

Investigation of 3T3-L1 Cell Differentiation to Adipocyte, Affected by Aqueous Seed Extract of *Phoenix Dactylifera L*

Behrouz Etesami¹, Sara Ghaseminezhad², Azin Nowrouzi*¹,
Marzieh Rashidipour³, Razieh Yazdanparast⁴

Abstract

Background: Obesity, often associated with insulin resistance and type 2 diabetes, is a metabolic disease that can result in dyslipidemia and hyperglycemia. Many reports describe the hypoglycemic and hypolipidemic properties of the *Phoenix dactylifera L.* seed extract in STZ-induced diabetic rat models, however, its anti-diabetic effects in other diabetic models are less characterized in the literature. This study set out to determine the possible effects of the *Phoenix dactylifera L.* seed extract on adipogenesis and glucose homeostasis.

Methods: 3T3-L1 cells were cultured in adipocyte differentiation media with or without varying doses of *Phoenix dactylifera L.* extract (0.312-1 mg/ml). Assays were performed on days 5, 8, and 12 after induced differentiation.

Results: Our results demonstrate that the triglyceride content in treated groups was significantly lower compared to controls. Further, treating 3T3-L1 cells with *Phoenix dactylifera L.* seed extract reduced adipogenesis through the downregulation of PPAR- γ and CEBP- α , and adipocyte-specific genes involved in fatty acid metabolism including ap2, ACACA, and FAS.

Conclusions: *Phoenix dactylifera L.* seeds have the potential to inhibit adipogenesis and obesity. Overall, this study explored the inhibitory effects of *Phoenix dactylifera L.* seed extract on adipogenesis in 3T3-L1 cells on the molecular level.

Keywords: Adipocyte Differentiation, Adipogenesis, Glucose Homeostasis, Obesity, *Phoenix Dactylifera* Seed, 3T3-L1 Cell.

Introduction

Obesity is a universal health problem and its prevalence is on the rise. Adipocyte hypertrophy and dysfunction are associated with various metabolic diseases such as obesity, insulin resistance and inflammation (1). Apart from adipocyte hypertrophy, an increase in adipocyte number (hyperplasia) can also lead to obesity. Moreover, increased adipocyte numbers in obese individuals are correlated more vigorously with severe obesity (2). Within adipose tissue, preadipocytes proliferate and differentiate into

mature adipocytes. Adipocytes can emanate from the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes (1).

Adipocyte differentiation (adipogenesis) is a multi-step process that is highly regulated by many transcriptional factors (TFs) and cell cycle proteins (3). In addition to regulating adipocyte differentiation, the TFs, PPAR- γ , and C/EBP- α are also critical differentiation markers (4). Furthermore, these TFs are frequently present in

1: Department of Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

2: Department of Chemistry, Pharmaceutical Science Branch, Islamic Azad University, Tehran, Iran.

3: Department of Environmental Toxicology, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran.

4: Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran.

*Corresponding author: Azin Nowrouzi; Tel: +98 21 64053318; E-mail: nwr29@yahoo.com.

Received: 21 Dec, 2019; Accepted: 26 Dec, 2019

mature adipocytes and are involved in activating the transcription of adipocyte-specific genes, such as the adipocyte protein 2 (ap2), glucose transporter type 4 (GLUT4), stearoyl-CoA desaturase-1 (SD1), phosphoenolpyruvate carboxykinase (PEPCK) and leptin genes (1).

The primary function of adipocytes is to maintain energy balance but also provide energy through the storage of triglycerides (TG) (5). Together, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACACA) are involved in fatty acid synthesis; however, ACACA is also known to regulate fatty acid oxidation in adipocytes. To emphasize their importance in metabolic disease, scientists know now that increased *de novo* lipogenesis and reduced fatty acid oxidation can lead to both lipid accumulation and obesity (6).

Adipocytokines secreted by adipocytes, such as leptin and adiponectin, are known to regulate insulin sensitivity globally within the body (7). GLUT4, found in skeletal muscle and adipose tissue, is an insulin-regulated glucose transporter that permits the uptake of glucose in cells, and plays a central role in insulin resistance and diabetes type 2 (8). Additionally, Akt2, belonging to the Akt (PKB) subfamily of serine/threonine kinases, mediates signal transduction in adipocytes downstream of insulin, and overall is crucial for maintaining glucose homeostasis (9).

The carrier protein for fatty acids, ap2, is abundantly expressed in mature adipocytes. AP2 is also known as the fatty acid-binding protein 4 (FABP4) and is primarily involved in fatty acid, cholesterol, and phospholipid metabolism. Studies show that high levels of ap2 correlate to insulin resistance and dyslipidemia (10). Thermogenin also referred to as UCP1, is an uncoupling protein, found in the inner mitochondrial membrane (IMM) that generates heat by uncoupling oxidative phosphorylation. Increased expression of PPAR- γ can lead to increased expression of UCP1, browning of white adipose tissue, reduced insulin sensitivity and, in some cases, obesity (11). Expression of brown adipose tissue genes in white adipose tissue is essential for glucose tolerance, energy expenditure, and treating obesity and insulin resistance (12).

Date palm consumption was found to be beneficial for lipid and glucose control in diabetic individuals (13). *Phoenix dactylifera* L. (*P. dactylifera*), commonly known as the date palm, belongs to the palm plant genus and is largely found within southwest Asia and North Africa (14). Plants species within this genus are widely used to treat various infectious diseases, and health conditions, such as atherosclerosis, diabetes, hypertension, and cancer (15). Today, decocted *Phoenix dactylifera* L. seeds are mainly used to heal scorpion stings, cancer, kidney and bladder stones (16). The seeds contain high levels of phenolic compounds, antioxidants, and fibers. Moreover, the phenolic compounds consist of phenolic acids and flavonoids (17). Flavonoids can promote anti-cancer, anti-inflammatory and anti-obesity effects, and act as an antioxidant (18-21). Both alcoholic and aqueous extracts in *P. dactylifera* seeds have been shown to decrease blood sugar and lipid levels (22-24) and protect against diabetic nephropathy (25) and CCl₄-induced hepatotoxicity in rats (26).

Further, aqueous extracts from *P. dactylifera* seeds may produce hypoglycemic effects in diabetic rats when administered with insulin (27). Interestingly, *P. dactylifera* seeds may also stimulate endogenous insulin secretion in streptozotocin-induced type I diabetic rats (28). Lastly, combining insulin and date seed extracts were found to alleviate the negative consequences of diabetes on the liver and kidneys (29). Despite numerous studies illustrating the anti-glycemic, anti-inflammatory, antioxidant and anti-lipidemic effects of date palm seeds (30-31), others have denied the hypoglycemic effect of aqueous compounds extracted from the tops of date palm leaves in diabetic rats (32).

