



Exploring the potential of pomegranate (*Punica granatum*) peel extract and punicalagin as novel anti-hypercholesterolemic agents

Parva Dehghani¹; Motahareh Masjedi², Laleh Shariati^{3,4}, Golnaz Vaseghi⁵, Nasim Dana⁶, Mehrdad Zeinalian⁷, Sedigheh Asgary^{8*}

¹Physiology Research Center, Department of Physiology, Isfahan University of Medical Sciences, Isfahan, Iran; ²Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran; ³Biosensor Research Center, School of Advanced Technologies in Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; ⁴Department of Biomaterials, Nanotechnology and Tissue Engineering, School of Advanced Technologies in Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; ⁵Applied Physiology Research Center, Cardiovascular Research Institute, Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; ⁶Physiology Research Center, Department of Physiology, Isfahan University of Medical Sciences, Isfahan, Iran; ⁷Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran; ⁸Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran

***Corresponding Author:** Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran.

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ABSTRACT

Background: Hypercholesterolemia, characterized by elevated low-density lipoprotein (LDL) cholesterol levels, is a significant risk factor for cardiovascular diseases. Familial and behavioral factors contribute to its onset, and while lifestyle changes offer some benefits, medical interventions are essential to manage this condition effectively. Statins, the primary medications used to lower LDL cholesterol, have limitations, including the unintended upregulation of proprotein convertase subtilisin/kexin type 9 (PCSK9). Therefore, exploring alternative approaches to enhance LDL receptor expression while targeting PCSK9 is crucial, with herbal medicine showing promise in this endeavor.

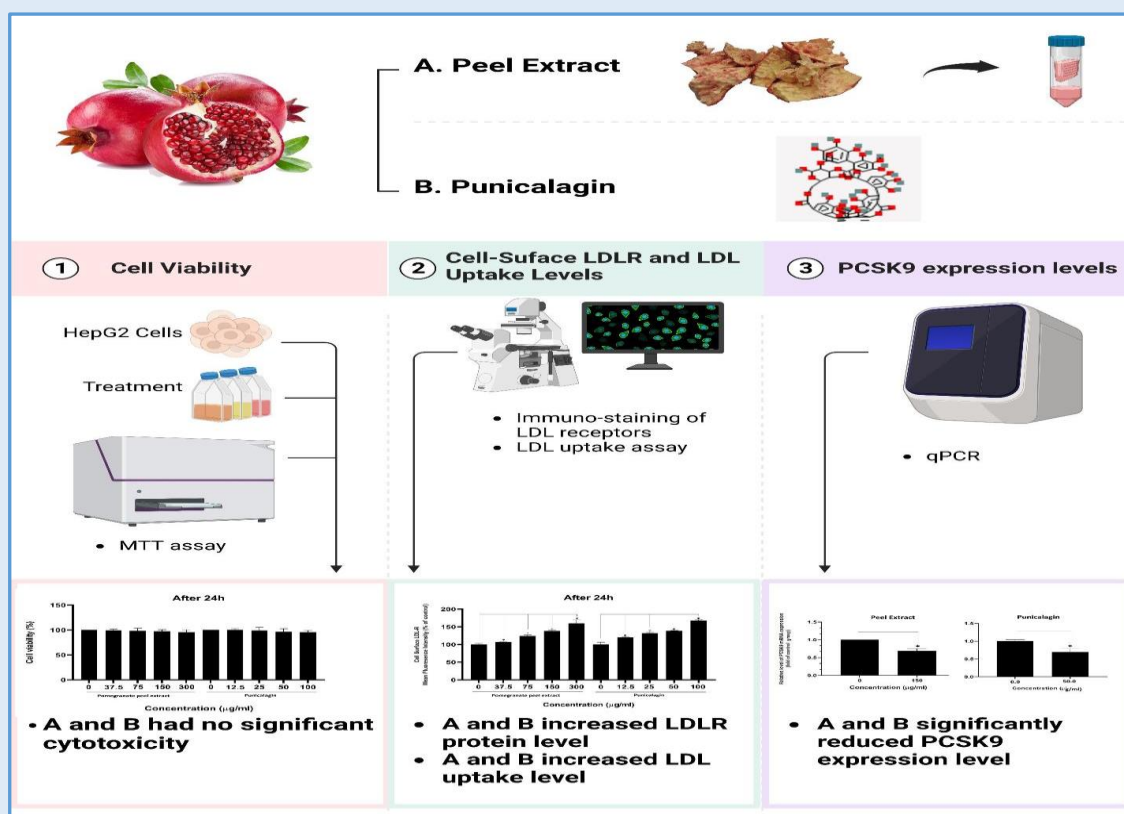
Objective: This study investigates the potential of pomegranate peel extract and punicalagin, a key pomegranate component, in lowering LDL cholesterol in HepG2 cells. The research explores their effects on LDL receptor (LDLR) expression, LDL uptake, and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene expression.

Methods: HepG2 cells were treated with different concentrations of pomegranate peel extract (37.5, 75, 150, and 300 $\mu\text{g/ml}$) and punicalagin (12.5, 25, 50, and 100 $\mu\text{g/ml}$). Next, the viability of HepG2 cells, the protein levels of LDL receptors and LDL uptake, and the expression levels of *PCSK9* were measured.

