International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Anti-Obesity Effects of Punicalagin and *Punica granatum* Fruit Juice Extract in Adipocytes Differentiated from Human Bone Marrow Mesenchymal Stem Cells

Tahani Nader Almuzaini[1], P. SubasH-Babu[2], Ali Abdullah Alshatwi[3], Laila Naif Al-Harbi[4], Doha Mustafa Al-Nouri[5], Manal Abdulaziz Binobead[6]

[1]Ministry of Health (MOH), General Administration of Nutrition Deputy Ministry for Therapeutic Services, Kingdom of Saudi Arabia (KSA)
Corresponding Author Email: TnAlmuzaini[at]moh.gov.sa
[2]Department of Food and Nutrition Sciences, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia, (KSA)
Email: sbpandurangan[at]ksu.edu.sa
[3]Department of Food and Nutrition Sciences, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia (KSA)
Email: alshatwi[at]ksu.edu.sa
[4]Department of Food and Nutrition Sciences, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia, (KSA)
Email: lalharbi1[at]ksu.edu.sa
[5]Department of Food and Nutrition Sciences, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia, (KSA)
Email: dr_nouri[at]ksu.edu.sa
[6]Department of Food and Nutrition Sciences, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia, (KSA)
Email: mbinobead[at]ksu.edu.sa

Abstract: ***Punicalagin (PUN) is an ellagitannin, a type of phenolic compound. It is found as alpha and beta isomers in pomegranates. This study investigates the anti-obesity effects of PUN and pomegranate fruit juice extract on adipocyte differentiation, lipid accumulation, and obesity-related inflammation. The research utilized adipocytes differentiated from human bone marrow-derived mesenchymal stem cells. Results demonstrated that, 50% of the cells were inhibited by treatment with 400 µM PUN and 1.8 mg/dL pomegranate fruit juice. The expression levels of various genes associated with obesity, such as PPARGC1, UCP-1, PPARG, and PRDM16, were decreased by treatment with 100 µM PUN ($p \leq 0.05$). PUN and pomegranate fruit juice inhibited adipocyte differentiation, lipid accumulation, and obesity-related inflammation. In Conclusion, the present findings suggest that PUN and pomegranate fruit juice reduce adipocyte differentiation and lipid accumulation, further suggesting that, they may be role in treatment of obesity.***

Keywords: Punicalagin, Pomegranate fruit juice extract, Adipocyte differentiation, Lipid accumulation, Obesity-related inflammation, Mesenchymal stem cells

1. Introduction

Obesity involves accumulation of fat, and is generally defined as the accumulation of more than 20% normal body fat [1]. Obesity is an inflammatory disorder that results in the secretion of inflammatory factors by adipocytes, including tumor necrosis factor alpha (**TNF-α**) and interleukin 6 (**IL-6**) [2, 3]. The World Health Organization (**WHO**) estimated that, there are more than 1.9 billion overweight individuals with obesity worldwide [3]. Saudi Arabia ranks third globally in terms of the spread of obesity [3, 4]. In 2015, WHO reported that 36% of the population in Saudi Arabia suffers from obesity, which is estimated to cause 20, 000 deaths annually [4]. Obesity has been classified by the WHO as an epidemic, owing to its spread and association with up to 48 diseases [4]. Moreover, obesity treatment imposes an economic burden on the Ministry of Health [5].

Punica granatum (pomegranate) fruit is a functional food that consists of three parts: seeds, juice (approximately 3% and 30% of the fruit weight, respectively), and peels (approximately 60% of the fruit) [6]. Two types of polyphenols are found in pomegranate fruit, namely flavonoids (anthocyanins, catechins, cyanidin, kaempferol, and other complex flavonoids) and hydrolysable tannins (PUN, punicalin, gallic acid, ellagic acid, and complex polysaccharides), as well as various minerals, including potassium, calcium, magnesium, phosphorus, and sodium [7]. The peel of pomegranate fruit is particularly rich in PUN. Punicalagin reportedly inhibits lipid peroxidation and exerts antioxidant effects in elderly individuals [8]. In addition to its anti-inflammatory effects, PUN has anti-cancer, anti-atherosclerotic, and anti-obesity properties [9].

The aim of this study was to evaluate the effects PUN and pomegranate fruit juice on adipocyte differentiation, lipid accumulation, and obesity-related inflammation.

2. Methods

2.1 Culture of human bone marrow mesenchymal stem cells

Human bone marrow mesenchymal stem cells (**hMSCs)** were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in 75-cm^2 flasks in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (both from Gibco-Invitrogen, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere and 95% humidity, until 80% confluence was reached [10]. Culture and maintenance of cells in vitro were performed in compliance with the guidelines from the Institutional Research Committee of King Saud University, Riyadh, Kingdom of Saudi Arabia (Approval number: E-20-4542).

2.2 Counting cells

Cells were counted using an automated cell counter after adding 10 µL of Trypan blue stain (0.4%) to the cell suspension. For each sample, the total number of TUNEL-positive cells in 10 representative fields was measured following a previously described method [11]. The number of TUNEL-positive cells were 112.5×10^4.

2.3 Cytotoxicity assay

PUN was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pomegranate juice was prepared in the laboratory through several steps. Two pomegranates, each weighing 100 g, were cut and the seeds were collected. Following this, the seeds were placed in gauze and crushed, and the extracted pomegranate juice was retained. The cytotoxicity of PUN and pomegranate juice at different concentrations was determined using a tetrazolium-based colorimetric assay MTT [12]. In brief, adipocytes were seeded at a density of 1×10^4 cells/mL in cell culture media. Thereafter, we plated 200 µL of the cell culture (10, 000 cells) in the wells of a flat-bottomed 96-well culture plate. The cells were then treated with PUN (0, 50, 100, 200, 400, 500, and 600 µM) and pomegranate juice (0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/dL) for 24 and 48 hours, respectively. The control cells were not treated with any PUN or pomegranate juice.