Our study aimed to determine whether *P. dactylifera* seed extracts could produce hypoglycemic and hypolipidemic effects in an *in vitro* model. We specifically investigated the effects of aqueous extracts from *P. dactylifera* seeds on adipogenesis and glucose homeostasis, on the molecular level, in a 3T3-L1 cell model that is well-known to differentiate into mature adipocytes.

Materials and methods

All the experiments were approved by the ethical committee of Tehran University of Medical Sciences.

Preparation of aqueous extract of P. dactylifera seed

Seeds obtained from *P. dactylifera* were washed in water, roasted and crushed. We added distilled water to form a 50 g/L solution. Then, the solution was brought to the boiling point for 90 minutes, and after cooling to room temperature (RT), the solution was filtered and lyophilized. This process yielded ~3.8 g of brown powder that was stored at -20°C for future experiments (29).

Cell culture and maintenance

The 3T3-L1 cell line (preadipocytes derived from mouse embryonic fibroblasts) was purchased from the Iranian Biological Resource Center (IBRC). The cells were grown in Dulbecco's Medium Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin at 37°C in a 5% CO₂ incubator.

Cell viability

Cell viability was assessed using an MTT assay. 3T3-L1 cells were seeded to a 96-well plate (15x10³ cells/well) for 24 h at 37°C in a 5% CO₂ incubator. The cells were then treated with various concentrations of extract and incubated for 72 h. MTT stock solution (5 mg/ml, 20 µl) was added to each well and the plate was incubated for 4 h. After discarding the MTT solution, 100 µl of DMSO was added to each well and the plate was left in the dark for 15 minutes with gentle rocking. Finally, the absorbance was measured at 540 nm using a microplate reader (33).

Cell culture and differentiation

Differentiation of 3T3-L1 cells to adipocytes was performed according to a previous protocol with minor modifications (34). 3T3-L1 cells were seeded in 6-well plates (30 × 10³ cells/well) or T25 flasks (10 × 10⁴ cells per well). After reaching confluence, the medium was changed, and the cells were incubated for 48 h. To induce differentiation, the cells were incubated for 72 h in DMEM

supplemented with a differentiation cocktail (0.5 mM IBMX, 1 µM dexamethasone, 2 µM rosiglitazone, 10 µg/ml insulin) and 10% FBS with or without the extract (day 0). Then, the medium was changed to DMEM containing 10 µg/mL of insulin every other day with or without the extract. It is to be noted that for this study, cell differentiation did not continue past day 12.

Oil-Red O staining

On days 5, 8 and 12 the cultured cells were washed three times with phosphate-buffered saline (PBS) and fixed in 10% formalin for 60 minutes at RT. After washing with deionized water, and an incubation period in 60% isopropanol (10 minutes), the cells were stained with Oil-Red O for 15 minutes at RT (35). To quantify the number of cells, they were washed twice with 60% isopropanol, for 5 minutes between each wash, with gentle rocking. Then, Oil-Red O stain was extracted using 100% isopropanol and the absorbance was measured at 492 nm (33).

Triglyceride assay

On days 5, 8 and 12 the cells in T25 flasks were washed with PBS, harvested and placed in 0.5% NP-40 solution and RIPA buffer separately. Next, cell suspensions were sonicated until a homogenized mixture was formed. NP-40 homogenate was incubated in a water bath at 80°C for 5 minutes to allow TG to precipitate out of solution. TG content was measured using the lipase glycerol kinase enzymatic method and Pars Azmoon kit (Cat. No. 97006). RIPA homogenate was used to determine protein content using the BCA method and Pars Tous kit (Cat. No. A101-251). Finally, TG content was normalized against the total protein concentration.

Adiponectin and leptin quantification

Adiponectin and leptin levels were measured in cell culture medium using the enzyme-linked immune-sorbent-assay kit (adiponectin; cat. No. KA1025 and leptin; cat. No. KA0026, mouse Abnova ELISA kit). On days 5, 8 and 12 the media in T25 flasks were removed and centrifuged for 5 minutes at 1500 rpm. The supernatant was then diluted 10-fold. Finally,

adiponectin and leptin levels (ng/mg) were determined.

Quantitative real-time PCR

Total RNA was extracted from cells on days 5, 8 and 12 following induced differentiation using RiboEx (GeneAll, Cat. No.302-001). *cDNA* was synthesized using the Primer Script RT Reagent

kit (Takara, Cat. No. RR037A) with 1 μ g of *total RNA*. The real-time PCR assay was performed using a Rotor-Gene Q and Quanti Fast SYBR Green PCR kit (QIAGEN, Cat. No. 204054). The relative amount of mRNA, normalized to β -Actin levels, was calculated using the delta-delta method (36). The primer sequences used can be found in Table 1.

Table 1- Sequences of primers used for real-time PCR

Gene name	Accession No.	Forward primer	Reverse primer
PPAR- γ	NM_001127330	CAAGAATACCAAAGTGCATCAA	GAGCTGGGTCTTTTCAGAATAATAAG
C/EBP α	NM_001287514	AGCAACGAGTACCGGGTACG	TGTTTGGCTTTATCTCGGCTC
aP2	NM_024406	AGTGAAAACCTTCGATGATTACATGAA	GCCTGCCACTTTCCTTGIG
FAS	NM_007988	GAGGGTGTGCCATTCTGTCA	TACTGCTGCATGGGTTCCAG
ACACA	NM_133360	CGATCTATCCGTCGGTGGTC	ATCCCTTTCCCTCCTCCTCC
UCP1	NM_009463	CCTGCCTCTCTCGAAACAA	TGTAGGCTGCCCAATGAACA
ACTB	NM_007393	TAAGAGGAGGATGGTCGCGT	CTCAGACCTGGGCCATTGAG

Glucose uptake assay

A glucose uptake assay was performed using a colorimetric kit (Sigma-Aldrich, Cat. No. MAK083). On day 12, the cultured cells in 96-well plates were washed twice with PBS and incubated in serum-free media for 24 h at 37°C. Then, cells were washed twice with PBS and incubated with Krebs-Ringer HEPES (KRPH) buffer (118 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄ and 30 mM HEPES, pH 7.4) for 40 minutes at 37°C. The wells containing cells were divided into two groups. The first group was stimulated with insulin (1 μ M), while the other group received no insulin. Within each group, one subgroup received the *P. dactylifera* seed extract at the time of insulin addition. All groups were incubated for 20 minutes. Following the addition of 10 mM 2-DG (Deoxy-Glucose), cells were incubated for another 20 minutes. After a third PBS wash, cell lysates were prepared in lysis buffer. Using these lysates, the assay was performed according to the manufacturer's instructions. The results were presented as pmol 2-DG6P/mg protein.