Results: Pomegranate peel extract (37.5, 75, 150, and 300 $\mu\text{g/ml}$) and punicalagin (12.5, 25, 50, and 100 $\mu\text{g/ml}$) did not exhibit significant cytotoxicity and significantly increased LDLR protein levels ($P\text{-value}= 0.00$, after 24 hours) and LDL cholesterol uptake ($P\text{-value}= 0.00$, after 24 hours). Furthermore, they exhibited a dose-dependent reduction in the expression of *PCSK9* (pomegranate: $P\text{-value}= 0.01$; punicalagin: $P\text{-value}= 0.00$).

Conclusion: These findings suggest that pomegranate peel extract and punicalagin have the potential to serve as effective agents in managing hypercholesterolemia by enhancing LDL receptor expression and reducing *PCSK9* expression in HepG2 cells. Further research is required to explore their effects on transcription factors and *PCSK9* function.

Keywords: Pomegranate; Punicalagin; HepG2 cell; LDL; Cardiovascular; *Punica granatum*



INTRODUCTION

Hypercholesterolemia, among the most important risk factors contributing to the development of cardiovascular diseases (CVDs), is characterized by a marked increase in low-density lipoprotein (LDL) cholesterol levels, as well as either standard or lower levels of high-density lipoprotein (HDL) cholesterol in the blood [1]. Factors exacerbating the condition include familial hypercholesterolemia, diabetes, high blood pressure, smoking, and unhealthy eating habits [2]. Most of these risk factors, including familial hypercholesterolemia, cannot be addressed by changing lifestyle. For this reason, medical interventions are required to manage the condition and reduce mortality and morbidity [3].

The first-line medication is statins, which are safe and can reduce LDL-C levels by 30% [4]. Additionally, statins could increase proprotein convertase subtilisin/kexin type 9 (PCSK9) production in hepatic cells, which binds to LDL receptors (LDLRs) and degrades them proteolytically. This, in turn, causes the blood to contain more LDL cholesterol [5-6]. It is, therefore, a priority to identify new anti-hypercholesterolemic agents that increase LDLR expression on cell surfaces while

simultaneously reducing PCSK9 expression in the blood.

There are promising therapeutic options in herbal medicine for hypercholesterolemia [7-8]. According to mounting research, different herbs are proven to lower cholesterol [9–11]. In recent studies, it has been demonstrated that the fruit pomegranate (*Punica granatum* Linn.) holds substantial potential for the food industry, including its ability to manage inflammation, ulcers, brain ischemia, high blood cholesterol, and high blood sugar levels [12–14]. Additionally, researchers have found punicalagin, gallic acid, anthocyanin, and ellagic acid in traditional pomegranate waste components, such as peel, flower, and seed [15] (Figure 1). The food industry can benefit from an untapped resource by extracting and utilizing health-beneficial components from these waste parts.

Research suggests that punicalagin, a key component of pomegranate extract, contributes to lipid metabolism balance and mitigates metabolic disorders [16-17]. Pomegranate active compounds, therefore, hold potential as novel anti-hypercholesterolemic agents for food if we explore the molecular mechanisms underlying their effects on reducing LDL cholesterol levels.

