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PUNICALAGIN AND PUNICALIN SUPPRESS THE ADIPOCYTE DIFFERENTIATION THROUGH THE TRANSCRIPTION FACTORS

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Abstract

Background. Pomegranate is a rich source of many polyphenolic compounds including ellagitannins (punicalagin, punicalin and others).

Aim. The effects of punicalagin and punicalin on adipogenesis were investigated in this study.

Materials and Methods. To examine the effect of punicalagin and punicalin on adipocyte differentiation, various concentrations of punicalagin and punicalin (2-10 μM) were applied to differentiated 3T3-L1 cells. Glyceraldehyde-3-phosphate dehydrogenase (GPDH) activity, Oil red O staining, intracellular triglyceride levels, and gene expressions of transcription factors (Peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT-enhancer-binding proteins-α (C/EBPα), Sterol regulatory element-binding protein 1c (SREBP-1c)) and lipolysis-associated genes (hormone-sensitive lipase (HSL), Perilipin A, tumor necrosis factor-α (TNF-α)) were examined in order to investigate the effects of punicalagin and punicalin on adipocyte differentiation.

Results. Punicalagin and punicalin applications caused a continuous decrease in cell size and intracellular triglyceride accumulation. GPDH activity and transcription gene expressions decreased significantly in groups that were applicated punicalagin and punicalin at high concentrations. Punicalagin, but not punicalin, down-regulated the expression of HSL and perilipin A and up-regulated the expression of TNF-α in a dose-dependent manner. In conclusion, both punicalagin and punicalin were able to inhibit the adipocyte differentiation.

Keywords: 3T3-L1, adipogenesis, punicalagin, punicalin, transcription factors, cell differentiation.

INTRODUCTION

The prevalence of overweight and obesity has risen dramatically during the past few decades in the world (1,2). Corresponding with this increase in obesity has been a clustering of metabolic pathologies, including cardiovascular disease (CVD), type 2 diabetes (T2DM), and endometrial, breast, pancreatic, kidney, and colon cancers. Obesity is not only implicated as a primary cause of these degenerative diseases, but also represents a major component of the metabolic syndrome, which represents a clustering of risk factors known to precede the development of CVD and T2DM (3).

Adipose tissue dysfunction, characterized by an increased release of free fatty acid and proinflammatory cytokines, is associated with the development of insulin resistance and metabolic syndrome. In obesity, the primary defect contributing to these metabolic pathologies appears to be an impairment of adipogenesis, which results in greater adipocyte hypertrophy and dysfunction (4). This process is regulated by a number of adipogenic transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR-γ), CCAAT/enhancer binding protein (C/EBP), and sterol regulatory element binding protein-1 (SREBP-1). Many phytochemicals have been found to suppress PPARγ by disrupting the functions of C/EBPα and C/EBPγ genes, and finally controls the process of adipogenesis (5). In the literature, it is seen that various plant extracts prevent the adipocyte differentiation (6).

Pomegranate (*Punica granatum* L.) is an erect shrub and belongs to the family "Punicaceae". The pomegranate is one of the oldest medicinal plants known to humanity. It is native to South West Asia and was extensively cultivated in the Mediterranean region. Different parts of pomegranate have different chemical composition. For this reason, each part of the pomegranate has medically different effects (7). Pomegranate seed oil shows anti-diabetic, antibacterial, antifungal and antitumoral properties. It has been

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proved experimentally that pomegranate flower has anti-diabetic, antihistaminic, cardioprotective, and hepatoprotective properties. Pomegranate juice has been shown to have antioxidant and estrogenic activity in both pre-clinical and clinical trials. Pomegranate husk is used in dysentery and diarrhea caused by parasites due to the anti-helminthic and vasoconstrictive effects. It is also known to be good against mucosal damage such as ulcers and aphthae. Besides, it has antitumoral, antibacterial, anti-inflammatory, immunostimulatory and weight-reducing properties (8, 9). Although pomegranate husk has been found to cause reduction in body weight and fat mass, it is not exactly known which phytochemicals in the pomegranate husk are responsible for this activity. Among the phytochemicals found in pomegranate husk, punicalagin and punicalin have attracted considerable attention in recent years (10). Punicalagin and punicalin are in the form of ellagitannin structure and show a very strong antioxidant activity (11). Although there have been few studies reporting that punicalagin and punicalin show anti-obesity activity *in vivo*, no studies investigating the anti-obesity activity of these phytochemicals in *in vitro* conditions have been found in the literature (8,9,12). Therefore, in this study, we aimed to detect whether punicalagin and punicalin, substances found in pomegranate husk, are able to inhibit the conversion of pre-adiposits into mature adiposits.

MATERIALS AND METHODS

Materials

Punicalagin and punicalin were obtained from Sigma-Aldrich, USA. All cell culture chemicals were bought either from Sigma-Aldrich, Merck or other standard suppliers. Mouse Glycerol-3-phosphate dehydrogenase (GPDH) ELISA kit was commercially available from Elabscience. 3T3-L1 mouse embryo preadipocyte cell line was purchased from the Health Protection Agency (HPA) cell culture collection (Sigma 86052701). Specific PCR primers for PPAR-γ, C/EBPα, SREBP-1c, hormone-sensitive lipase (HSL), Perilipin A, tumor necrosis factor-α (TNF-α), and β-actin were designed using primer3web program (v. 4.1.0) and synthesized by PRZ BioTECH (Bilkent, Ankara, Turkey).