Western blot analysis

On day 12, cultured cells in T25 flasks were washed with PBS, harvested and lysed in RIPA buffer containing the anti-protease inhibitor cocktail, PMSF and sodium orthovanadate. The total protein concentration was measured in cell

lysates using the BCA method and Pars-Tous kit (Cat. No. A101-251). For each sample, 30 μ g of protein was mixed with 4X sample buffer [240mM Tris-HCl pH 6.8, 8 % SDS, 40% glycerol, 5% β -mercaptoethanol, 0.04% bromophenol blue]. The samples were heated for 5 minutes at 95°C, separated by 10% SDS-PAGE and transferred to PVDF membranes. PVDF membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween-20 for 24 h at 4°C. They were then incubated overnight at 4°C with the appropriate primary antibodies, including mouse anti- β -actin (1:3000; Abcam), rabbit anti-glucose transporter GLUT4 (1:1000; Abcam), mouse anti-Akt-2 (1:500; Abnova) and mouse anti-phospho-Akt (Ser473; 1:1000; Cell Signaling). On the next day, PVDF membranes were incubated with the appropriate HRP-secondary antibody (1:2500; Abnova) for 1h at RT. Immunoblots were developed using the Amersham ECL Prime Western Blotting Detection Reagent (Cat. No. RPN 2232) and exposed to x-ray film. Finally, protein expression was analyzed using ImageJ software and normalized against β -actin.

Statistical Analysis

Statistical differences between groups were determined using the one-way analysis of variance (ANOVA). We used a post-Hoc Dennett's test to

analyze multiple comparisons. Data analysis was performed using GraphPad Prism version 8.0, and statistical differences were expressed as the mean \pm standard deviation (SD). P values < 0.05 were considered statistically significant.

Results

Effect of extract on 3T3-L1 cell viability

The effect of aqueous extract from *P. dactylifera*

seeds on cell viability was assessed using an MTT assay. Cells treated with the extract for 72 h showed cytotoxic effects at high doses (2.5 mg/ml, 5 mg/ml, and 10 mg/ml with $58.33\pm 2\%$, $42.3\pm 2\%$ and $36.6\pm 1.5\%$ viability, respectively). However, at low doses, 0.315 mg/ml, 0.625 mg/ml, and 1.25 mg/ml, cells had $96.3\pm 1.1\%$, $91.6\pm 1.5\%$ and $86.6\pm 2.5\%$ viability, respectively. Thus, we used low extract concentrations for subsequent analysis (Fig. 1).

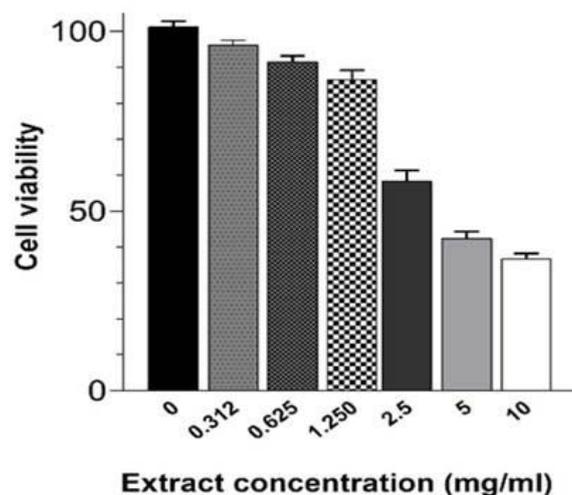


Fig. 1. Effect of aqueous extract from *P. dactylifera* seed on 3T3-L1 cell viability. 3T3-L1 cells, cultured in 96-well plates, were treated with a pre-determined extract concentration between 0.312 and 10 mg/ml for 72 h. Then, cell viability was assessed using an MTT assay. Low doses, 0.315 mg/ml, 0.625 mg/ml, and 1.25 mg/ml, resulted in cell viability around, $96.3\pm 1.1\%$, $91.6\pm 1.5\%$ and $86.6\pm 2.5\%$, respectively. Cell viability in the control group was 100%. The results are represented as the mean \pm SD of three independent experiments.

Effect of extract on adipogenesis

On day 5 we detected the first signs of TG accumulation within the cell cytoplasm as lipid droplets. To visualize lipid droplets, cells were stained with Oil-Red O on days 5, 8 and 12 which are depicted as red areas within the cytoplasm (Fig. 2). On days 8 and 12, we

found that both the number and size of lipid droplets in the extract-treated group (0.315, 0.625, and 1.25 mg/mL) were lower compared to controls, despite maintaining a normal phenotype and shape of a mature adipocyte (spherical) (Fig. 3).

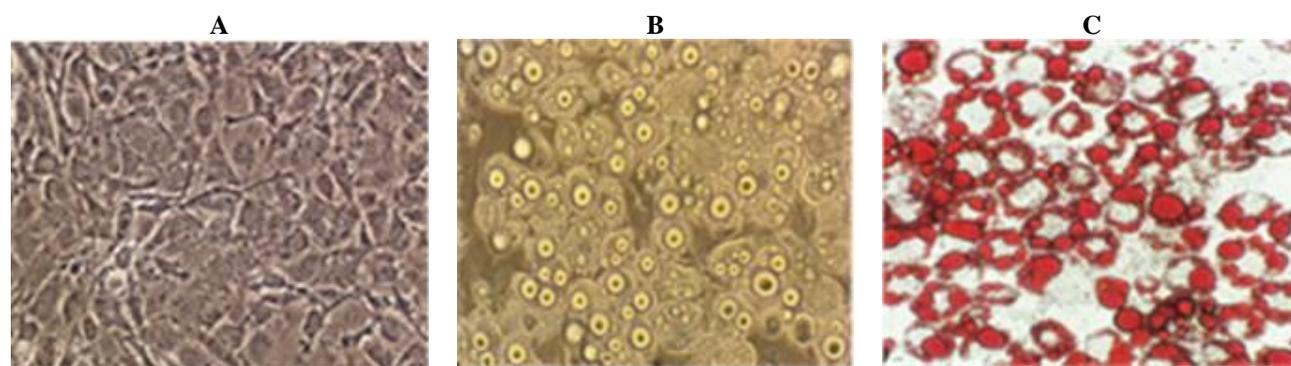


Fig. 2. Morphology of 3T3-L1 cells before and after differentiation to mature adipocytes. (A) Undifferentiated 3T3-L1 cells on day 0, differentiated 3T3-L1 cells on day 12 following induced differentiation before (B) and after (C) Oil-Red O staining. Lipid droplets, depicted as red areas, can be observed within the cell cytoplasm (Magnification: 20x).