The cells were labeled with MTT solution (1 mg/mL) in phosphate-buffered saline (**PBS**) for 4 hours and the resulting formazan was solubilized in 10% dimethyl sulfoxide. The absorbance of the samples was measured at 560 nm using an enzyme-linked immunosorbent assay reader (Sigma-Aldrich, Germany). To study the dose-response relationship, three doses of PUN (50, 100, and 200 µM) and pomegranate fruit juice (0.2, 0.4, and 0.8 mg/dL) were used.

2.4 Anti-obesity evaluation

2.4.1 Adipocyte differentiation

The hMSCs were plated at a density of 50, 000 cells/cm^2 as a monolayer and cultured in basal media, followed by treatment with adipocyte differentiation factors: 5 µg/mL insulin, 0.4 µg/mL dexamethasone (DEX), and 0.5 µg/mL 1-methyl-3-isobutyl xanthine (IBMX), all purchased from Sigma-Aldrich (St. Louis, MO, USA). When the cells reached 70% confluence, adipocyte differentiation was induced by standard induction/maintenance media. Furthermore, after 72 hours, the induction media were replaced with maintenance media containing recombinant human insulin alone, and the cells were incubated for a further 48 hour [13]. Differentiation-induced cells were further maintained by changing the media once every 3 days.

2.4.2 Oil Red O staining

Accumulated triglycerides (**TGs**) in the cells were measured after 14 days by staining with Oil Red O. Briefly, the cells were washed with PBS, fixed in 3.7% formaldehyde, and stained with Oil Red O solution (0.5 g in 100 mL of isopropanol). Adipocytes were stained for 1 hour with Oil Red O, which made the fat droplets red. Adipogenesis was monitored through morphological examination of the cells for accumulation of lipid droplets and Oil Red O (0.2%) staining [14].

2.4.3 Nile Red staining

Accumulated TGs in the cells were measured after 14 days by staining with Nile Red. Briefly, the cells were washed with PBS, fixed in 3.7% formaldehyde, and stained with Nile Red O solution (0.5 g in 100 mL of isopropanol). Adipocytes were stained for 1 hour with Nile Red, which made the fat droplets red. Adipogenesis was monitored through morphological examination of the cells for the accumulation of lipid droplets and Nile Red (0.2%) staining [15].

2.5 Gene expression analysis

2.5.1 Analysis of obesity-related gene expression

For gene expression analysis, adipocytes were cultured and treated with PUN and pomegranate fruit juice for 24 and 48 hour. After 48 hour of treatment, the Fastlane® Cell cDNA kit (QIAGEN, Hilden, Germany) was used to synthesize complementary DNA (cDNA) from the cells, which was subjected to quantitative polymerase chain reaction (qPCR). The mRNA levels were analyzed using gene-specific SYBR Green-based QuantiTect® Primer assay kits (QIAGEN, Germany) of target genes, namely, *PPARGC1*, *LTB4R1*, *LPL*, *ADIPOR1*, *PPARG*, *C/EBPα*, *SREBP-1*, *FABP4*, *UCP-1*, STAT6, and PRDM1. The expression levels of the target genes were normalized to that of actin-β (ACTB), as the internal reference gene. The qPCR was performed in a 25 µL reaction mixture comprising 12.5 µL master mix, 1 µL template cDNA (100 µg), 1 µL primer (see **Table 1**), and 10.5 µL RNase-free water in each well of a 96-well PCR plate. The reaction mixture was subjected to real time-PCR for 40 cycles as previously described [16]. The obtained products were analyzed using the comparative cycle threshold (**Ct**) method, and fold changes were compared with the control. The Ct cycle was used to determine gene expression levels in the control, PUN-, and pomegranate juice-treated adipocytes. The $2^{-\Delta\Delta Ct}$ values were used to plot the expression of target genes. Thus, mRNA expression level ranges are presented as the n-fold difference with respect to the expression level of the reference control.