Cell culture

Frozen 3T3-L1 cells were immediately thawed and transferred into a 10 cm^2 culture dish with culture media (DMEM, 10% bovine calf serum (BCS), and 1% penicillin/streptomycin). The cells were incubated in a humidified cell culture incubator with 5% CO_2 at 37°C. The media was replaced the following day. When the 3T3-L1 cells reached 70% confluence, cells were subcultured by adding 1 mL Trypsin-EDTA solution into the culture dish and incubated for 5 minutes at 37°C. Culture media was added to the culture dish, and then cell suspension was transferred into a new 10 cm^2 culture dish, which included 10 mL of culture media (13). To determine the numbers of cells that needed to be seeded for experiments, 10 µL of the cell suspension was loaded onto a hemocytometer. Cells in the four squares of the hemocytometer were counted, averaged, and multiplied by 10^4 (14).

Preparation of punicalagin and punicalin solutions in different concentrations

To examine the effect of punicalagin and punicalin on 3T3-L1 cells, various concentrations of punicalagin and punicalin solutions (2, 4, 6, 8, 10, 20, 30, 40, 50, 100, 150, 200, and 250 µM) were prepared using 1 mM punicalagin and punicalin stock solutions.

Effect of punicalagin and punicalin applications in different concentrations on cell viability

To determine the concentrations of punicalagin and punicalin that did not exhibit harmful effects on cell viability (≤10% cytotoxicity), MTT assay was performed. This assay is a quantitative colorimetric method that determines cell viability based on the reduction of yellow MTT into purple formazan by nicotinamide adenine dinucleotide (NADH) in the mitochondria of proliferating cells. 3T3-L1 cells were seeded at 10,000 cells/cm^2 in a 96-well plate and incubated for 24 hours in the cell culture incubator. The media was replaced with fresh culture media containing 2-250 µM concentrations of punicalagin and punicalin. After 48 hours of incubation, cell viability was determined according to the manufacturer's instruction (Sigma-Aldrich, USA). Briefly, the media was removed and replaced with 100 µL of fresh culture media including 0.5 mg/mL of MTT reagent, and cells were incubated for 3 hours at 37°C in the cell culture incubator. During the incubation period, the yellow MTT reagent was reduced and resulted in the formation of purple formazan precipitation at the bottom of the wells. The media was gently removed and 100 µL dimethyl sulfoxide (DMSO) was added to solubilize the precipitated formazan. Absorbance was measured at a wavelength of 570 nm using VersaMax Microplate

Reader (15). The treatments were performed in triplicates.

Differentiation of 3T3-L1 cells to mature adipocytes

The cells were then seeded at 50,000 cells/mL in 6-well, 12-well, 96-well plates with culture media, and this is day 0 of the experiment. The cells were incubated in the cell culture incubator. On day 2, when the cells reached 100% confluence, the culture media was switched to differentiation media (DMEM, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 10 µg/mL insulin, 0.5 mM IBMX, 0.25 mM dexamethasone, and 100 ng/mL biotin) including 2, 4, 6, 8, and 10 µM of punicalagin and punicalin (non-cytotoxic concentrations) were provided to the cells and then cells were incubated in a cell culture incubator. As negative control, only differentiation media was applied to the cells. On day 4, the differentiation media was removed and replaced with post-differentiation media (DMEM, 10% FBS, 1% penicillin/streptomycin, and 1 µg/mL insulin). The post-differentiation media was refreshed every other day until day 18 (13).

Lactate dehydrogenase (LDH) release assay

At the end of the differentiation period, LDH release assay was performed to determine the cytotoxic effects of punicalagin and punicalin in mature adipocytes using LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany) (Catalog No: 11644793001). The media in the cell culture plates were taken into the falcon tubes and centrifuged at 1250 rpm for 5 minutes. In order to determine the sample absorbance, 50 µL of the supernatant was transferred to a 96-well plate. 100 µL of LDH reaction mixture was added to the each well. In order to find the control absorbance value, 50 µL DMEM was added to the plates and 100 µL LDH reaction mixture was added. To determine the maximal absorbance, 50µL of supernatant from each sample was taken and 100 µL of 1% Triton X-100 was added over it. All samples were incubated at room temperature for 30 minutes and their absorbance at 492 nm was read on the ELISA plate reader. The percentage of cytotoxicity was calculated using the following formula (16):

Cytotoxicity (%) = [(Sample absorbance-Control absorbance) / (Maximal absorbance/Control absorbance)] x 100.

Oil Red O staining

During the differentiation process, lipids are accumulated as a single lipid droplet in the cytosol of the cell. This indicates that white pre-adipocytes have developed into mature white adipocytes. To examine whether the treatment of punicalagin and punicalin in the different growth stages of 3T3-L1 cells affects the differentiation process, lipid droplets in fully differentiated mature white adipocytes (day 18) were stained using Oil Red O. Oil Red O is an oil-soluble dye used to stain lipids, which visualizes lipid droplets in red color. At day 18, mature white adipocytes were washed twice with PBS and incubated in 10% formalin for 5 minutes at room temperature. The formalin was removed, replaced with fresh formalin, and then incubated at room temperature for 1 hour. After removing formalin, the cells were washed with 60% isopropanol and completely dried at room temperature. Oil Red O solution (6 parts Oil Red O and 4 parts deionized water) was added to each well and then incubated for 30 minutes at room temperature. The cells were rinsed four times with tap water. Images of the stained lipids were taken with an Euromex inverted microscope at 10x, 20x and 40x magnification. After the imaging, stained oil droplets were dissolved in isopropyl alcohol, and relative triglyceride content was quantified by reading the absorbance at 490 nm on a microplate reader (17).