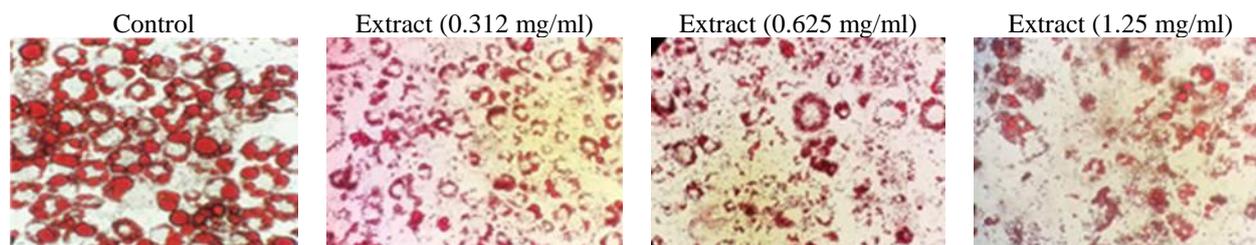


Fig. 3. Effect of aqueous extract from *P. dactylifera* L. seeds on TG accumulation in 3T3-L1 cells on day 12 following induced differentiation. 3T3-L1 cells were cultured in an adipocyte differentiation cocktail with or without extract treatment at the following doses: 0.315, 0.625 and 1.25 mg/mL. On days 5, 8, and 12 the cells were stained with Oil-Red *O* and imaged using an inverted microscope. Images (Magnification: 20x) show that both the number and size of lipid droplets in groups treated with extract were lower compared to controls.

We performed an Oil-Red *O* stain to also quantify TG and lipid accumulation, formerly expressed as absorbance values. A higher absorbance indicates increased lipid droplet formation and greater differentiation potential. Next, the relative lipid content (%) was compared

to controls (Fig. 4A). Moreover, cytoplasmic TG content was measured on days 5, 8 and 12 and normalized to the total protein concentration (Fig. 4B). Relative lipid and TG content were reduced significantly in treated groups in a dose-dependent manner (Fig.4A and B).

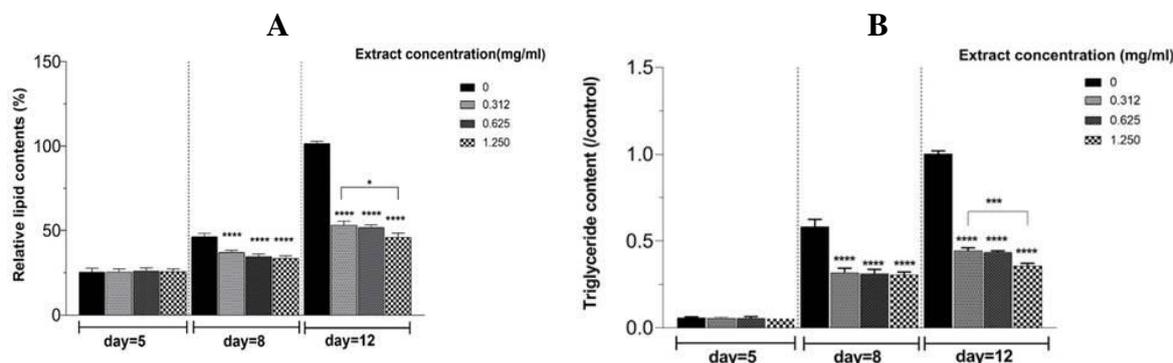


Fig. 4. The relative lipid and TG content in treatment groups. 3T3-L1 cells were cultured in adipocyte differentiation media with or without extract treatment at the following doses: 0.315, 0.625 and 1.25 mg/mL. Oil-Red *O* staining was quantified on days 5, 8 and 12. (A) Lipid content in the control group on day 12 was considered 100%. The cytosolic TG content was measured using the lipase glycerol kinase enzymatic method. TG content was normalized to the total protein concentration: (B) on day 12, the control group had a value of one. Relative lipid and TG content were reduced significantly in treated groups compared to controls on days 8 and 12. The results are represented as the mean \pm SD of three independent experiments (* p < 0.05, **** p < 0.0001).

Effect of extract on leptin and adiponectin secretion

Both leptin and adiponectin are adipokines secreted by adipocytes. These proteins were measured during 3T3-L1 differentiation in cell culture medium on days 5, 8 and 12. There was a significant decrease in leptin and adiponectin levels in groups treated with extract at the following doses, 0.312, 0.625 and 1.25 mg/ml, compared to controls (Fig. 5A and B). Collectively, our results demonstrate that the presence of aqueous extract at low doses (0.312, 0.625, and 1.25mg/ml) could result in reduced levels of adipokines during differentiation. Thus, by increasing the extract concentration, we see an increase in adiponectin and leptin levels.

Effect of extract on the expression of adipogenic transcription factors and adipocyte-specific genes

PPAR- γ and C/EBP- α are adipogenic TFs involved in the differentiation of preadipocytes to adipocytes. We investigated whether aqueous extract could influence the expression of PPAR- γ and C/EBP- α during adipogenic differentiation. As shown in Fig. 6, the presence of aqueous extract significantly reduced PPAR- γ and C/EBP- α expression in a dose-dependent manner, compared to controls. The results strongly indicate that the extract can suppress adipogenic differentiation by decreasing the expression of PPAR- γ and C/EBP- α .

Given the inhibitory effect of the aqueous extract on adipocyte differentiation, we further explored its anti-adipogenic effect through the expression of adipocyte-specific genes including aP2, ACACA, and FAS. Furthermore, we investigated the browning effect of the extract on 3T3-L1 adipocytes by examining UCP1 expression. As shown in Fig.

6, the aqueous extract treatment significantly decreased the mRNA expression of aP2, ACACA, FAS and UCP1 in 3T3-L1 differentiated adipocytes. These results suggest that the extract may trigger a down-stream regulatory pathway that suppresses the expression of adipocyte-specific genes, leading to decreased TG accumulation in adipocytes.