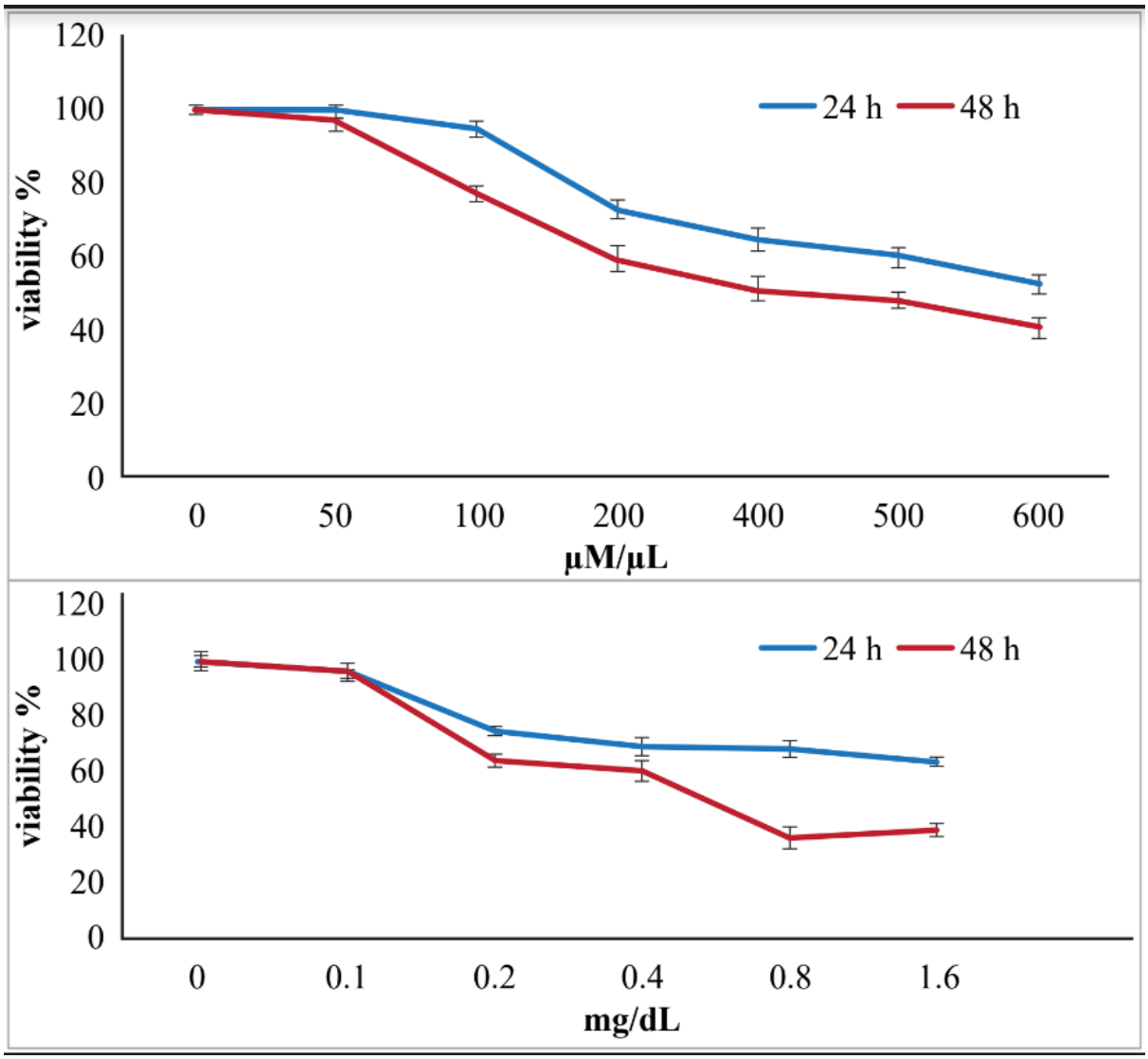
2.6 Statistical analysis

Experiments were performed in three replicates for each experimental group. The obtained data were expressed as the mean ± standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test in the statistical package for social science (**SPSS**) version 22 software package. Treatment means of the gene expression analysis and the cytotoxicity assay were compared using the least significant difference method (*$p < 0.05$, **$p \leq 0.01$). Two-way ANOVA was used because it considers both concentration and time, and the results were compared using the least significant difference method (**$p \leq 0.05$). All graphs were generated using Graph Pad Prism 8 software.