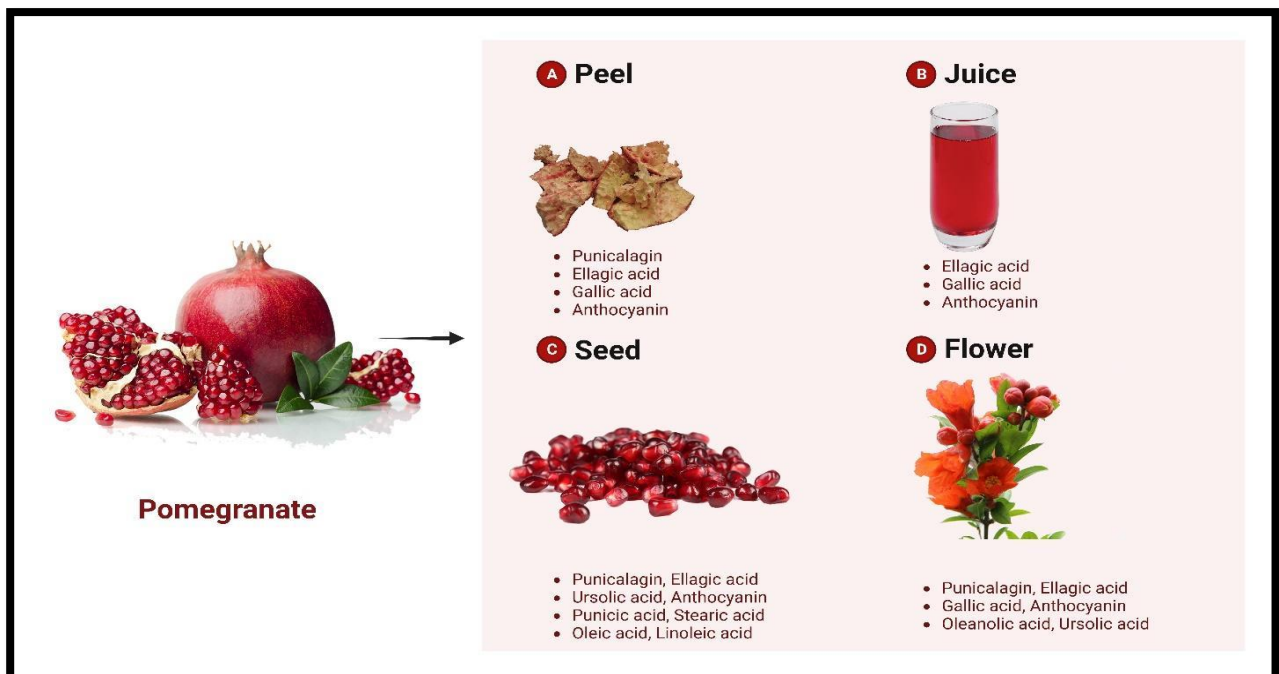


Figure 1. Active components in various parts of the pomegranate.

Therefore, this study was designed to assess the effects of various concentrations of pomegranate peel extract and punicalagin on HepG2 cells. Specifically, we will evaluate whether these substances could exert their LDL-lowering effect by increasing the LDL receptor expression while decreasing PCSK9 gene expression. By uncovering the health-beneficial compounds within pomegranate waste parts, this research enhances the value of pomegranate among other functional foods, highlighting its potential as a sustainable and health-promoting ingredient.

METHODS

Reagents: GIBCO (BRL Life Technologies, USA) supplies phosphate-buffered saline (PBS). Chemicals used in this study include punicalagin (PubChem ID: 16129869), dimethyl sulfoxide (DMSO), and 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Thermo Fisher Scientific, Inc. (Rockford, IL, USA) provided Roswell Park Memorial Institute Medium (RPMI-1640) and Gibco fetal bovine serum (FBS).

Preparation of Peel Extract: Air-drying and crushing fresh pomegranate peels were undertaken. Afterward, 100 g of crushed particles were macerated in 500 ml of 80% (v/v) ethanol in the dark for 72 hours. Whatman No.1 filter paper was used to remove particles from the extracts. The solvent was evaporated by a mini-rotary evaporator (N-N series, EYELA) at 40°C under vacuum. Finally, semi-dried extracts (10-13% moisture content) were stored at -20 °C until used.

Cell Culture: The National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) provided the human hepatoma cell line, HepG2. RPMI-1640, supplemented with 10% FBS, was used as the growth medium to maintain the desired growth rate. A humidified incubator (95%) with 5% (V/V) CO₂ was used to incubate the cells after they

were treated with 1% penicillin-streptomycin. The initial thawed vial of HepG2 cells was sub-cultured at 80-90% confluence. In assays, cultured cells were serum-starved for 24 h before being incubated for 24/48 h in different concentrations of peel extract (37.5 µg/ml, 75 µg/ml, 150 µg/ml, and 300 µg/ml), punicalagin (12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml), or DMSO (0.1% as a control).

HepG2 Cells Viability Assay: The viability of the cells was quantified by MTT colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). The culture medium was removed when HepG2 cells reached 80-90% confluence in a 96-well plate (1×10⁴ cells/well). Various concentrations of peel extract (37.5 µg/ml, 75 µg/ml, 150 µg/ml, and 300 µg/ml), punicalagin (12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml), or DMSO was used to treat cells for 24 h and 48 h. The plates were incubated at 37°C for four hours with MTT labeling reagent (final concentration 1 mg/ml). DMSO was then used to dissolve the formazan crystals after removing the media. Shaking the plates for 20 minutes was performed. An ELISA reader (BioTek, USA) was used to measure optical density (OD) at 570 nm for each well, and cell viability was reported as the percentage of non-treated controls as follows:

$$\text{Percentage of cell viability} = \frac{(A_{\text{Treatment}} - A_{\text{Blank}})}{(A_{\text{Control}} - A_{\text{Blank}})} \times 100$$
 where A= absorbance.

Parallel experiments were conducted by three independent groups, and the results were expressed as mean ± SD.

LDL Uptake: An LDL uptake cell-based kit (Abnova, Taiwan) was used to quantify LDL uptake in peel extract/punicalagin-treated HepG2 cells. Cell treatments were done as described earlier. Then, the culture medium was replaced with diluted LDL-DyLight™ 550 working solution in a serum-free medium. Cell nuclei were counterstained by adding Hoechst 33342 directly to wells at a final concentration of 5 mM after 6 h incubation at 37 °C. After 30 minutes, the PBS was used twice for 5

minutes each to wash the cells after removing the medium. Using a plate reader capable of detecting excitation (540 nm) and emission (570 nm) wavelengths, the fluorescence intensity of each well was measured. Images were captured at 200 X by a fluorescent microscope. LDL receptors were then detected on the stained cells.

