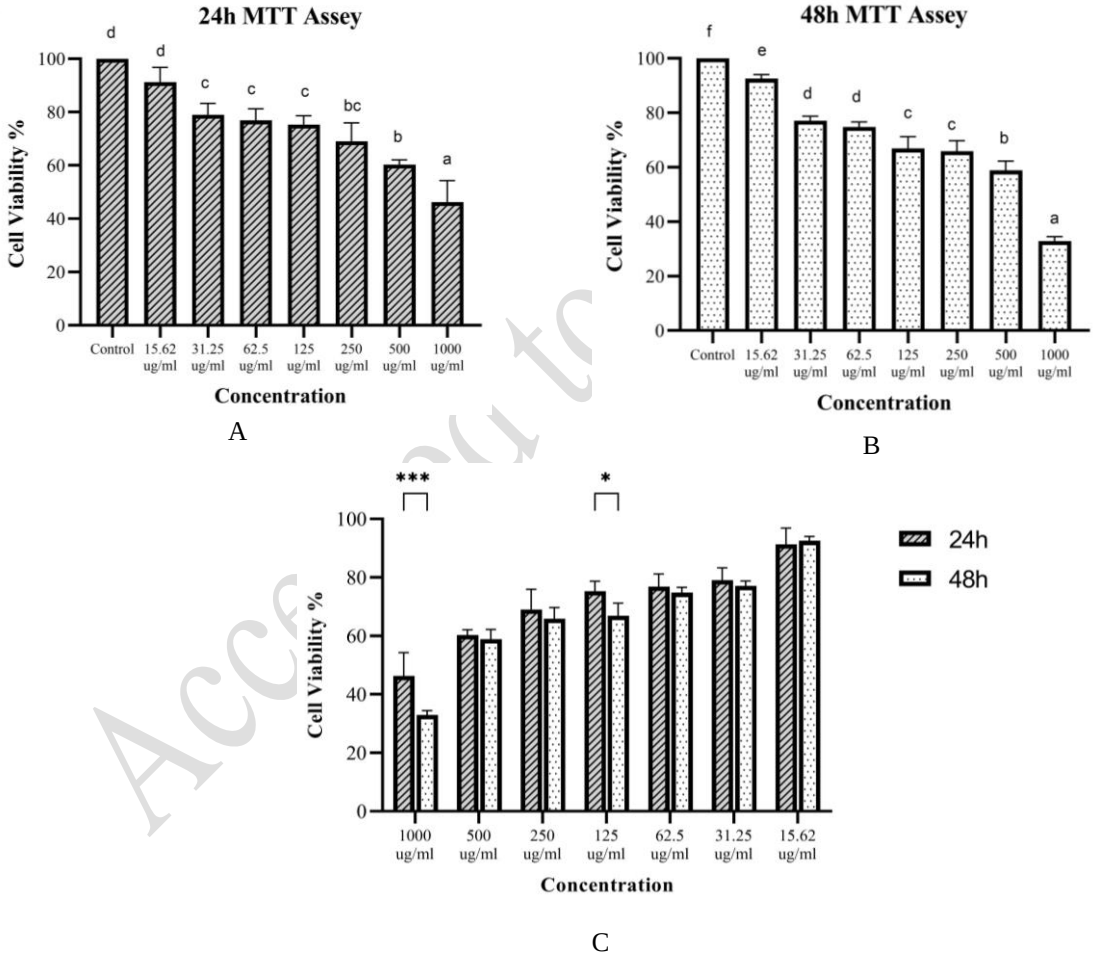

Fig. 1 The effect of Persian Shallot on cell viability in 3T3-L1 preadipocytes. A) 3T3-L1 preadipocytes were incubated for 24 h with various concentrations of Persian Shallot extract (0–1000 µg/mL). Concentrations with significant differences are indicated by different English letters. One-way analysis of variance was used. B) 3T3-L1 preadipocytes were incubated for 48 h with various concentrations of Persian Shallot extract (0– 1000 µg/mL) Concentrations with significant differences are indicated by different English letters. One-way analysis of variance was used. C) Comparison of cell viability percentage in the treatment with different concentrations of Persian Shallot in 24 hours and 48 hours. Tukey's post hoc test was used to make pairwise comparisons between concentrations. The significant difference between 24 h and 48 h is indicated by * *(P*<0.05*)* and *** *(P*<0.001*)*.