3. Results

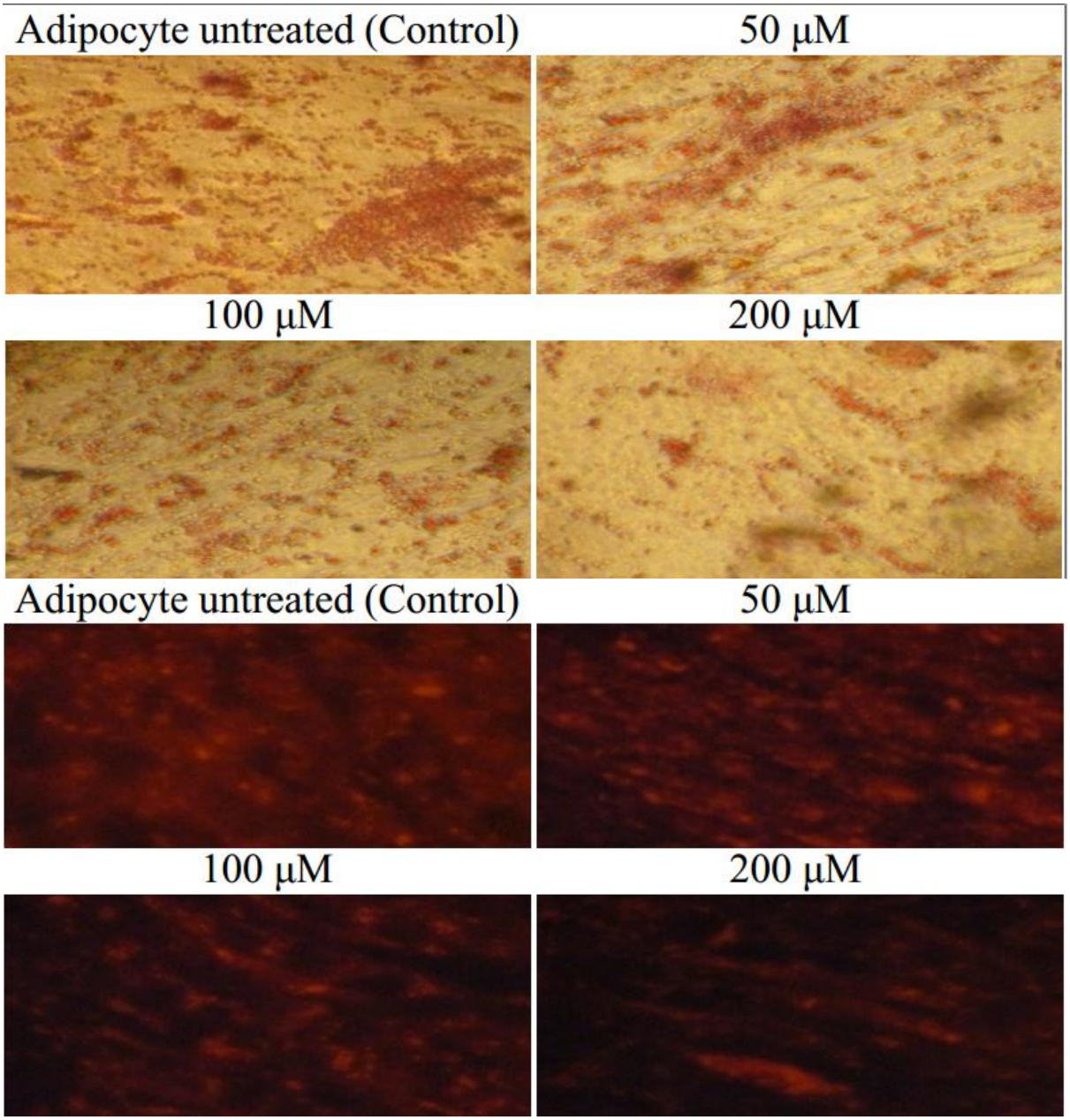
3.1 Toxicity of PUN on adipocytes

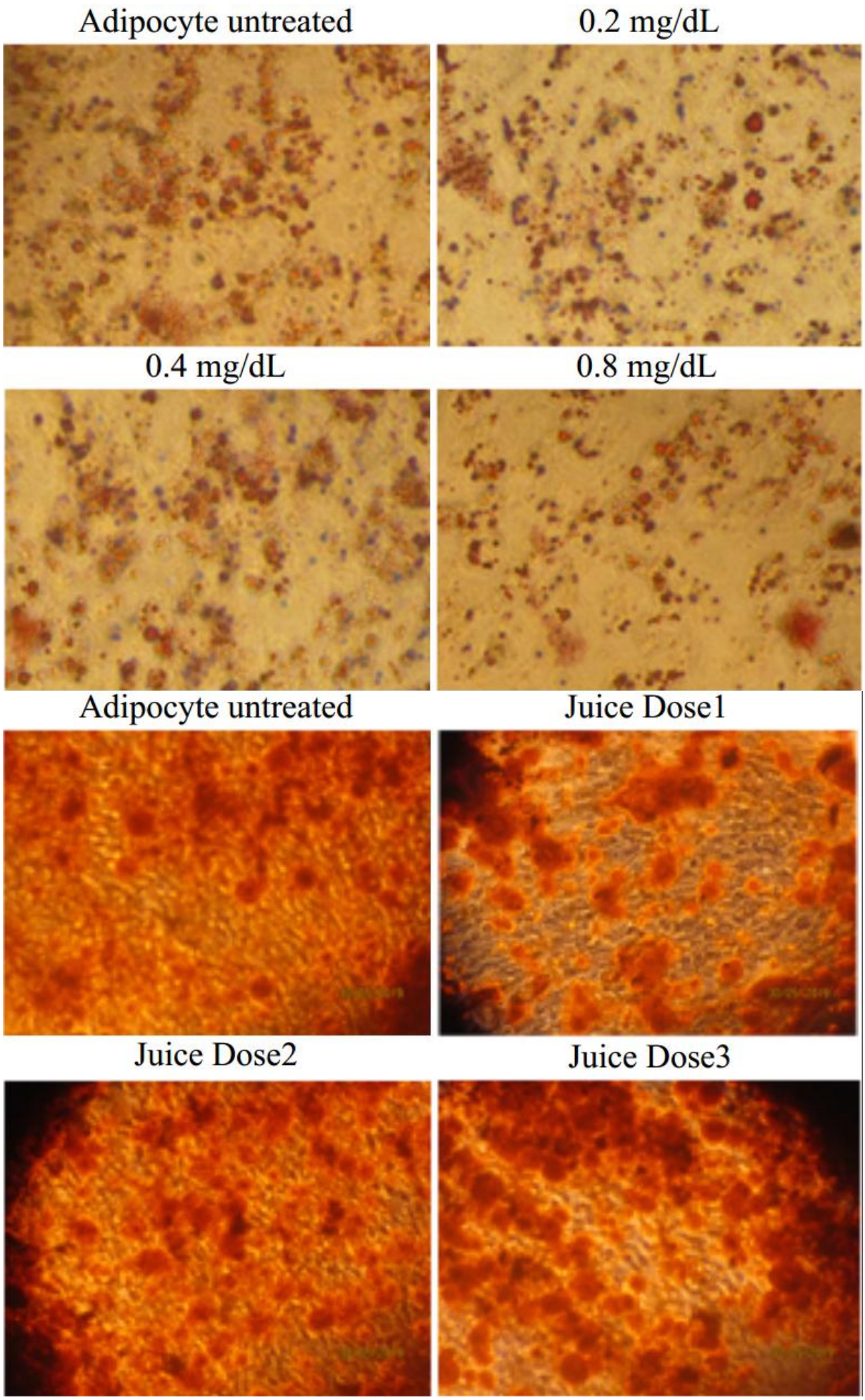
The viability of cells treated with 100 µM PUN for 24 and 48 h was reduced ($p \leq 0.01$) compared with that of untreated adipocytes (**Figure** 1). Additionally, the viability of cells treated with; 0, 2, 0.4, and 0.8 mg/dL; and 0.4 and 0.8 mg/dL pomegranate juice for 24 h and 48 h, respectively, were significantly reduced ($p \leq 0.01$) compared with that of untreated adipocytes. The half-maximal inhibitory concentration (IC_{50}) of PUN was 400 µM, indicating that the growth of 50% of the cells was inhibited by the 48 h treatment at this concentration. Additionally, growth of 48% of cells was inhibited by treatment with 600 µm PUN for 24 h. Similarly, the IC_{50} value of pomegranate juice was 1.8 mg/dL with 48 h treatment. However, pomegranate juice inhibited the growth of 46% of cells when treated at a concentration of 3.2 mg/dL for 24 h (**Figure 1**).