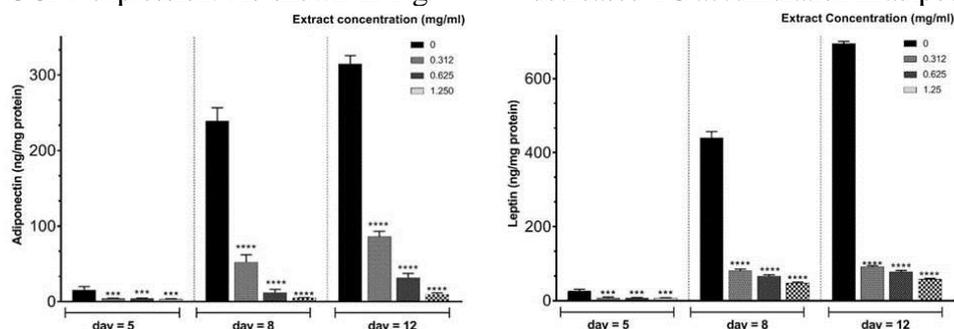


Fig. 5. Effect of extract on adiponectin and leptin secretion from 3T3-L1 cells during adipocyte differentiation. During differentiation, adiponectin (A) and leptin (B) levels were low in groups treated with the extract (0.312, 0.625, and, 1.25 mg/ml) compared to controls. There was a significant difference in adiponectin and leptin levels between treated groups on day 8 and 12. Data are represented as the mean \pm SD of three independent experiments and expressed as adiponectin or leptin ng/mg protein (** $p < 0.001$, **** $p < 0.0001$).

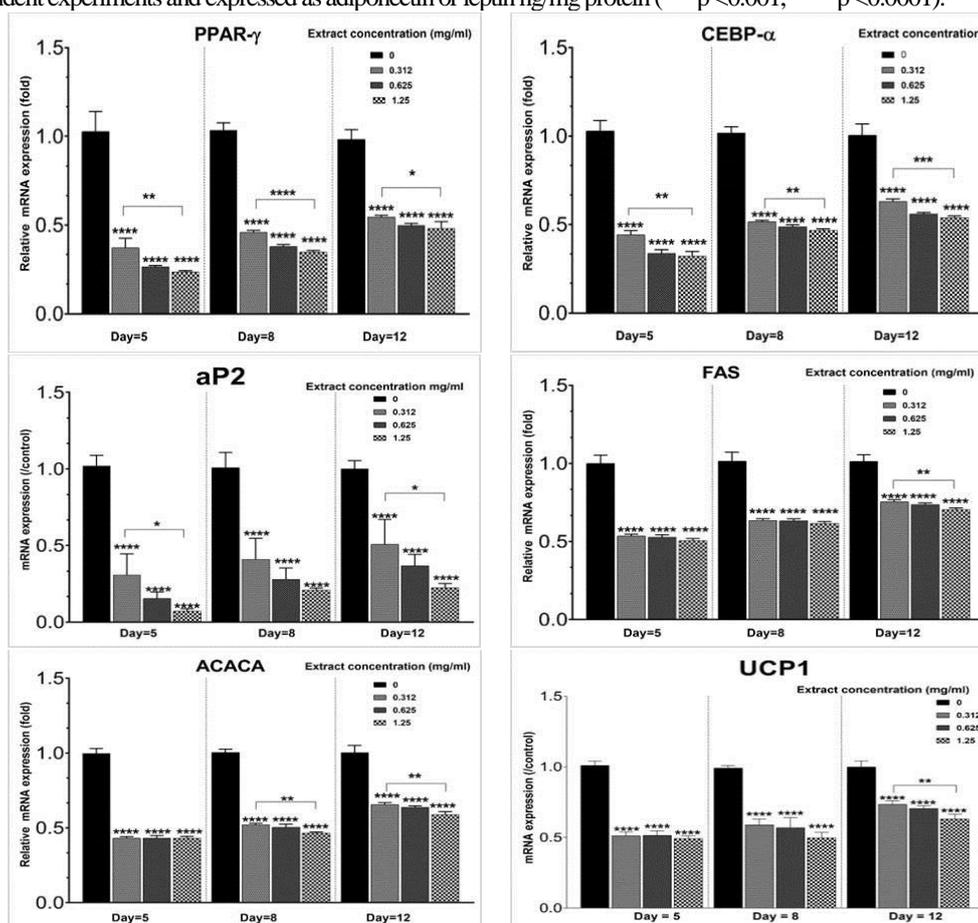


Fig. 6. Effect of extract on the gene expression of adipocyte-specific genes during differentiation of 3T3-L1 cells into mature adipocytes. 3T3-L1 cells differentiation in medium with or without extract (0.312, 0.625, and 1.25 mg/ml). On day 5, 8, 12, mRNA was extracted and the gene expression of PPAR- γ , CEBP- α , aP2, FAS, ACACA, and UCP1 genes were evaluated using Q-PCR; PPAR- γ : peroxisome proliferator-activated receptor- γ ; CEBP- α : CCAAT/enhancer-binding protein- α ; aP2: Adipocyte protein 2; ACACA: Acetyl-CoA Carboxylase- Alpha; FAS: Fatty acid synthase; UCP1; uncoupling protein. Data are represented as the mean \pm SD of three independent experiments and were normalized to β -actin levels (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Effects of extract on glucose uptake

We then investigated the effect of the extract on basal glucose uptake of mature 3T3-L1 adipocytes. The results show that there was no significant change in basal glucose uptake in the presence of

the extract at the following concentrations, 0.312, 0.625, and 1.25 mg/ml when compared to controls. Furthermore, the extract did not affect insulin-stimulated glucose uptake (Fig. 7).

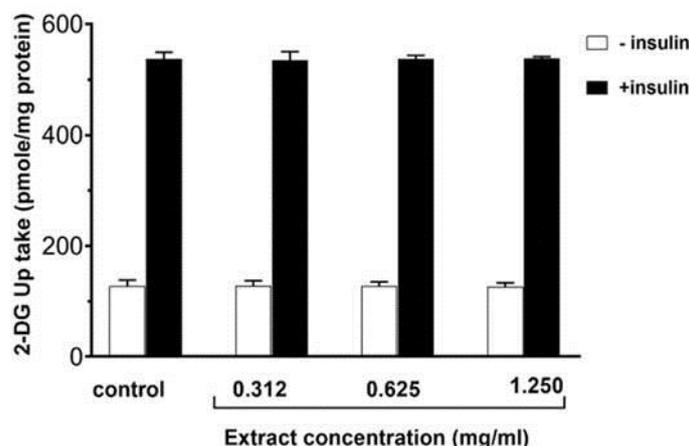


Fig. 7. Effect of extract on glucose uptake in mature 3T3-L1 adipocytes on day 12. Basal and insulin-stimulated glucose uptake were investigated in the presence of extract (0.312-1.25mg/ml). The results are represented as the mean \pm SD of three independent experiments and expressed as 2-DG uptake pmol/mg protein.