Immunocytochemical Staining of LDL Receptor

A dark room was used during all procedures to ensure that LDL-DyLight™ 550 staining remained intact (Abnova, Taiwan). Tris-Buffered Saline (TBS, pH 7.4) was used to wash the cells after removing the culture medium from the wells. A fixative solution was applied for 10 minutes, followed by three 5-minute washings with TBS. Cells were incubated with Rabbit Anti-LDLR primary antibody at room temperature for 1 hour after being blocked for 30 minutes using 100 µL/well blocking solution. The plates were washed three times with TBS, then diluted DyLight™ 488-Conjugated Secondary Antibody was added and kept at 4 °C in darkness. After the previous washing steps, the cells were washed three times with TBST (Tris-buffered saline with Tween 20) for 5 minutes each time. The cells were then observed under a fluorescent microscope (IX70, Olympus) equipped with specific filter sets to detect fluorescein (excitation/emission = 485/535 nm) and rhodamine (excitation/emission = 540/570 nm). To quantify the images, Image J software (NIH, USA) was utilized for analysis. The corrected total cell fluorescence (CTCF) was calculated using Equation (1).

$$\text{CTCF} = \text{ID} - (\text{Area of SC} \times \text{MFBR}) \quad (1)$$

Where ID is the integrated density, SC refers to the

selected cell, and MFBR denotes the mean fluorescence of background readings.

The Mean fluorescence intensity (MFI) was calculated using Equation (2) in the next step.

$$\text{MFI} = \text{CTCF}/\text{Area} \quad (2)$$

PCSK9 Expression Quantification by qRT-PCR Analysis

In this study, HepG2 cells were examined for PCSK9 mRNA expression using quantitative reverse transcription PCR (qRT-PCR). Cells were treated with pomegranate peel extract (150 µg/ml) / punicalagin (50 µg/ml) and DMSO for 24 h and 48 h. The total RNA was extracted from cells using the “EXTRACTME RNA & DNA” kit (EM15, Blirt). A high-capacity kit (Biofact, China) was used to reverse-transcribe 1 microgram of total RNA into cDNA. Using a Nanodrop (Nanodrop Technologies, Wilmington, DE), cDNA quality was confirmed. Real-time PCR was performed with a 20 µl total volume of Maxima SYBR Green/ROX qPCR Master Mix (Biofact, China), cDNA, and gene-specific primers (Table 1). The PCR amplification was conducted in a StepOne™ Real-Time PCR system (Applied Biosystems, USA). The following conditions were used: 95 °C for 15 min, 45 cycles of 95 °C for 20 sec, and 60 °C for 1 min. Lastly, agarose gel electrophoresis was used to confirm the quality of amplicons. Each sample was examined three times. The relative quantitation method (2-ΔΔCT) was used to determine the fold change in gene expression, having glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control.

Table 1. qRT- PCR primer sequences.

Gene	Forward	Reverse
GAPDH	5'-ATGGGGAAGGTGAAGGTCG-3'	5'-GGGGTCATTGATGGCAACAATA-3'
PCSK9	5'-TCAGCTCCCAGGTCATC-3'	5'-TCGGCAGACAGCATCATG-3'

Statistical Analysis: Statistical analysis and data presentation were performed using SPSS Statistics

(version 21.0, IBM, US) and GraphPad Prism (version 9.2, GraphPad Software, CA, USA), respectively. All data were

analyzed using one-way analysis of variance (ANOVA), followed by an LSD posthoc test to determine specific differences between the groups. Means \pm standard deviations were used to express the results. The results were considered statistically significant if the P-value was less than 0.05.

RESULTS

Viability of HepG2 Cells in Response to Pomegranate Peel Extract and Punicalagin:

To assess the potential cytotoxic effects of pomegranate peel extract and punicalagin on HepG2 cells, various concentrations of

peel extract (37.5 $\mu\text{g/ml}$, 75 $\mu\text{g/ml}$, 150 $\mu\text{g/ml}$, and 300 $\mu\text{g/ml}$) and punicalagin (12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$) were administered to the cells for 24 and 48 hours. The MTT assay was performed to determine the viability of the cells. The findings revealed that after 24 hours of treatment, the peel extract and punicalagin exhibited no noticeable cytotoxicity. However, after 48 hours, the concentration of 300 $\mu\text{g/ml}$ for peel extract and 100 $\mu\text{g/ml}$ for punicalagin resulted in a reduction in cell viability to 89.46% and 90.43%, respectively (Figure 2).