3.2 Effects of PUN and pomegranate juice on lipid accumulation in adipocytes

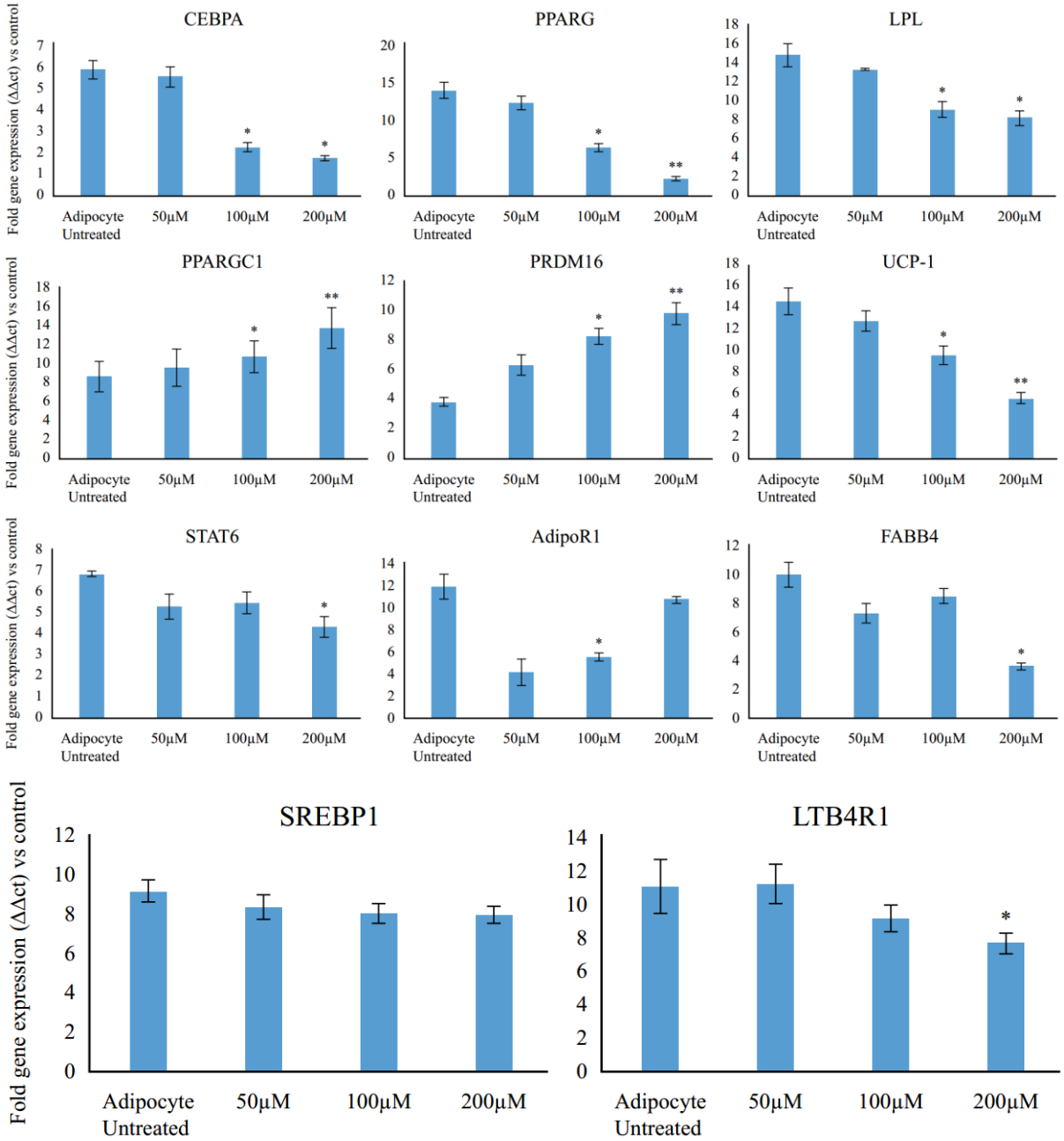
Figure 2 shows the lipid accumulation in the control and PUN-treated matured adipocytes after 14 days, as determined by Oil Red O staining. PUN treatment considerably decreased the Oil Red O staining intensity in a dose-dependent manner; 100 and 200 µM PUN decreased lipid accumulation in differentiation-induced mature adipocytes as compared with that of untreated control cells. Additionally, as shown in **Figures 2**, 0.5 and 0.8 mg/dL pomegranate juice inhibited lipid accumulation in differentiation-induced maturation adipocytes as compared with that of the control. Nile Red staining also showed a considerably reduced lipid content (droplets) in adipocytes treated with 100 and 200 µM PUN as compared with that in untreated cells. Furthermore, as shown in **Figure** 2, illustrated that, 0.4 and 0.8 mg/dL pomegranate juice considerably reduced lipid content in cells, compared with that in untreated differentiated adipocytes.





3.3 Effects of PUN on adipogenesis-and mitochondrial biosynthesis-related gene expression in mature adipocytes

PUN treatment decreased the lipid accumulation-related gene levels, including *PPARGC1*, *LTB4R1*, *LPL*, *ADIPOR1*, *PPARG*, *C/EBPα*, *FABP4*, *UCP-1*, *STAT6*, and *PRDM16*, compared with those in untreated adipocytes. **Figure 3** shows the expression levels of various obesity-associated genes. Expression of *PPARGC1*, *UCP-1*, *PPARG*, and *PRDM16* was significantly inhibited by treatment with 100 μM PUN ($p \leq 0.05$), and it significantly decreased further by treatment with 200 μM PUN ($p \leq 0.01$), compared with those of untreated adipocytes. Additionally, expression levels of *LTB4R1*, *FABP4*, and *STAT6* were significantly decreased by treatment with 200 μM PUN ($p \leq 0.05$). Expression levels of *C/EBPα* and *LPL* were significantly decreased by 100 and 200 μM PUN ($p \leq 0.05$). Furthermore, expression of *ADIPOR1* was significantly inhibited by 100 μM PUN ($p \leq 0.05$). However, expression of *SREBP-1* was unaffected by PUN at all doses.