Glycerol-3-phosphate dehydrogenase (GPDH) activity assay

Cells were lysed in buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, and 1 mM β-mercaptoethanol. GPDH activity was measured using a commercial kit (Elabscience Biotechnology Inc., Texas, USA) (Catalog No: E-EL-M0424) and by monitoring the dihydroxyacetone phosphate dependent oxidation of nicotinamide adenine dinucleotide (NAD) at 340 nm. GPDH activity was expressed as units/mg of protein (18).

Estimation of total protein

Protein content in cell lysate was determined using Bradford's dye. Briefly, 100 µL cell lysate was reacted with 1 mL of Bradford reagent (2 parts stock Bradford reagent and 7 parts deionized water) and absorbance was recorded at 595 nm. Protein concentration was expressed as bovine serum albumin (BSA) equivalent/mL (19).

RNA preparation and quantitative real-time PCR

Total RNA was extracted using TriPure

RNA isolation kit (Roche Diagnostics, Mannheim, Germany) (Catalog No: 11667165001), according to the manufacturer's protocol. The total RNA (2 µg) was used for cDNA synthesis using First Strand cDNA synthesis kit (Promega Corporation, Wisconsin, USA) (Catalog No: E6560S). Then mRNA expression was quantitatively determined by Roche LightCycler 480 II Real-Time PCR device using Invitrogen Universal EXPRESS SYBR GreenER qPCR SuperMixes and Two-Step qRT-PCR kit. GAPDH was the invariant control. The primer sequences used were: β-actin, sense (5'- AGG TCA TCA CTA TTG GCA AC -3') and antisense (5'- ACT CAT CGT ACT CCT GCT TG -3') (20); PPARγ, sense (5'- ACC CCC TGC TCC AGG AGA T -3') and antisense (5'- TGC AAT CAA TAG AAG GAA CAC GTT -3') (21); C/EBPα, sense (5'- CGC AAG AGC CGA GAT AAA GC -3') and antisense (5'- GCG GTC ATT GTC ACT GGT CA -3') (21); SREBP-1c, sense (5'- GAC GCT CAT TGG CCT GG -3') and antisense (5'- CTC TGG AGG CAG ACG ACA AG -3') (22); HSL, sense (5′- GCT GGG CTG TCA AGC ACT GT -3′) and antisense (5′- TAC CGT CGG ATG GGT CAA TG -3′); Perilipin A, sense (5′- GGC CTG GAC GAC AAA ACC -3′) and antisense (5′- CAG GAT GGG CTC CAT GAC -3′) (23); and TNF-α, sense (5′- CCT GTA GCC CAC GTC GTA G -3′) and antisense (5′- GGG AGT AGA CAA GGT ACA ACC C -3′) (24). In addition, relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold.

Statistical analysis

Statistical analyses were done in SPSS for Windows v16.0 package program. ANOVA was used to test the statistical differences between the groups for all parameters and Tukey multiple comparison tests were used for subgroup comparisons. Mean ± standard deviation values were given as descriptive statistics and an error bar graph was drawn for variables. Differences with a value of $p<0.05$ were considered statistically significant.

RESULTS

To assess cytotoxic effect of punicalagin and punicalin, cell viability in 3T3-L1 preadipocytes was evaluated using MTT assay. The effects of different concentrations of punicalagin and punicalin on cell viability were shown in Figure 1. Punicalagin and punicalin treatment at concentrations of 2-10 µM for 48 hours, these compounds had no remarkable inhibitory effects on cell viability (≤10% cytotoxicity). At the concentration of 20-250 µM, punicalagin and punicalin showed an inhibition activity on the cell growth in 48 hours (>10% cytotoxicity). The IC50 value of punicalagin and punicalin on 3T3-L1 cells was found to be 248.07 µM and 385.53 µM, respectively.

LDH release assay was performed to determine cytotoxicity after the differentiation process. The

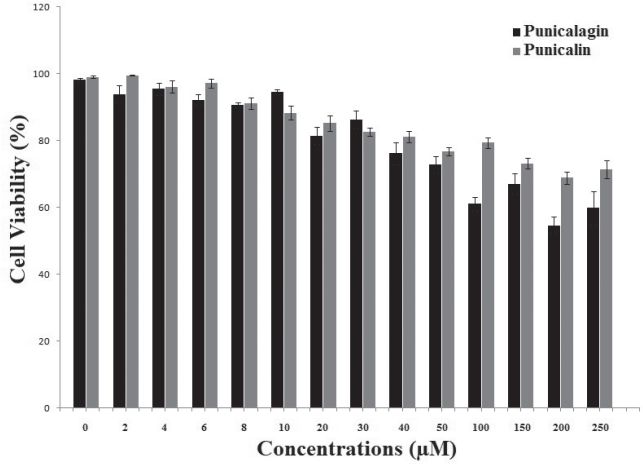

Figure 1. Effects of punicalagin and punicalin administration at different concentrations on cell viability in undifferentiated 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated with various concentrations of punicalagin and punicalin (0–250 µM) for 24 hrs and cell viability was measured by MTT assay. Data are shown as the means ± SD (n = 3).