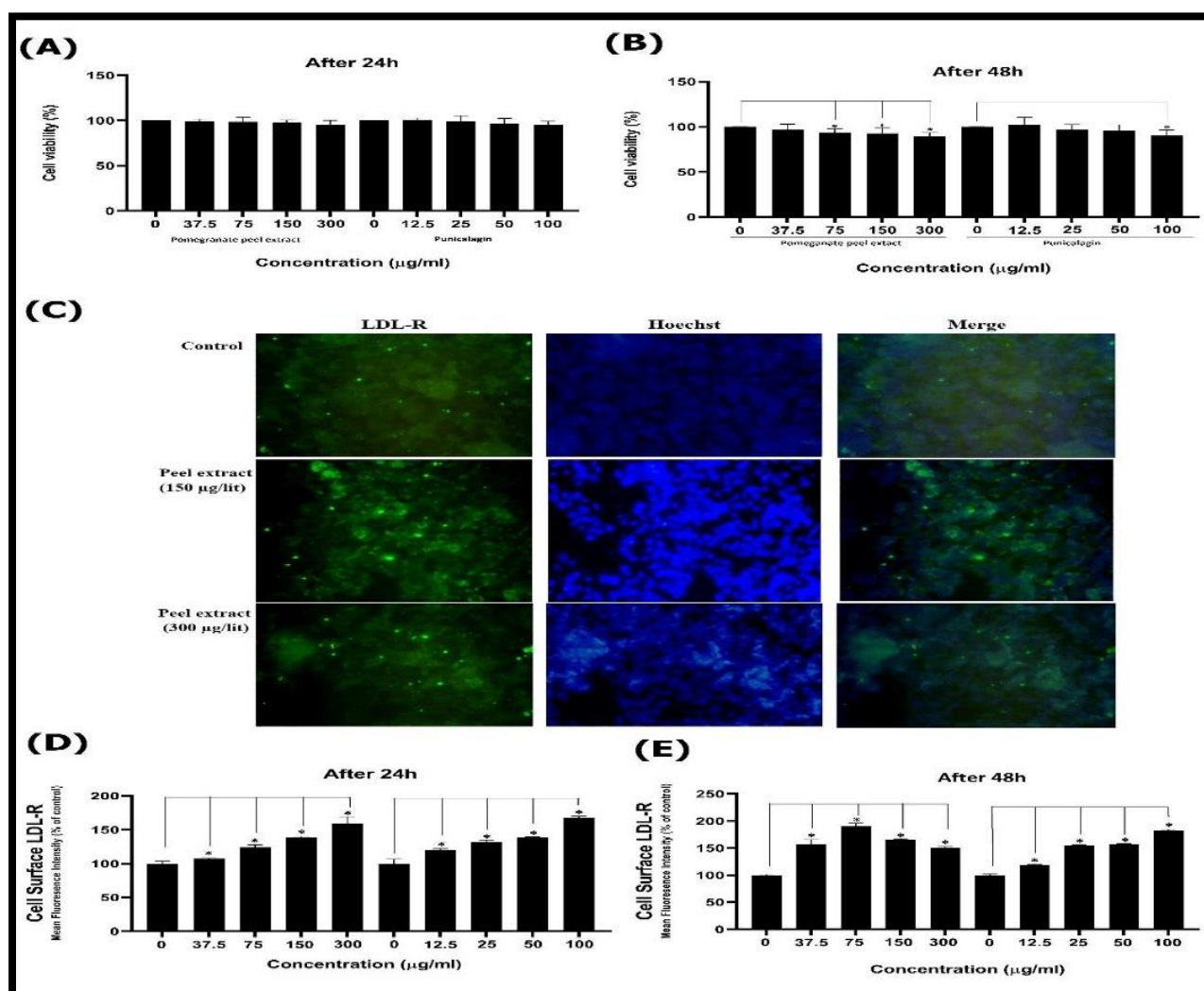


Figure 2. (A) Viability of HepG2 cells treated with peel extract (37.5 $\mu\text{g/ml}$, 75 $\mu\text{g/ml}$, 150 $\mu\text{g/ml}$, and 300 $\mu\text{g/ml}$) and punicalagin (12.5 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$) for 24 h and (B) 48 h. The MTT assay was used to measure cell viability. (C) The effects of pomegranate peel extract on the number of cell-surface LDLRs after 48 h. The extract increases the number of LDLRs on the cell membrane (magnification $\times 200$). (D) Effects of peel extract and punicalagin on LDLR expression in HepG2 cell surfaces: A 24-hour and a (E) 48-hour treatment was performed on the cells. The expression of LDLR was significantly higher in the experimental group than in the control group. The LDLR expression was detected by immunofluorescence assay. Three independent experiments are presented statistically as a mean \pm standard deviation. To determine whether there was a significant difference between the control group and the experimental group, a P-value < 0.05 was used, denoted by *.

Effects of Pomegranate Peel Extract and Punicalagin on LDLR levels and LDL Uptake in HepG2 Cells: LDL receptors are oligomeric surface glycoproteins that promote LDL uptake from plasma and degradation in hepatocytes, thus contributing significantly to LDL clearance. Therefore, increasing LDLR levels and LDL uptake on cell surfaces could be a reliable indicator of a compound's ability to decrease LDL cholesterol in hepatic cells. We explored LDLR expression and LDL uptake levels in pomegranate peel extract/punicalagin-treated HepG2 cells. According to immunofluorescence analysis, LDLRs increased significantly with increasing concentration after 24 h and 48 h of treatment (Figure 2).

Afterward, we examined the impact of peel extract and punicalagin on LDL uptake in HepG2 cells after 24 and 48 hours of treatment. To do so, treated cells were labeled with LDL-DyLight™ 550, and after 3-4 h incubation, their fluorescent intensity was investigated by a confocal microscope (Figure 3). A concentration-dependent increase in LDL uptake was observed in Hep2G cells treated with peel extract, as shown in Figure 3. The increasing trend in LDL uptake with punicalagin, however, was interrupted after 48 h at a concentration of 100 µg/ml. Based on our findings, pomegranate peel extract and punicalagin can increase LDLR expression in HepG2 cells, thus increasing LDL cholesterol uptake.