3.4 Effect of pomegranate fruit juice on adipogenesis-and mitochondrial biosynthesis-related gene expression in mature adipocytes

The relative mRNA expression levels of *PPARGC1*, *LPL*, *ADIPOR1*, *PPARG*, and *C/EBPα* decreased through treatment with pomegranate juice at certain doses compared with those in untreated adipocytes.

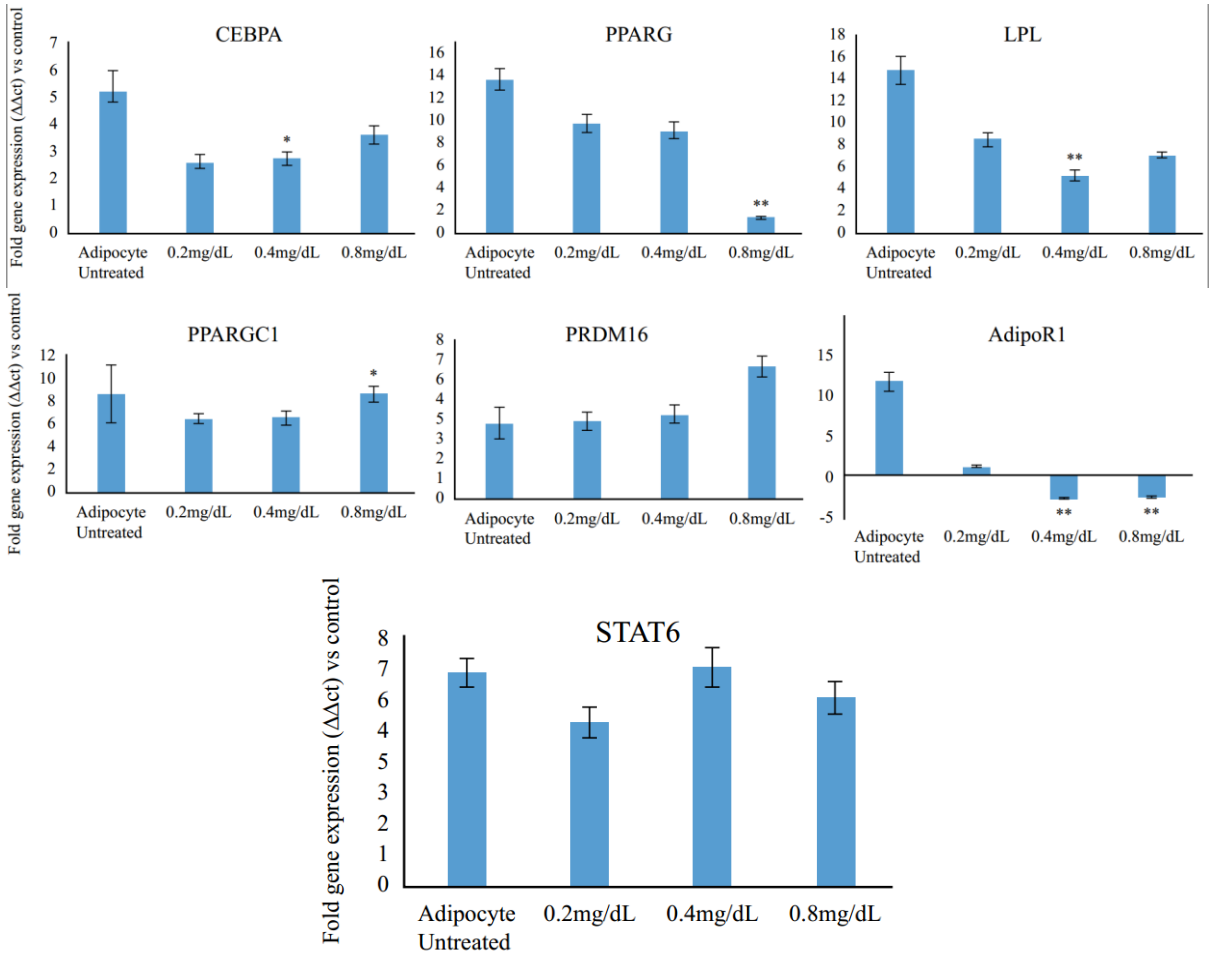


Figure 4: shows that expression of *C/EBPα* was significantly inhibited by treatment with 0.4 mg/dL pomegranate juice ($p \leq 0.05$). However, expression of *PPARG* was only significantly suppressed in pomegranate juice-treated adipocytes at 0.8 mg/dL ($p \leq 0.01$). Additionally, expression of *LPL* was significantly inhibited by 0.4 mg/dL pomegranate juice ($p \leq 0.01$). However, expression of *PPARGC1* was significantly inhibited by 0.8 mg/dL pomegranate juice ($p \leq 0.05$). Expression of *ADIPOR1* was significantly decreased by pomegranate juice at both 0.4 and 0.8 mg/dL ($p \leq 0.01$). However, expression of *STAT6*, *UCP-1*, *FABP4*, *PRDM16*, *SREBP-1*, and *LTB4R1* was unaffected by pomegranate juice at all doses.