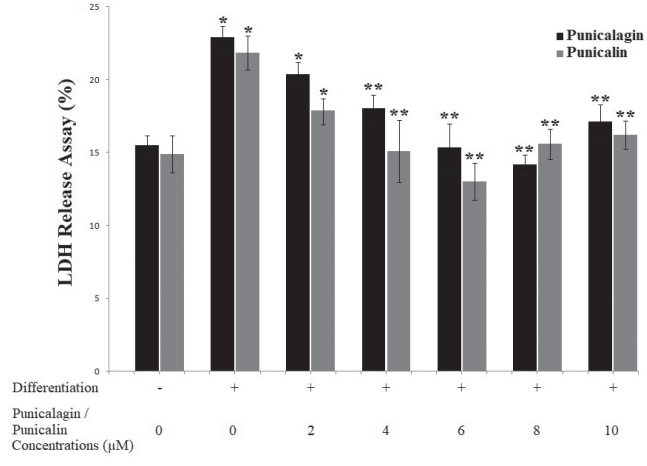

Figure 2. Effects of punicalagine and punicalin administration at different concentrations on LDH release in 3T3-L1 cells. Data are shown as the means ± SD (n = 3). (LDH; Lactate dehydrogenase)
* $p<0.05$ in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** $p<0.05$ in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

effects of different concentrations of punicalagin and punicalin on LDH release were shown in Figure 2. Cytotoxic effect of punicalagin and punicalin in mature adipocytes was statistically higher than in preadipocytes. 4-10 µM punicalagin and 4-6 µM punicalin treatments decreased the cytotoxicity compared with untreated mature adipocytes ($p<0.05$). The other concentrations of punicalagin and punicalagin also decreased the cytotoxicity but these decreases were not statistically significant ($p>0.05$).

Microscopic image of 3T3-L1 cells after Oil Red O staining was shown in Figure 3 and Figure 4. As a result of the morphological examination, punicalagin and punicalin applications caused a continuous

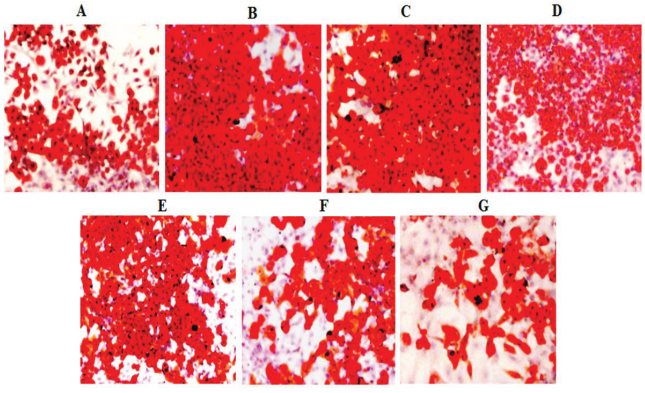

Figure 3. Effects of punicalagin administration at different concentrations on intracellular triglyceride storage in 3T3-L1 cells by Oil Red O staining. The cells stained with Oil Red O were visualized using an Euromex inverted microscope at ×40 magnification (n=3). (A; Undifferentiated - untreated cells, B; Differentiated - untreated cells, C; Differentiated - 2 µM punicalagin treated cells, D: Differentiated - 4 µM punicalagin treated cells, E: Differentiated - 6 µM punicalagin treated cells, F: Differentiated - 8 µM punicalagin treated cells, G: Differentiated - 10 µM punicalagin treated cells).

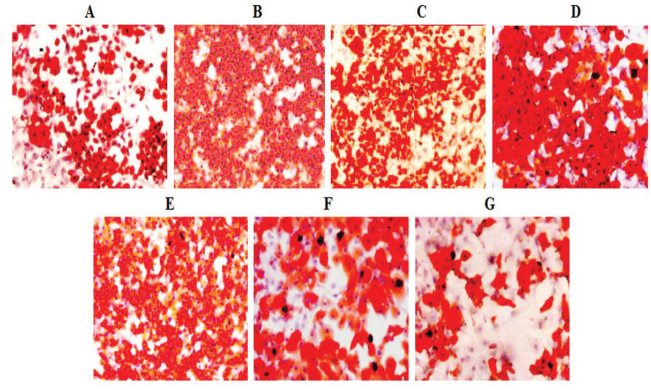

Figure 4. Effects of punicalin administration at different concentrations on intracellular triglyceride storage in 3T3-L1 cells by Oil Red O staining. The cells stained with Oil Red O were visualized using an Euromex inverted microscope at ×40 magnification (n=3).(A; Undifferentiated - untreated cells, B; Differentiated - untreated cells, C; Differentiated - 2 µM punicalin treated cells, D: Differentiated - 4 µM punicalin treated cells, E: Differentiated - 6 µM punicalin treated cells, F: Differentiated - 8 µM punicalin treated cells, G: Differentiated - 10 µM punicalin treated cells).way ANOVA followed by Tukey's test for multiple comparison.

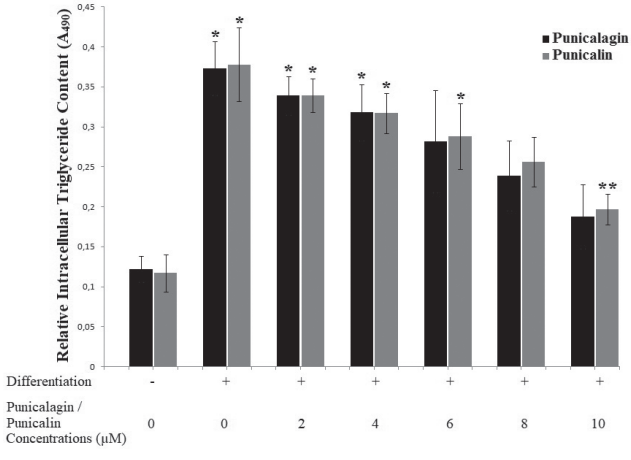

Figure 5. Effects of punicalagin and punicalin administration at different concentrations on relative intracellular triglyceride content in 3T3-L1 cells. Stained oil droplets were dissolved in isopropyl alcohol and measured by reading absorbance at 490 nm using a VersaMax Microplate Reader. Data are shown as the means ± SD (n = 3).