MTT Assay

MTT assay was conducted on 24 h and 48 h in 7 doses of Persian Shallot extract for detecting the effects of extraction cell viability. The extract was dissolved in PBS (15.62 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml) five times (Figure 1). After controlling and confirming the normality of the data distribution by the Shapiro-Wilk test; In order to compare the percentage of cell viability between different concentrations at 24 and 48 hours, one-way analysis of variance (ANOVA) was used. Tukey's post hoc test was used to make pairwise comparisons between concentrations. The results of this test are shown in Figure 1. In this figure, concentrations with significant differences are marked with different English letters. According to Figure 1A, in 24 hours, the percentage of cell viability in the concentrations of 31.25 µg/ml to 1000 µg/ml was significantly lower than the concentration of 15.62 µg/ml and the control (P<0.001). At 48 hours, the percentage of survival in all concentrations was significantly lower than the control (P<0.001) (Figure 1B). Based on the comparison of concentrations at 24h and 48h, there was a significant difference in concentrations of 125 µg/ml (P<0.05) and 1000 µg/ml(P<0.001) (Figure 1C). The doses less than 250 µg/ml (with over 70% cell viability) were utilized during 3T3-L1 cell adipogenesis as the cells spent 8 days to differentiate completely into adipocyte cells. Using cells treated with 125 ug/ml Persian Shallot extract, the citable results were achieved over the differentiation.

Oil-Red O Staining

After 80% pre-confluency, the cells are treated with differentiation medium for 2 days. Altering the medium to an expansion medium for six days was the next step. Every 48 hours, the Expansion medium was changed. During differentiation (Day 8), droplets of accumulated lipids were found. Following by differentiation of the cells, the lipid accumulation was qualitatively measured (Figure 2). Based on the control cells treated with PBS (10ul), lipid droplets were found in about 80% to 90% of the cells with a strong red color and overlapped cells. However, the cells treated with 125 µg/ml of extract were light red with no excessively noticeable droplets. There was a visible change in morphology in control cells with an additional rounded shape. However, a more fibroblastic-like morphology was found in treated cells. The cell count in treated cells was reduced owing to the extract in their medium for 8 days, which is well-suited with the results of MTT Assay.

Inhibition of Adipogenesis by Iranian Shallot Extract

The effect of Persian Shallot extract on adipogenesis was determined by treating 3T3-L1 cells with 125 µg/ml during the differentiation procedure. For PPARγ gene expression, mature adipocytes were studied (Figure 3). PPARγ mRNA level was significantly reduced than the untreated cells (77%, P value= 0.028).

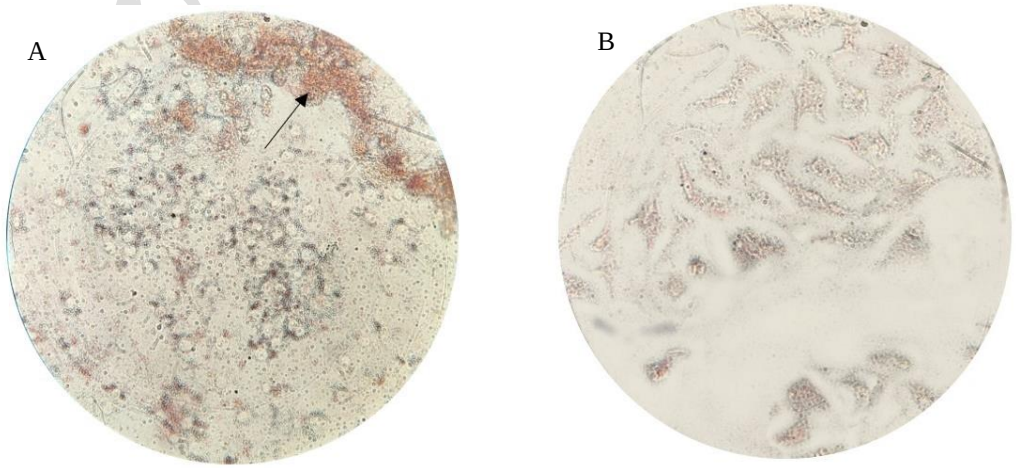

Fig. 2 Oil- red O staining of the 3T3-L1 preadipocyte cells. A) In control cells treated with 20µl PBS, droplets of lipid accumulated are seen in the picture and the general morphology of the cells is changed to a rounder shape and it differs from fibroblastic morphology, compactness of the cells and the red color resemblance is acknowledgeable. ➔ indicates the accumulation of fat in adipocytes. B) Cells treated with 125 µg/ml Persian Shallot extract. Microscopic pictures were taken at 100 X magnification

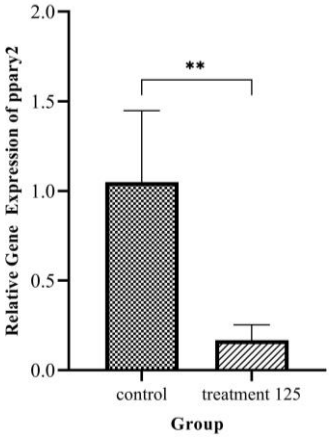

Fig. 3 Calculating gene expression with Average Fold Change between control cells and treated cells which shows a significant decrease in PPARγ2 expression in treated adipocytes. *P* value based on independent *t* test

DISCUSSION

Obesity is considered a universal health crisis, affecting millions of individuals worldwide as a severe health concern. Due to the increasing number of obese subjects, this epidemic poses a significant economic burden on healthcare systems, with costs associated with obesity-related diseases such as diabetes, cardiovascular diseases, hypertension, and certain cancers [17-19].