Effect of extract on glucose transportation and insulin signaling pathway

GLUT4 is the insulin-regulated glucose transporter in adipocytes. Akt2 plays a key role in signal transduction downstream of insulin and is activated when phosphorylated. On day 12, we investigated whether aqueous extract (0.312-1.25 mg/ml) could

influence GLUT4 and Akt2 (inactive form, dephosphorylated; active form, ser473phosphorylated) expression levels in 3T3-L1 adipocytes. Data analysis demonstrated that the aqueous extract did not affect GLUT4 and Akt2 expression levels in treated groups (Fig. 8A and B).

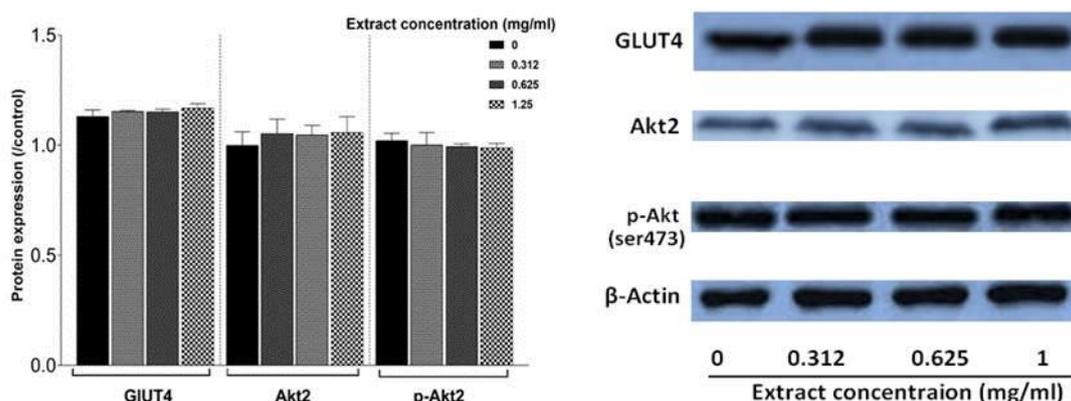


Fig. 8. Effect of extract on protein expression of GLUT4, Akt2, and phospho-Akt2. Differentiated 3T3-L1 cells in medium with or without extract (0.312-1.25 mg/ml). On day 12, GLUT4, Akt2, and phospho-Akt2 levels were assessed via western blot on PVDF membranes. (A) Representative blots are shown. Results demonstrate no effect of extract (0.312, 0.625, and 1.25 mg/ml) on GLUT4, Akt2 and phospho-Akt2 levels in treated groups (B). Data are represented as the mean \pm SD of three independent experiments and were normalized to β -actin levels.

Discussion

Numerous studies have shown that various species of the palm may have hypoglycemic and hypolipidemic properties. For instance, the

aqueous extract from the leaves of *Europeanpalm*, *Chamaeropshumilis* was shown to reduce plasma glucose, total cholesterol and triglyceride levels in

obese rats (37). Moreover, hypoglycemic compounds were found in the roots of American palm, *Arocomiaaculeate* (38). *P. dactylifera*, commonly known as the date palm, is reported to be beneficial for lipid and glucose regulation in diabetic individuals (13). In the past, specific parts of the *P. dactylifera* plant have been used to treat various infectious diseases, and other health conditions including atherosclerosis, diabetes, hypertension, and cancer (15). In streptozotocin-induced diabetic rats, palm oil tocotrienol-rich fractions improved dyslipidemia and reduced serum glucose and glycated hemoglobin concentrations (39). Furthermore, ethanol extracts from *P. dactylifera* leaves were shown to have antioxidant, anti-inflammatory and anti-diabetic effects (40).

Interestingly, phenolic compounds in *P. dactylifera* seed extracts, including phenolic acids and flavonoids, (17) is known to promote anti-cancer (18), antioxidants (19), anti-inflammatory (20) and anti-obesity (21) effects. Moreover, alcoholic extracts from *P. dactylifera* seeds were shown to decrease blood glucose and lipid concentrations in male diabetic rats (24). Another study showed that *P. dactylifera* aqueous extracts may produce hypoglycemic effects when administered with insulin in diabetic rats (27).

To date, the impact of *P. dactylifera* seeds on lipid and glucose metabolism has not been fully investigated. In our study, the hypoglycemic and hypolipidemic effects of aqueous extracts were evaluated *in vitro* using 3T3-L1 cells. Firstly, we evaluated the effect of aqueous extract on TG accumulation. A prominent feature of adipocytes is the presence of lipid droplets in the cellular cytoplasm. We found that lipid droplets began to appear on day 5 following induced differentiation. The results of the Oil-Red *O* stain on days 8 and 12 revealed that when 3T3-L1 cells were treated with aqueous extract, they maintained a normal adipocyte phenotype (spherical). However, the number and size of lipid droplets in extract-treated groups (0.315-1.25 mg/mL) were lower compared to controls (Fig. 3). Further, quantifying the Oil-Red *O* stain confirmed a reduction in lipid droplets in treated groups (Fig. 4A). Additionally, the average TG content in treated groups was significantly lower, in a dose-dependent manner,

compared to the control group (Fig. 4B). Overall, these results show that the aqueous extract reduced TG accumulation while the 3T3-L1 cells maintained a normal adipocyte phenotype during differentiation.

It is a well-known fact that a decrease in TG accumulation is considered to be a promising therapeutic strategy against obesity, insulin resistance and type 2 diabetes (41). Therefore, we aimed to investigate a possible mechanism to support the anti-adipogenic effect observed in the presence of the aqueous extract. Firstly, we studied two key adipogenic TFs, specifically, PPAR- γ and C/EBP- α . We compared the mRNA levels of both PPAR- γ and C/EBP- α on days 5 (early stage), 8 (intermediate stage) and 12 (final stage) during adipogenic differentiation. We found that the extract inhibited adipogenic differentiation in 3T3-L1 cells by decreasing C/EBP- α and PPAR- γ expression (Fig.6).