* $p<0.05$ in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

** $p<0.05$ in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

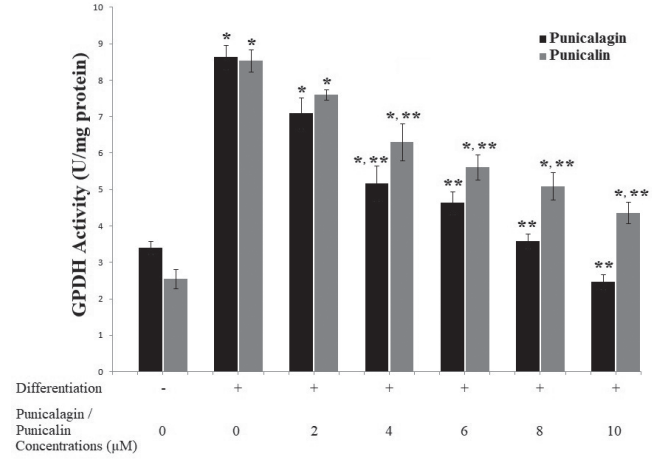

Figure 6. Effects of punicalagin and punicalin administration at different concentrations on GPDH activity in 3T3-L1 cells. Data are shown as the means ± SD (n = 3). (GPDH; Glycerol-3-phosphate dehydrogenase).

* $p<0.05$ in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

** $p<0.05$ in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

decrease in cell size and intracellular triglyceride accumulation in a dose-dependent manner. Relative intracellular triglyceride contents were shown in Figure 5. Although relative intracellular triglyceride contents exhibited a dose-related decrease compared with untreated mature adipocytes, only 10 μM punicalin treatment significantly reduced the relative intracellular triglyceride contents ($p<0.05$).

GPDH activity was measured in order to determine the terminal differentiation after the differentiation process. GPDH activities were shown in Figure 6. GPDH activity of mature adipocytes was statistically higher than of preadipocytes. 4-10μM punicalagin and punicalin treatments decreased the GPDH activity compared with untreated mature adipocytes ($p<0.05$). The other concentrations of punicalagin and punicalagin also decreased the GPDH activity but these decreases were not statistically significant ($p>0.05$).

Transcription gene expressions were shown in Figure 7, Figure 8, and Figure 9. Transcription gene expressions (PPARγ, C/EBPα, and SREBP-1c) of mature adipocytes were statistically higher than of preadipocytes. The application of punicalagin and punicalin at different concentrations showed a dose dependent lowering effect on expression of all three transcription genes. However, this decrease was found to be significant in groups that were applicated 6-10 μM punicalagin and 8-10 μM punicalin for PPARγ, 6-10 μM punicalagin for C/EBPα and 10 μM punicalagin for SREBP-1c ($p<0.05$). The other concentrations of punicalagin and punicalagin also decreased the gene expressions but these decreases were not statistically significant ($p>0.05$).

Lipolysis-associated gene expressions (HSL, perilipin A, and TNF-α) were shown in Figure 10, Figure 11, and Figure 12, respectively. The application of punicalagin at different concentrations showed a dose dependent lowering effect on HSL and perilipin A, but an increasing effect on expression of TNF-α ($p<0.05$). However, 10 μM punicalagin treatment significantly suppressed HSL expression (Fig. 10), whereas only 8-10 μM punicalagin treatment significantly suppressed perilipin A expressions (Fig. 11) ($p<0.05$). On the other hand, 8-10 μM punicalagin treatment induced TNF-α expressions significantly (Fig. 12) ($p<0.05$). But, punicalin did not show a significant effect on expression of all three genes ($p>0.05$).

DISCUSSION

Mesenchymal stem cell-derived pre-adipocytes have the potential to differentiate into adipocytes. The adipocyte life cycle contains changes in cell shape and growth arrest, clonal amplification and a complex sequence of alterations in gene expression leading to lipid accumulation and finally to cell death. Obesity is a condition associated with excessive growth of adipose tissue resulting from an increase in the number and/or size of adipocytes differentiated from pre-

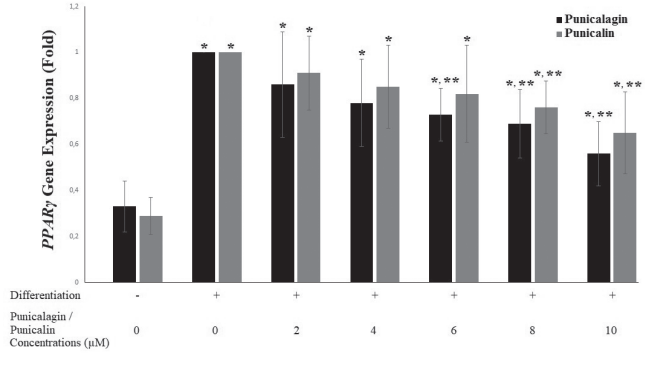

Figure 7. Effects of punicalagin and punicalin administration at different concentrations on relative PPARγ gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4). (PPARγ; Peroxisome proliferator-activated receptor-γ).
* $p<0.05$ in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** $p<0.05$ in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