4. Discussion

In obesity, various inflammatory mediators are produced in the adipose tissue, and these mediators are involved in mechanisms responsible for the exacerbation of chronic obesity-related diseases. In addition, the inflammatory response in the adipose tissue affects the pathogenesis of metabolic disorders in people with obesity [3]. In line with our results, Bounihi et al. [17] showed that pomegranate may prevent obesity and exert anti-inflammatory and anti-adiposity effects in male Wistar rats. Hosseini et al. [2] reported that supplementation with pomegranate extract significantly decreased levels of total cholesterol and low-density lipoprotein cholesterol. In the current study, our results demonstrated that PUN, natural pomegranate fruit extract, has an anti-obesity effect on adipocytes (Figure 2). In addition, data identified the effective doses for potential treatment of obesity using PUN and pomegranate juice. Moreover, the results from the cell viability assay suggested that PUN and pomegranate juice induced acute cytotoxicity in a dose-or time-dependent manner. Time appeared to be a particularly important factor in mediating the effects of PUN and pomegranate juice. The effect on viability at 48 hours was greater than that at 24 hours. In addition, the IC_{50} of PUN and pomegranate juice was 400 µM 1.8 mg/dL at 48 hours respectively. A previous study reported that the IC_{50} of PUN was 4.50 µM [18]. The results of this study confirmed that PUN treatment at 100 and 200 µM considerably decreases lipid accumulation in differentiated mature adipocytes, based on the Oil red O staining intensity in a dose-dependent manner. A dose of 0.4 and 0.8 mg/dL pomegranate juice inhibited lipid accumulation in differentiation-induced maturation adipocytes (Figure 2). Nile Red staining clearly showed the presence of lipid droplets in differentiated adipocytes after 14 days and a decrease in the proportion of differentiated adipocytes treated with 0.4 and 0.8 mg/dL pomegranate juice. Thus, the present study confirmed that PUN and pomegranate juice suppresses lipid accumulation in differentiation-induced mature adipocytes in the adipose tissue. This result is consistent with a previous study showing that pomegranate extract is effective in reducing TG content and intracellular lipid accumulation [19]. Another study reported that PUN significantly decreased the accumulation of red droplets and lipids, as determined by Oil Red O staining [20]. The gene expression analysis results are also consistent with those of previous studies, which indicates the role of pomegranate extract on gene expression inhibition (*SREBP* and *PPAR-γ*) [2, 17, 19]. The results **also** indicated that, the inhibitory effect of pomegranate fruit juice on gene expression was less

than that of PUN. This difference may be due to the high total polyphenol content in the pomegranate peels (67%), whereas that in pomegranate juice is only 29.7% [20].

5. Conclusions

PUN and pomegranate juice were found to inhibit adipocyte differentiation, lipid accumulation, and obesity-related inflammation. Thus, they may be effective in treating obesity. Therefore, we recommend further research regarding the use of PUN as a nutrition formula or drug candidate for the treatment of obesity among humans.

Acknowledgements
The authors would like to thank King Abdulaziz City for Science and Technology (KACST), Kingdom of Saudi Arabia for finding [Award number 1-18-04-001-0019]. Authors would like to thank Dr. B ELbashir for his valuable assistant.

References

[1] Wang Y, Beydoun MA, Liang L, Caballero B, Kumanyika SK. Will all Americans become overweight or obese? Estimating the progression and cost of the US obesity epidemic. Obesity (Silver Spring) 2008; 16 (10): 2323–30. https: //doi. org/10.1038/oby.2008.351

[2] Hosseini B, Saedisomeolia A, Wood LG, Yaseri M, Tavasoli S. Effects of pomegranate extract supplementation on inflammation in overweight and obese individuals: A randomized controlled clinical trial. Complement Ther Clin Pract 2016; 22: 44–50. https: //doi. org/10.1016/j. ctcp.2015.12.003

[3] Sung J, Ho CT, Wang Y. Preventive mechanism of bioactive dietary foods on obesity-related inflammation and diseases. Food Funct 2018; 9 (12): 6081–95. https: //doi. org/10.1039/c8fo01561a

[4] Soriano JB, Abajobir AA, Abate KH, Abera SF, Agrawal A, Ahmed MB, et al. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Respir Med 2017; 5 (9): 691–706. https: //doi. org/10.1016/S2213-2600 (17) 30293-X

[5] Cancer Research UK. Cancer statistics reports for the UK, http: //www.cancerresearchuk. org/aboutcancer/statistics/cancerstatsreport/; 2003 [accessed 13 March 2003].

[6] Lansky EP, Newman RA. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. J Ethnopharmacol 2007; 109 (2): 177–206. https: //doi. org/10.1016/j. jep.2006.09.006

[7] Viuda-Martos M, Fernández-López J, Pérez-Álvarez JA. Pomegranate and its many functional components as related to human health: a review. Compr Rev Food Sci Food Saf 2010; 9 (6): 635–54. https: //dx. doi. org/10.1111/j.1541-4337.2010.00131. x

[8] Kumari A, Dora J, Kumar A, Kumar A. Pomegranate (*Punica granatum*) —Overview. Int J Pharm Chem Sci 2012; 1 (4): 1218–22.

[9] Sahebkar A, Simental-Mendía LE, Giorgini P, Ferri C, Grassi D. Lipid profile changes after pomegranate consumption: A systematic review and meta-analysis of randomized controlled trials. Phytomedicine 2016; 23 (11): 1103–12. https: //doi. org/10.1016/j. phymed.2015.12.014

[10] Zhan J, Li Y, Yu J, Zhao Y, Cao W, Ma J, et al. Culture medium of bone marrow-derived human mesenchymal stem cells effects lymphatic endothelial cells and tumor lymph vessel formation. Oncol Lett 2015; 9 (3): 1221–1226. https: //doi. org/10.3892/ol.2015.2868

[11] Ooi A, Wong A, Esau L, Lemtiri-Chlieh F, Gehring C. A guide to transient expression of membrane proteins in HEK-293 cells for functional characterization. Front Physiol 2016; 7: 300. https: //doi. org/10.3389/fphys.2016.00300

[12] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65 (1-2): 55–63. https: //doi. org/10.1016/0022-1759 (83) 90303-4