The increase in the number and size of differentiated adipocytes from preadipocytes is known to result in obesity (42-44). Our results show that the extract at high doses (2.5-10 mg/mL) could significantly reduce the viability of 3T3-L1 preadipocytes (Fig.1). The extract (0.315-1.25 mg/mL) also inhibited adipogenic differentiation, in a dose-dependent manner, as shown by fewer mature adipocytes with decreased lipid droplets and reduced levels of accumulated intracellular TG. The reduction in secretory markers in mature adipocytes in treated groups, specifically adiponectin and leptin, demonstrates a lower differentiation potential in extract-treated 3T3-L1 cells (Fig. 5A and B).

In this study, we found that both C/EBP- α and PPAR- γ induced the expression of adipocyte-specific genes, which are key regulators of fatty acid metabolism. These genes include aP2, the fatty acid-binding protein, FAS, a major enzyme responsible for fatty acid synthesis, and ACACA, which regulates the fatty acid synthesis and fatty acid oxidation in adipocytes. Several studies show that high aP2 levels correlate to insulin resistance and dyslipidemia (10). Indeed, in the absence of these important proteins, there is an increase in *de novo* lipogenesis and reduced fatty acid oxidation resulting in lipid accumulation and obesity (6).

Hence, we investigated the effect of aqueous extracts on aP2, FAS and ACACA mRNA levels during differentiation. We found that the extract reduced mRNA expression of FAS and ACACA, proteins involved in the late stage of adipogenesis. Furthermore, aP2 gene expression was also decreased (Fig.6). Taken together, we show that the anti-adipogenic effect of the aqueous extract was mediated by down-regulating PPAR- γ , CEBP- α , aP2, FAS and ACACA expression.

The mechanism and molecular events mediating the hypoglycemic and hypolipidemic effects induced by *P. dactylifera* seed extracts remain poorly characterized within the literature. An *in vivo* study proposed that components, such as oleic acid and linoleic acid, found in *P. dactylifera* alcoholic extracts could produce hypolipidemic effects. Further, the study suggests that elements such as magnesium (Mg) and zinc (Zn) in *P. dactylifera* seeds can produce a hypoglycemic effect through insulin production and secretion (24).

Another study showed that *P. dactylifera* aqueous extract combined with exogenous insulin promotes hypoglycemic effects in diabetic rats (27). Interestingly, it has been reported that the extract may stimulate endogenous insulin secretion in streptozotocin-induced type-I diabetic rats (28). The present study explored the hypoglycemic property of the *P. dactylifera* aqueous extract, and glucose homeostasis on day 12 (late differentiation stage). The results show that the extract had no effect on cellular glucose uptake with or without insulin stimulation (Fig.

7). Furthermore, the extract did not affect GLUT4, Akt2 and p-Akt2 (ser473) protein expression in treated groups (Fig. 8A and B). Our findings demonstrate that although *P. dactylifera* seeds produced an anti-adipogenic effect, it had no significant impact on cellular glucose transport and insulin signaling.

To explain why there was no effect on glucose homeostasis, we considered the presence of insulin in the culture medium. As we know, insulin has direct effects on glucose metabolism and transport. We speculate that the extract could not overcome the effects of insulin. However, the extract could play a role in inhibiting the lipogenic activity of insulin in 3T3-L1 cells.

To gain a more accurate picture regarding the relationship between aqueous extract and glucose homeostasis, further investigations should consider *in vivo* models. This is the first study to illustrate the inhibitory effect of *P. dactylifera* aqueous extract on adipogenesis in 3T3-L1 cells. Moreover, *P. dactylifera* seeds demonstrate the potential to reduce adipogenesis through the downregulation of C/EBP- α and PPAR- γ and fatty acid metabolic genes. All things considered, *P. dactylifera* seed may provide a novel treatment strategy against obesity.

The authors declare no conflict of interest.

Acknowledgment

This work was financially supported by Grant No. 94-04-30-30882 provided by Tehran University of Medical Sciences and Health Services.

References

1. Moreno-Navarrete JM, Fernández-Real JM. Adipocyte Differentiation. In: Symonds M. (eds.), Adipose Tissue Biology. Springer, New York. 2012;17-38.
2. Hirsch J, Batchelor B. Adipose tissue cellularity in human obesity. Clin Endocrinol Metab. 1976; 5(2): 299-311.
3. Lefterova MI, Lazar MA. New developments in adipogenesis. Trends Endocrinol Metab. 2009; 20(3): 107-14.
4. Tang QQ, Grønberg M, Huang H, Kim JW, Otto TC, Pandey A, and Lane MD. Sequential phosphorylation of CCAAT enhancer-binding

protein β by MAPK and glycogen synthase kinase 3 β is required for adipogenesis. Proc Natl Acad Sci USA. 2005;102(28): 9766-9771.

5. Savage DB, Petersen KF, Shulman, GI. Mechanisms of insulin resistance in humans and possible links with inflammation. Hypertension. 2005;45(5): 828-33.

6. Grover LW, Jacqueline MS. Targeting Acetyl-CoA Carboxylase for Anti-obesity Therapy. Current Medicinal Chemistry- Immunology, Endocrine & Metabolic Agents. 2003;3(3): 229-234.

7. Pagliuca FW, Melton DA. How to make a functional β -cell. Development. 2013;140(12):

2472-83.