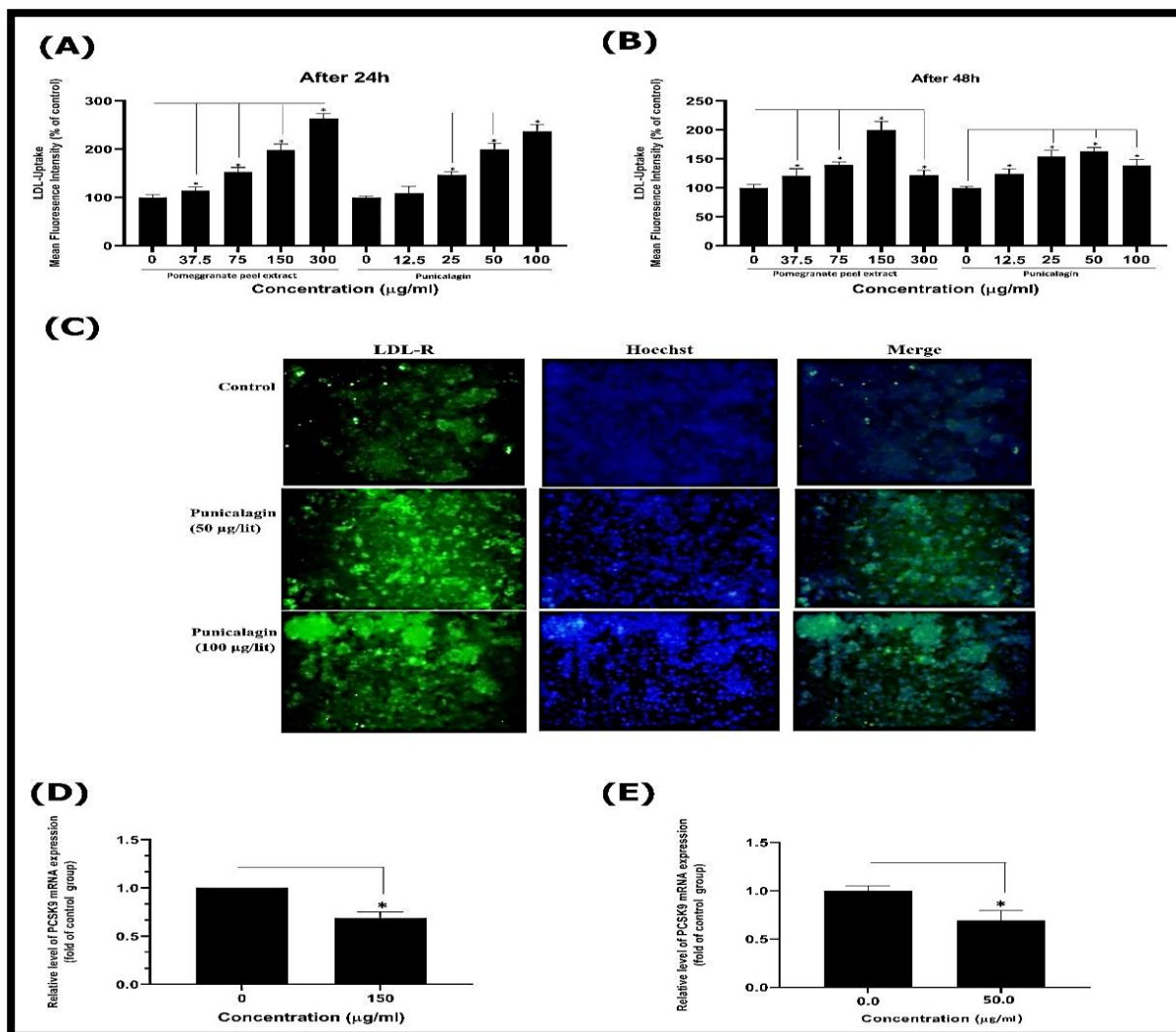


Figure 3. (A) Effect of pomegranate peel extract/punicalagin on LDL uptake in HepG2 cells after 24 h and (B) 48 h incubation. Both increase the uptake of fluorescent-labeled LDL in a concentration-dependent manner. (C) Effects of punicalagin on the number of cell-surface LDLRs after 24h. As shown in the figure, the green fluorescence intensity increased with the increasing punicalagin concentrations (magnification ×200). (D) Effects of pomegranate peel extract (150 µg/ml) and (E) punicalagin (50 µg/ml) on PCSK9 mRNA expression levels after 48 h. The figure shows that both compounds decrease PCSK9 expression levels in HepG2 cells. The expression levels were detected by analysis of real-time PCR data. *P<0.05 was considered statistically significant.