[13] Scott MA, Nguyen VT, Levi B, James AW. Current methods of adipogenic differentiation of mesenchymal stem cells. Stem Cells Dev 2011; 20 (10): 1793–1804. https: //doi. org/10.1089/scd.2011.0040

[14] BenSaad LA, Kim KH, Quah CC, Kim WR, Shahimi M. Anti-inflammatory potential of ellagic acid, gallic acid and punicalagin A&B isolated from *Punica granatum*. BMC Complement Altern Med 2017; 17 (1): 47. https: //doi. org/10.1186/s12906-017-1555-0

[15] Vishnubalaji R, Al-Nbaheen M, Kadalmani B, Aldahmash AM, Ramesh T. Comparative investigation of the differentiation capability of bone-marrow-and adipose-derived mesenchymal stem cells by qualitative and quantitative analysis. Cell Tissue Res 2012; 347 (2): 419–27. http: //dx. doi. org/10.1007/s00441-011-1306-3

[16] Yuan JS, Reed A, Chen F, Stewart Jr CN. Statistical analysis of real-time PCR data. BMC Bioinform 2006; 7 (1): 85. https: //doi. org/10.1186/1471-2105-7-85

[17] Bounihi A, Bitam A, Bouazza A, Yargui L, Koceir EA. Fruit vinegars attenuate cardiac injury via anti-inflammatory and anti-adiposity actions in high-fat diet-induced obese rats. Pharm Biol 2017; 55 (1): 43–52. https: //doi. org/10.1080/13880209.2016.1226369

[18] Wu D, Ma X, Tian W. Pomegranate husk extract, punicalagin and ellagic acid inhibit fatty acid synthase and adipogenesis of 3T3-L1 adipocyte. J Funct Foods 2013; 5 (2): 633–41. https: //doi. org/10.1016/j. jff.2013.01.005

[19] Sorrenti V, Randazzo CL, Caggia C, Ballistreri G, Romeo FV, Fabroni S, et al. Beneficial effects of pomegranate peel extract and probiotics on pre-adipocyte differentiation. Front Microbiol 2019; 10 : 660. https: //doi. org/10.3389/fmicb.2019.00660

[20] Zhao W, Li J, He X, Lv O, Cheng Y, Liu R. In vitro steatosis hepatic cell model to compare the lipid-lowering effects of pomegranate peel polyphenols with

several other plant polyphenols as well as its related cholesterol efflux mechanisms. Toxicol Rep 2014; 1: 945–54. https: //doi. org/10.1016/j. toxrep.2014.10.013

Figure Legends

Figure 1: Effect of punicalagin (PUN) and pomegranate juice on adipocyte viability. (a) PUN; (b) pomegranate juice. The data are presented as mean ± SD of experiments performed in triplicate. $^{**}p \leq 0.01$ versus the control.

Figure 2: Light microscopy of cells. (a) Results of Oil Red staining after 14 days of punicalagin (PUN) treatment. (b) Results of Nile Red staining after 14 days of PUN treatment. (c) Results of Oil Red staining after 14 days of treatment with pomegranate juice. (d) Results of Nile Red staining after 14 days of treatment with pomegranate juice.

Figure 3: Effect of punicalagin (PUN) on the expression of adipogenesis-and mitochondrial biosynthesis-related genes in mature adipocytes. Data are presented as mean ± SD (n = 6). ** $p \leq 0.01$ and *$p \leq 0.05$ compared with untreated adipocytes. PUN doses: 50, 100, and 200 µM.

Figure 4: Effect of pomegranate juice on the expression of adipogenesis-and mitochondrial biosynthesis-related genes in mature adipocytes. Data are presented as mean ± SD (n = 6). ** $p \leq 0.01$ and * $p \leq 0.05$ compared with untreated adipocytes. Juice doses: 0.2, 0.4, and 0.8 mg/dL.

Table 1: Primer sequences for target genes in qPCR

Symbol	Gene Name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
ACTB	Beta-actin	CCT CgC CTT TgC CgA TCC	ggA TCT TCA TgA ggT AgT CAg TC
PPARGC1	Peroxisome proliferator activated receptor gamma coactivator-1	CTGTCGGATCCACAAAAAGAGTAGA	CCCCACAGCAAGGCACTT
LTB4R1	Leukotriene B4 receptor	CCCTGCTGGTGCTGAACT	AGGCAGGTGTGTCCTTCG
LPL	Lipoprotein lipase	CCGAGAGTGAGAACATCCCATTCA	CCTTTCTGCAAATGAGACACTTTCTC
ADIPOR1	Adiponectin receptor 1	GCCATGGAGAAGATGGAGGA-3	5′-AGCACGTCGTACGGGATGA-3
PPARG	Peroxisome proliferators-activated receptor gamma	CTGTCGGTT TCAGAAGTGCCT	CCCAAACCTGATGGCATTGTGAGACA
C/EBPα	enhancer binding protein α	GTAACCTTGTGCCTTGGATACT	GGAAGCAGGAATCCTCCAAATA
SREBP-1	sterol regulatory element-binding protein-1	GATCGGATCCGAGAAGCGCA CAGCCCACAA-3	GATCCTCGAGGCTTTTGTG GACAGCAGTGC-3
FABP4	fatty acid binding protein 4	TTTTGCAGCAACATAGGTGAA	ACCTTCCCACCTTTTGGAGT
UCP-1	uncoupling protein	GTGAACCCGACAACTTCCGAA	TGAAACTCCGGCTGAGAAGAT
STAT6	Signal transducer and activator of transcription 6	GGC CCC AAC AAA CTT CTC ATC	TTT GGC GTT GTC TTG GTT A
PRDM16	PR domain containing 16	AAATGGCGTTACTTAAGCTAGCTTGC	AGGTTGGAGAACTGCGTGTAGG