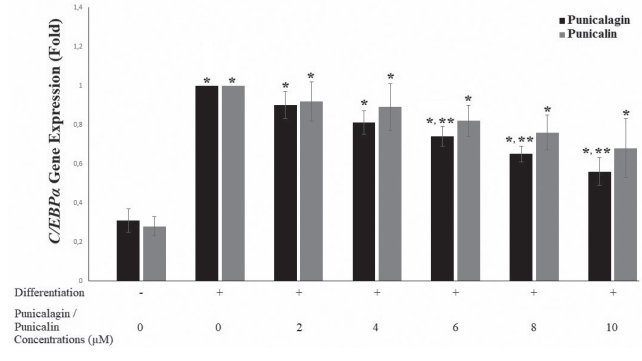

Figure 8. Effects of punicalagin and punicalin administration at different concentrations on relative C/EBPα gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4). (C/EBPα; CCAAT-enhancer-binding proteins-α).
* $p<0.05$ in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** $p<0.05$ in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

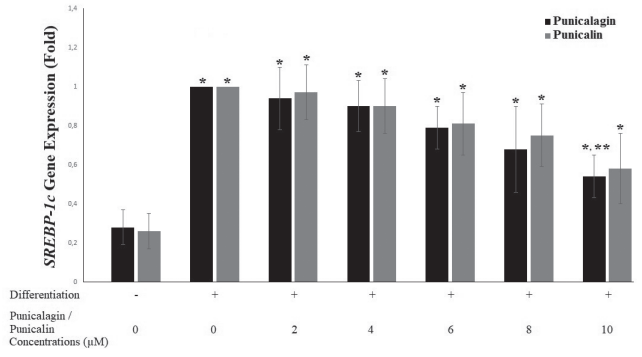

Figure 9. Effects of punicalagin and punicalin administration at different concentrations on relative SREBP-1c gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4). (SREBP-1c; Sterol regulatory element-binding protein-1c).
* p<0.05 in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** p<0.05 in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

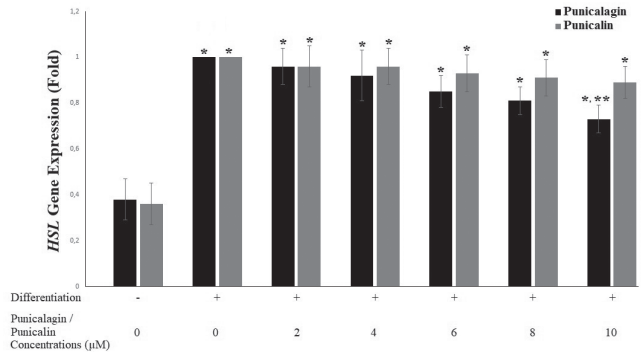

Figure 10. Effects of punicalagin and punicalin administration at different concentrations on relative HSL gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4). (HSL; hormone-sensitive lipase).
* p<0.05 in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** p<0.05 in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

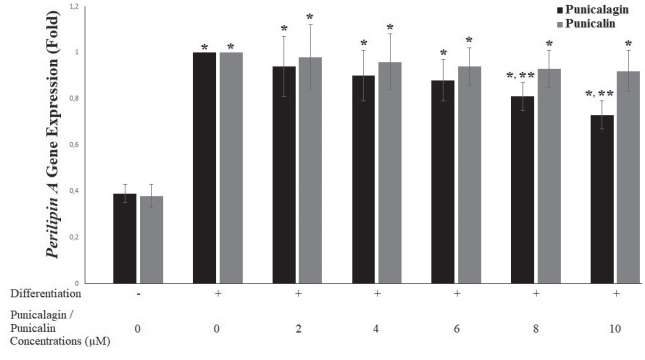

Figure 11. Effects of punicalagin and punicalin administration at different concentrations on relative perilipin A gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4).
* p<0.05 in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** p<0.05 in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

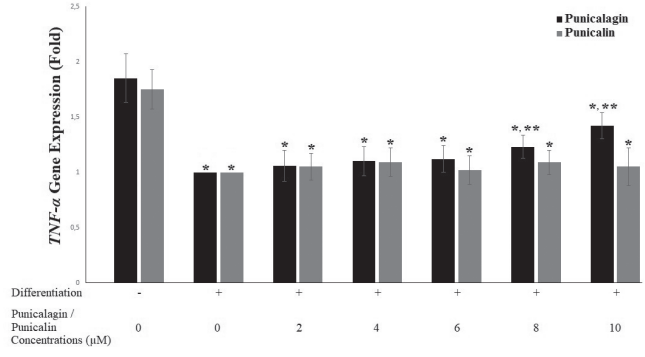

Figure 12. Effects of punicalagin and punicalin administration at different concentrations on relative TNF-α gene expression in 3T3-L1 cells. β-actin expression was used as an internal control. relative quantitation method was used to calculate expression changes and gene expression values in differentiated-untreated adipocytes were accepted as 1.00, and changes in gene expressions of the other cells were expressed as relative fold. Data are shown as the means ± SD (n = 4). (TNF-α; tumor necrosis factor-α).
* p<0.05 in comparison to undifferentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.
** p<0.05 in comparison to differentiated - untreated cells by one-way ANOVA followed by Tukey's test for multiple comparison.

adipocytes. Therefore, suppression of pre-adipocyte differentiation to adipocytes, thereby reducing the genesis and development of adipose tissue, may be useful to searching anti-obesity agents. The committed pre-adipocytes undergo initial alterations of cells and subsequent terminal differentiation into adipocytes, as well as changes in gene expression and the storage of lipids, and mature adipocytes can then undergo lipolysis changes under certain conditions (25).

The cellular and molecular mechanisms of adipocyte differentiation have been extensively studied using pre-adipocyte culture systems. Primary adipocyte cell cultures most accurately represent normal *in vivo* conditions; however, insurmountable disadvantages exist. The foremost disadvantage of primary adipocyte cell culture is the heterogeneity of the cell population.