8. Akash MSH, Rehman K, Liaqat A. Tumor Necrosis Factor-Alpha: Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes Mellitus. *J Cell Biochem.* 2018;119(1):105-110.
9. Tsuchiya A, Kanno T, Nishizaki T. PI3 kinase directly phosphorylates Akt1/2 at Ser473/474 in the insulin signal transduction pathway. *J Endocrinol.* 2013;220(1):49-59.
10. Xu A, Tso AW, Cheung BM, Wang Y, Wat NM, Fong CH, Yeung DC, Janus ED, Sham PC, Lam KS. Circulating adipocyte-fatty acid binding protein levels predict the development of the metabolic syndrome: a 5-year prospective study. *Circulation.* 2007;115(12):1537-43.
11. Poher AL, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. *Front Physiol.* 2015;6,4.
12. Jankovic A, Golic I, Markelic M, Stancic A, Otasevic V, Buzadzic B, Korac A, Korac B. Two key temporally distinguishable molecular and cellular components of white adipose tissue browning during cold acclimation. *J Physiol.* 2015;593(15): 3267-80.
13. Miller CJ, Dunn, EV, Hashim IB. The glycaemic index of dates and date/yoghurt mixed meals. Are dates 'the candy that grows on trees'? *Eur J Clin Nutr.* 2003;57(3):427-30.
14. Al-Farsi MA, Lee CY. Nutritional and functional properties of dates: a review. *Crit Rev Food Sci Nutr.* 2008;48(10):877-87.
15. Elgindi M, Singab AN, El-Taher MM, Kassem M. A comprehensive review of Phoenix (Arecaceae). *Research Journal of Pharmaceutical, Biological and Chemical Sciences.* 2015;6(3):966.
16. Morton JF, Dowling CF. *Fruits of warm climates.* Miami, FL; Winterville, N.C: J.F. Morton; Distributed by Creative Resources Systems; 1987.
17. Al-Farsi MA and Lee CY. Optimization of phenolics and dietary fibre extraction from date seeds. *Food Chem.* 2008;108(3):977-85.
18. Adhami VM, Syed DN, Khan N, Mukhtar H. Dietary flavonoid fisetin: a novel dual inhibitor of PI3K/Akt and mTOR for prostate cancer management. *Biochem Pharmacol.* 2012;84(10):1277-81.
19. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity.* 2009;2(5):270-78.
20. Rathee P, Chaudhary H, Rathee S, Rathee D, Kumar V, Kohli K. Mechanism of action of flavonoids as anti-inflammatory agents: a review. *Inflamm Allergy Drug Targets.* 2009;8(3):229-35.
21. Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R, Bapat P, Kwun I, Shen CL. Novel insights of dietary polyphenols and obesity. *J Nutr Biochem.* 2014;25(1):1-18.
22. Abdelaziz DH, Ali SA, Mostafa MM. Phoenix dactylifera seeds ameliorate early diabetic complications in streptozotocin-induced diabetic rats. *Pharm Biol.* 2015;53(6):792-9.
23. Mohamed NA, Ahme OM, Hozayen WG, Ahmed MA. Ameliorative effects of bee pollen and date palm pollen on the glycemic state and male sexual dysfunctions in streptozotocin-Induced diabetic wistar rats. *Biomed Pharmacother.* 2018; 97:9-18.
24. Mokhtari M, Sharifi A, Sabzevari Fard A. Effect of palm seed alcoholic extract on the blood glucose and lipids concentration in male diabetic rats. *Scientific Journal of Kurdistan University of Medical Sciences.* 2008;12(4):8-15.
25. El-Mousalamy MDA, Hussein AAM, Mahmoud SA, Abdelaziz A, Shaker G. Aqueous and methanolic extracts of palm date seeds and fruits (*Phoenix dactylifera*) protects against diabetic nephropathy in type II diabetic rats. *Biochemistry and Physiology.* 2016;5(2):1-9.
26. Abdelaziz DH, Ali SA. The protective effect of *Phoenix dactylifera L.* seeds against CCl4-induced hepatotoxicity in rats. *J Ethnopharmacol.* 2014;155(1):736-43.
27. El-Fouhil AF, Ahmed AM, Darwish HH. Hypoglycemic effect of an extract from date seeds on diabetic rats. *Saudi Med J.* 2010;31(7):747-51.
28. El Fouhil A, Ahmed A, Atteya M, Mohammad R, Moustafa A, Darwish H. An extract from date seeds stimulates endogenous insulin secretion in streptozotocin-induced type I diabetic rats. *Functional Foods in Health and Disease.* 2013;3(11):441-46.
29. El Fouhil AF, Ahmed AM, Darwish HH, Atteya M, Al-Roalle AH. An extract from date

seeds having a hypoglycemic effect. Is it safe to use?. Saudi Med J. 2011;32(8):791-6.

30. Hasan M, Mohieldein A. *In Vivo* Evaluation of Anti Diabetic, Hypolipidemic, Antioxidative Activities of Saudi Date Seed Extract on Streptozotocin Induced Diabetic Rats. J Clin Diagn Res. 2016;10(3):FF06-12.

31. Saryono S. Date Seeds Drinking as Antidiabetic: A Systematic Review. IOP Conference Series: Earth and Environmental Science. 2019;255:12-18.

32. Ismail MS, Abuzaid OI, El-Ashmawy IM. Effect of aqueous extract of tops of date palm leaves on blood glucose of diabetic rats. Pak J Pharm Sci. 2017;30(5(Supplementary)):20317-22037.

33. Azahari N, Khattak MMAK, Taher M, Ichwan SJA. Dose Water Extract of Cinnamon (*Cinnamomum Zeylanicum*) Exhibits Anti-Diabetic Properties in Cultured 3T3-L1 Adipocytes: A Concurrent Assessment of Adipogenesis, Lipolysis and Glucose Uptakes. Journal of Food and Nutrition Research. 2014;2(11):764-769.

34. Zebisch K, Voigt V, Wabitsch M, Brandsch M. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. Anal Biochem. 2012;425(1):88-90.

35. Shu G, Lu NS, Zhu XT, Xu Y, Du MQ, Xie QP, et al. Phloretin promotes adipocyte differentiation *in vitro* and improves glucose homeostasis *in vivo*. The Journal of Nutritional Biochemistry. 2014;25(12):1296-1308.

36. Wang S, Wang X, Ye Z, Xu C, Zhang M, Ruan B, et al. Curcumin promotes browning of white adipose tissue in a norepinephrine-dependent way. Biochem Biophys Res Commun. 2015;466(2):247-253.

2015;466(2):247-253.

37. Gaamoussi F, Israili ZH, Lyoussi B. Hypoglycemic and hypolipidemic effects of an aqueous extract of *Chamaerops humilis* leaves in obese, hyperglycemic and hyperlipidemic Meriones shawi rats. Pak J Pharm Sci. (2010) 23(2): 212-219.

38. Perez S, Perez RM, Perez C, Zavala MA, Vargas R. Coyolosa, a new hypoglycemic from *Acrocomia mexicana*. Pharmaceutica Acta Helvetiae. 1997;72(2):105-11.

39. Budin SB, Othman F, Louis SR, Bakar MA, Das S, Mohamed J. The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats. Clinics (Sao Paulo). 2009;64(3):235-44.

40. Laouini Salah E. Antioxidant, anti-inflammatory and diabetes related enzyme inhibition properties of leaves extract from selected varieties of *Phoenix dactylifera* L. Innovare Journal of Life Sciences. 2013;1(1):14-18.

41. Wang YW, Jones PJ. Conjugated linoleic acid and obesity control: efficacy and mechanisms. Int J Obes Relat Metab Disord. 2004;28(8):941-55.

42. Camp HS, Ren D, Leff T. Adipogenesis and fat-cell function in obesity and diabetes. Trends Mol Med. 2002;8(9):442-47.

43. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. 2006;444(7121):847-53.

44. Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, Baile CA. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sci. 2008;82(19-20):1032-9.