Adipogenesis, the process of cell differentiation by which preadipocytes become mature adipocytes, is tightly regulated by a network of transcription factors and signaling pathways. Peroxisome proliferator-activated receptor gamma (PPARγ) is a critical modulator of this process, but other factors such as CCAAT/enhancer-binding protein alpha (C/EBPα) and sterol regulatory element-binding protein 1 (SREBP1) also play essential roles [20].

Recent research has focused on the potential of natural products to combat obesity by inhibiting adipogenesis. Compounds such as resveratrol, found in grapes, and curcumin, the active ingredient in turmeric, have demonstrated significant anti-adipogenic effects in vitro and in vivo. These natural products exert their effects through various mechanisms, including the downregulation of PPARγ expression and activity. The exploration of such compounds offers promising avenues for the development of safer and more effective anti-obesity therapies [21]. Several natural products can suppress adipogenesis through the downregulation of PPARγ expression and/or activity [8]. Drugs such as sibutramine and orlistat with side effects including increased blood pressure, headache, dry mouth, constipation, and insomnia [22]. Numerous prominent therapeutic targets have attracted the attention of the scientific community notwithstanding various disappointments [23, 24]. Recently, natural alternatives exhibiting anti-obesity potential have been investigated widely. In this regard, the Allium family is known for a huge deal of organosulfur giving them a distinctive smell [25].

Persian Shallot, (Mooseer), a member of the Allium genus, is a native plant of Iran known for its distinctive organosulfur compounds. Traditionally used in Persian cuisine and medicine, Persian Shallot has been reported to possess antibacterial, anti-inflammatory, and lipid-lowering properties. Phytochemical analyses have identified several bioactive compounds in Persian shallot, including flavonoids, saponins, and alkaloids. These compounds contribute to its potential health benefits and warrant further investigation [10, 26, 27]. It has been indicated that the 3T3-L1 preadipocyte line has characterized the ability to experience complete differentiation within mature adipocytes [28].

The presented study focuses on investigating the molecular aspect of the differentiation process of adipogenesis. An innovative approach is being pursued through the investigation of a traditional medicinal plant, Persian Shallot, as a potential solution. With its natural origins, the medicinal properties of Persian Shallot offer promise as an effective intervention in combating obesity and managing this serious health concern.

In our study, the preadipocyte cells were treated with different concentrations of Mooseer extract for 24 h and 48 h to study the effects of Persian Shallot extract on viability in mature adipocytes. Persian Shallot extract did not display any cytotoxic effect at 15.62 µg/ml, 31.25 µg/ml, 62.5 µg/ml and 125 µg/ml. Hence, extract at 125 µg/ml was non-cytotoxic for mature 3T3-L1 adipocytes in this work (Figure1). Moreover, according to the Oil-Red O staining results, Persian Shallot extract might possess an anti-lipogenic effect in mature 3T3-L1

adipocytes (Figure 2). Persian Shallot extract also nearly contained 3.7% polyphenol compounds which seem to be able to affect fat mass and human and animal fat cells via suppression of adipocyte differentiation thus reducing lipogenesis [29]. Using 125 µg/ml of Persian shallot extract, the expression PPARγ levels were reduced by > 77% (*P* value= 0.028), (Figure 3) which is required to maintain mature adipocyte function, such as triglyceride storage. In the present work, the PPARγ mRNA expression was down-regulated significantly by treatment with Mooseer extract (125 µg/ml) in mature 3T3-L1 adipocytes. It was revealed that lipid accumulation was inhibited by Persian Shallot extract in mature 3T3-L1 adipocytes. The presence of organosulfur compounds was confirmed in different parts of this plant such as stem, flower, and onion while phytochemical analysis and assessing the transcriptome *Allium hirtifolium* [30]. Since we used shallots in this work, Persian Shallot extract seems to contain organosulfur compounds. It has been reported that numerous organosulfur compounds possess inhibitory effects on adipogenesis thus reducing lipogenesis in 3T3-L1 adipocytes [31].

In conclusion, it is demonstrated that Persian Shallot (Allium hirtifolium) extract seems to reduce the fat content of mature adipocytes, as well as the expression of the PPARγ gene, which is a crucial agent during adipogenesis. Therefore, it can be considered a natural food composition that helps manage obesity as a complementary nutrient. There were some limitations to the present study, including the need to examine other genes involved in the adipogenesis signaling pathway, such as FAS, C/EBPα, adiponectin, and leptin, as well as conducting cell apoptosis analysis and determining the activity of enzymes like SOD and LDH. Hence, further animal models and in vivo studies are required.

It needs to be noted that the expression of the PPARγ gene was measured at the mRNA level, which may not be completely accurate due to the substantial role of epigenetic factors at this level. It must be mentioned that tandemly repeated DNA sequences, which can be highly dispersed or restricted in their own locations, should be considered as they may interfere with the measurement of gene expression. Microsatellites, Minisatellites, or other types of RNAs have been studied and shown to cause disturbances in real-time PCR. Newer methods, such as immunoassays, can be utilized for the most accurate measurements and results possible.

Conflict of Interests

The authors have not declared any conflict of interest.

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