This creates differential cellular responses within the population to hormones and other various chemicals. Another disadvantage is the short survival time of adipocyte cells in primary culture, making any detailed biochemical studies extremely difficult to perform and evaluate (26). The use of secondary cell lines in culture provides a homogeneous cell population with uniform response, allowing for system stability as well as reproducibility. Cell lines also provide a continuous source of large numbers of cells necessary for the study of proliferation and differentiation. The 3T3-L1 cell line was selected for this study because it is a well characterized adipocyte cell line which accurately reflects mature adipocytes *in vivo*. The 3T3-L1 cell culture system is considered an excellent model for the study of adipocyte growth and differentiation *in vitro* (27). During a research for naturally occurring anti-adipogenic products from medicinal plants, we observed anti-adipogenic activity of punicalagin and punicalin isolated from husk and leaves of pomegranate in 3T3-L1 cells.

Zou *et al.* (27) showed that pomegranate extract was effective in preventing obesity-induced fatty liver disease, Cao *et al.* (29) reported that the application of pomegranate extract to obese rats did not cause a change in the total fat mass but caused a decrease in body weight, and Mousavinejad *et al.* (30) observed that application of pomegranate peel extract reduced the body weight, body fat content and nutrient uptake of mice fed high fat diet, and decreased triglyceride and total cholesterol levels and pancreatic lipase activity. Although many studies showing the anti-hyperlipidemic and anti-obesity effects of pomegranate were present in the literature, there were no studies showing the anti-adipogenic activity of punicalagin and punicalin.

Fat accumulation is correlated with systemic oxidative stress in humans and mice. Production of reactive oxygen species (ROS) increases selectively in the adipose tissue of obese mice, accompanied by increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and decreased expression of antioxidative enzymes. In addition, increased ROS levels are reported to be an important trigger for insulin resistance. Consistent with this, it has been shown that oxidative stress impairs glucose uptake in mature adipocytes and decreases insulin secretion from pancreatic β-cells. ROS generation during adipogenesis has also been observed, and it has been proposed that insulin and insulin-like growth factor-I are active stimulators of NADPH-dependent hydrogen peroxide generation. Furthermore, ROS facilitate adipocyte differentiation by accelerating mitotic clonal expansion (31). On the other hand, punicalagin and punicalin are very strong antioxidant molecules and considered as agents capable of restraining the effect of ROS in the cell (32). Therefore, we think that punicalagin and punicalin can suppress the mitonic clonal expansion in adipocytes and suppress adipogenesis process by reducing intracellular ROS levels.

Firstly, we determined the non-cytotoxic concentrations of punicalagin and punicalinin by using MTT test. While punicalagin and punicalin at concentrations of 2-10 μM did not show a cytotoxic effect, application of higher concentrations led to an excessive increase in cell death. In our study, we also calculated the IC10 and IC50 value of punicalagin and punicalin on 3T3-L1 cells. IC10 value of punicalagin and punicalin as 2.83 μM and 15.84 μM, respectively. IC50 value of punicalagin and punicalin as 248.07 μM and 385.53 μM, respectively. Hsu and Yen (33) found the IC50 of quercetin as 40.4 μM and capsaicin as 45 μM, Lee *et al.* (34) found the IC50 of shikonin as 1.1 μM, and Takahaski *et al.* (35) found the IC50 of carnosic acid as to be 0.86 μM. The cytotoxic effect of punicalagin and punicalin was much less than the cytotoxicity of the substances used in other studies. This shows that punicalagin and punicalin can be safely used without causing too much cell death.

LDH release assay was performed for measuring the cytotoxicity after the differentiation process. LDH release assay is the most widely used enzyme release cytotoxicity test *in vitro* models (36). The cytotoxic effect of punicalagin and punicalin in differentiated cells was found to be higher than the cytotoxicity in undifferentiated cells, due to the fact that increased triglyceride and fat vacuoles in differentiated adipocytes made the cells more fragile. The application of punicalagin and punicalin at different concentrations to differentiated cells decreased the cell death in a dose-dependent manner. We have predicted that this situation is due to the decrease in the amount of triglycerides and fat vacuoles in adipocytes and the decrease in cell fragility. We observed a slight increase in cell death after 10 μM punicalagin and 8 and 10 μM punicalin applications and we have thought that high dose punicalagin and punicalin applications may have a negative effect on cell viability.

In our study, Oil Red O staining technique was used for the determination of cellular morphology and intracellular triglyceride storage. A significant increase in triglyceride content of adipocytes after

differentiation has been observed. Furthermore, undifferentiated adipocytes were small, hollow, and numerous, but after differentiation, the volume of the cells and the triglyceride contents were increased and the numbers decreased. We believe that the number of undifferentiated adipocytes were high due to the proliferation activity of these cells, the decrease in the number of differentiated fat cells was due to the fact that the differentiated fat cells lost their capacity of cell division and became more fragile and easily damaged due to the increase in lipid content. Triglyceride accumulation from dietary sources and endogenous lipogenic signalling increase the size of adipocytes, whereas increased proliferation and differentiation increase the adipocyte number. Therefore, treatments that modulate both the number and size of adipocytes could be a fundamental therapeutic approach for obesity remedies (37). The application of punicalagin and punicalin at different concentrations to differentiated cells decreased the triglyceride content in differentiated adipocytes in a dose-dependent manner, and also resulted in decreased cell size and intracellular triglyceride storage depending on concentration. This situation shows that punicalagin and punicaline reduced anabolic activity in differentiated cells. Application of 8 and 10 μM punicalagin and punicalin to mature fat cells significantly decreased the cell size and intracellular triglyceride content, so we have estimated that these concentrations may be the most effective ones.