Effect of Pomegranate Peel Extract and Punicalagin on PCSK9 mRNA Expression in HepG2 Cells:

PCSK9 negatively regulates the expression of LDLRs on the surfaces of hepatic cells, so we examined how pomegranate peel extract and punicalagin affect PCSK9 gene expression in HepG2 cells. Cell treatments were done by DMSO, 150 µg/ml peel extract, and 50 µg/ml punicalagin for 48 h. Different treatments normalized all results to GAPDH, a stable housekeeping gene. Peel extract and punicalagin-treated HepG2 cells showed significant reductions in PCSK9 mRNA levels by 68.70% and 69.59%, respectively, compared to the control.

DISCUSSION

Over the centuries, pomegranate, belonging to the *Punica* genus, has been consumed worldwide for many years. There have been continuous increases in pomegranate production and consumption; its production in 2017 was estimated at 3.8 million tons [18]. As a valuable by-product, pomegranate peel contains a wide range of bioactive compounds, such as vitamins, minerals, flavonoids, and polyphenols, with a broad range of biological activities and health benefits [19-20]. The abundance of beneficial substances in pomegranate peel extracts has been demonstrated in various studies. Furthermore, these extracts possess antibacterial and anticancer properties in addition to being anti-inflammatory [21-23]. Furthermore, their presence is known to reduce the risk of cardiovascular disease [13,24].

In a research study conducted by Sadeghipour et al. (2014), the antilipidemic effects of pomegranate peel extract (80% v/v hydroethanol) were investigated in male rats fed a high-fat diet. The rats were administered various concentrations of the extract via intraperitoneal injection. To this, factors like the levels of serum cholesterol, triglycerides, LDL, and HDL, as well as the

activity of alkaline phosphatase (AP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured in rats. Comparing pomegranate peel extract treatment with a saline control group, the authors found that these factors decreased. As a result, they recommended pomegranate as a suitable candidate for future studies on dyslipidemia [25].

In a review by Hou et al. (2019), the authors explored the advantageous effects of various pomegranate fractions (including peel, seeds, juice, and flowers) on lipid metabolism in metabolic disorders. This study reviewed studies examining pomegranate's lipid-lowering effects in atherosclerosis and nonalcoholic fatty liver disease. They emphasized that pomegranate extracts regulate lipid metabolism by combating oxidative stress and inflammation. While the review provides a helpful basis for researching and developing pomegranate-related drugs or nutrients, there is no information about molecular mechanisms underlying the lipid-lowering process in hepatic cells [13].

Our findings shed light on previous studies showing the LDL cholesterol-lowering effects of pomegranate. This study aimed to investigate the ability of pomegranate peel extract and its main constituent, punicalagin, to reduce LDL cholesterol (LDL-C). To this, their effects on LDLR expression levels, LDL-uptake, and PCSK9 mRNA levels, as the main molecular biomarkers for cholesterol metabolism, were explored. According to the cell viability results, pomegranate peel extract and punicalagin have no cytotoxic effects on HepG2 cells. Compared to the control, 300 µg/ml of peel extract and 100 µg/ml of punicalagin resulted in a mild reduction in cell viability by 89.46% and 90.43%, respectively. The findings for pomegranate peel extract revealed that it effectively increased the levels of LDLR protein in a time- and dose-dependent manner after 24 hours. However,

after 48 hours, this increasing trend was altered for 150 and 300 µg/ml concentrations. Not surprisingly, such a trend could be seen for LDL-C uptake in pomegranate peel extract-treated HepG2 cells. Treating HepG2 cells with different concentrations of punicalagin shows similar results, too. In HepG2 cells, both pomegranate peel extract and punicalagin reduce PCSK9 expression. Based on the results, punicalagin and pomegranate peel extract improved hypercholesterolemia in HepG2 cells by increasing LDLR levels and downregulating PCSK9 expression.

Landmark discoveries in human studies have revealed the crucial roles of LDLR and PCSK9 in maintaining cholesterol homeostasis [26–28]. LDLRs located on the surface of hepatic cells interact with lipoproteins containing apoB100 and apoE, thereby reducing circulating LDL cholesterol levels [29]. Based on mechanistic investigations, it has been shown that LDLRs are degraded in lysosomes by PCSK9 binding to the epidermal growth factor precursor homology domain A (EGF-A) of LDLRs. This leads to decreased LDLR clearance and improved cardiovascular events [30]. Therefore, activated PCSK9 and the molecular mechanisms underlying its expression and cell surface LDLRs are considered significant drug targets in CVD medicine [31]. As a result of our findings, we can conclude that both pomegranate peel extract and its principal constituent, punicalagin, have the potential to serve as promising candidates for upregulating LDLRs and downregulating PCSK9 mRNA expression.

This study had some limitations. It should be noted whether pomegranate peel extract and punicalagin affect HNF1α and SREBP2, transcriptional factors that regulate the expression of the LDLR and PCSK9 genes. The focus of this study was primarily on evaluating the LDLR expression and LDL uptake in HepG2 cells, as well as the impact on PCSK9 mRNA expression. Further research is necessary to investigate the potential influence of

pomegranate peel extract and punicalagin on HNF1α and SREBP2 in regulating LDLR and PCSK9 gene expression. Furthermore, it would be valuable to elucidate the molecular interactions between pomegranate peel extract, punicalagin, and active PCSK9 to assess their inhibitory potential on PCSK9 activity. Understanding how pomegranate constituents interact with PCSK9 could provide insights into their ability to modulate PCSK9 function and further support their potential as therapeutic agents for managing LDL cholesterol levels. Additional research in this area would gain an in-depth understanding of the mechanisms involved. Furthermore, it could aid in the development of PCSK9-targeted interventions.