In adipocyte differentiation studies, GPDH activity is frequently used as a marker of terminal differentiation. Pu and Veiga-Lopez (38) reported an increase in GPDH activity and increased triglyceride synthesis in adipocytes during the differentiation process. In the current study, GPDH activity of differentiated adipocytes was found to be higher than the undifferentiated ones. The application of punicalagin and punicalin to differentiated cells decreased the GPDH activity in a dose-dependent manner. This result proves that punicalagin and punicalin inhibit the differentiation of adipocytes. In addition, according to our results, it is also possible to say that the inhibitory effect of punicalagin on adipocyte differentiation is much higher than of punicalin. Furthermore, there was no significant decrease in the viability of 3T3-L1 cells treated with punicalagin and punicalin, therefore it is possible to say that the inhibitory effect of punicalagin and punicalin on adipocyte differentiation was independent of cytotoxicity.

It is known that the process of adipocyte differentiation is induced by transcriptional activation of adipose-specific genes. The transcription factors involved in the activation and suppression of transcription of genes involved in this process play an important role in the differentiation and maturation of adipocytes. Within 3 days following adipogenic stimulation, cells are finally separated from the cell cycle after being subjected to the last two mitotic divisions called clonal expansion and become suitable for differentiation. These cells begin to express the genes of late marker genes of differentiation (PPARγ, C/EBPα and SREBP-1c) from day 3. Following this time, the cells accumulate oil droplets within 5-7 days and begin terminal differentiation to form mature adipocytes. In this study, we have also investigated the effects of punicalagin and punicalin treatment at different concentrations on gene expression of transcription factors in mature adipocytes (39).

PPARγ is the most important transcription factor responsible for adipocyte differentiation (40). Many phytochemicals are natural agonists or antagonists of PPAR and may cause different effects on metabolism. (-) - catechin is a full agonist of PPARγ-dependent receptor genes, stimulating the differentiation of adipocytes from human bone marrow mesenchymal stem cells by increasing the expression of the target genes of PPAR (41). Genistein stimulates adipogenesis in 3T3-L1 cells by increasing gene expression of PPARγ (42). On the other hand, hydroxy citric acid found in the bark of *Garcinia cambogia*, capsaicin in hot pepper, curcumin in turmeric, resveratrol in grape, ethyl acetate extract of ginger rhizomes and ethanol extract of olive leaf blocks the adipogenesis by inhibiting the expression of PPARγ in 3T3-L1 cells (43). In the current study, PPARγ gene expression of differentiated adipocytes was found to be higher than of the undifferentiated cells. This demonstrates that 3T3-L1 cells have undergone complete terminal differentiation. The application of punicalagin and punicalin to differentiated cells decreased PPARγ gene expression in a dose-dependent manner. However, administration of punicalagin at a concentration of 6-10 μM and punicalin at a concentration of 8-10 μM significantly decreased PPARγ gene expression in the differentiated cells. This indicates that only high concentrations of punicalagin and punicalin sufficiently inhibit PPARγ gene expression.

C/EBPα and SREBP-1c are the other transcription factors exhibiting an increased activity during the terminal differentiation such as PPARγ. C/EBPα and SREBP-1c also regulates fatty acid synthesis and intracellular triglyceride storage (44,45).

In the current study, C/EBPα and SREBP-1c gene expressions of differentiated adipocytes were found to be higher than the undifferentiated cells. Only 6-10 μM punicalagin and 10 μM punicalin administrations reduced C/EBPα gene expression in the differentiated cells at the desired level. It has been found that no concentration of punicalin effectively reduced SREBP-1c expression among the studied concentrations. As seen, C/EBPα and SREBP-1c gene expressions have been inhibited only at high concentrations of punicalagine.

Breakdown of triglycerides in adipocytes and the release of fatty acids are essential for the regulation of energy homeostasis (46). Mature adipocytes may also undergo lipolysis and apoptotic cell death under certain conditions (47). Effects on lipolysis-associated target genes were assessed by measuring the transcriptional expression of perilipin A and HSL upon treatment of mature adipocytes with punicalagin and punicalin. In the curreny study, punicalagin, but not punicalin down-regulated the expression of HSL and perilipin A and up-regulated the expression of TNF-α in a dose-dependent manner. TNF-α increases adipocyte lipolysis and down-regulates the expression of the lipid droplet-associated protein, perilipin A, which is thought to modulate the accession of HSL to the surface of the fat droplet (48). The phosphorylation of HSL and perilipin A is critical in lipolysis, and the expression levels of perilipin A and HSL are a part of the lipolytic response (49). TNF-α can suppress expression and function of PPARγ, which is known to promote phosphorylation and down-regulation of perilipin A. These data suggest that punicalagin enhances the lipolytic response in mature adipocytes via modulation of lipolysis-associated gene expression.

In conclusion, the results showed that both punicalagin and punicaline prevented adipocyte differentiation and induced the lipolytic response, but punicalagine might be slightly more effective in this regard. Therefore, we think that punicalagin and punicalin can be useful in preventing obesity. Pomegranate peel extract, which contains these phytochemicals, can be useful in the treatment of obesity. How punicalagin and punicalin inhibit adipocyte differentiation by a cellular and molecular mechanism, and how it affects inflammation and oxidative stress in mature fat cells is unknown, so new studies are needed to clarify these issues.

Conflict of interest

The authors declare that they have no conflict of interest.

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