This study concludes by showing that pomegranate peel extract and punicalagin exhibit hypolipidemic effects on HepG2 cells. Furthermore, it was observed that neither pomegranate peel extract nor punicalagin had cytotoxic effects on HepG2 cells. Additionally, their treatment increased LDLR expression on HepG2 cell surfaces, resulting in an increased uptake of LDL. Additionally, pomegranate peel extract and punicalagin downregulated PCSK9 mRNA expression levels in HepG2 cells. Based on these results, it can be inferred that pomegranate peel extract and punicalagin can potentially serve as functional ingredients to help improve circulating LDL cholesterol levels. Additionally, their beneficial effects could contribute to the added value of the pomegranate industry.

CONCLUSION

In conclusion, our research underscores the significant potential of pomegranate peel extract and punicalagin in improving lipid metabolism. With pomegranate peel rich in bioactive compounds, our study reveals its ability to enhance LDL uptake and reduce PCSK9 mRNA levels in HepG2 cells. Notably, neither pomegranate peel extract nor punicalagin exhibited cytotoxic effects on these cells

(except for 300 µg/ml of peel extract and 100 µg/ml of punicalagin after 48h). Moreover, their treatment increased LDLR protein in a time- and dose-dependent manner after 24 hours. However, after 48 hours, this trend was altered at higher concentrations. The same trend could be seen for LDL-C uptake in treated HepG2 cells. These findings are particularly relevant given the global prevalence of dyslipidemia and its association with cardiovascular disease. While our study focused on molecular biomarkers, further research is warranted to elucidate the underlying mechanisms and potential therapeutic implications. Overall, our findings support the utilization of pomegranate peel extract and punicalagin as functional ingredients to mitigate dyslipidemia and enhance cardiovascular health, thus offering promising avenues for future research and food industry application.

List of Abbreviation: CTCF: Corrected total cell fluorescence; CVD: Cardiovascular Diseases; EGF-A: Epidermal Growth Factor Precursor Homology Domain; FBS: Fetal bovine serum; HDL: High-density lipoprotein; HepG2: Hepatoma G2; HNF1α: Hepatocyte nuclear factor 1α; LDL: Low-density lipoprotein; LDL-C: Low-density

lipoprotein cholesterol; LDLR: Low-density lipoprotein Receptor; MFI: Mean fluorescence intensity; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCSK9: Proprotein convertase subtilisin/kexin type 9; RPMI-1640: Roswell Park Memorial Institute- 1640; RT-qPCR: Reverse transcription quantitative real-time PCR; SREBP: Sterol regulatory element binding protein; TBS: Tris-buffered saline; TC: Total cholesterol

Declaration of Competing Interest: The authors declare that they have no competing interests.

Author contributions: Parva Dehghani: Analyzed the data, wrote the initial draft, edited the manuscript, and designed the figures; Motahareh Masjedi: performed the in vitro experiments; Laleh Shariati: validated and analyzed the data; Golnaz Vaseghi: validate, and analyze the data; Nasim Dana: perform the in vitro experiments; Mehrdad Zeinalian: validate and analyze the data; Sedigheh Asgary: design and direct the study, supervise the project, acquire funds, and revise the manuscript.

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REFERENCES

1. Glavinovic T, Thanassoulis G, de Graaf J, Couture P, Hegele RA, Sniderman AD. Physiological Bases for the Superiority of Apolipoprotein B Over Low-Density Lipoprotein Cholesterol and Non-High-Density Lipoprotein Cholesterol as a Marker of Cardiovascular Risk. *J Am Heart Assoc* 2022,11: e025858. DOI: <https://doi.org/10.1161/JAHA.122.025858>.
2. Stone NJ, Smith SC, Orringer CE, Rigotti NA, Navar AM, Khan SS, Jones DW, et al. Managing Atherosclerotic Cardiovascular Risk in Young Adults: JACC State-of-the-Art Review. *J Am Coll Cardiol*,2022,79:819–36. DOI: <https://doi.org/10.1016/j.jacc.2021.12.016>.
3. Krzemińska J, Młynarska E, Radzioch E, Wronka M, Rysz J, Franczyk B. Management of Familial Hypercholesterolemia with Special Emphasis on Evinacumab. *Biomedicines* 2022,10. DOI: <https://doi.org/10.3390/biomedicines10123273>.
4. Strilchuk L, Fogacci F, Cicero AF. Safety and tolerability of injectable lipid-lowering drugs: an update of clinical data. *Expert Opin Drug Saf* 2019,18:611–21. DOI: <https://doi.org/10.1080/14740338.2019.1620730>.
5. Waiz M, Alvi SS, Khan MS. Potential dual inhibitors of PCSK-9 and HMG-R from natural sources in cardiovascular risk management. *EXCLI J* 2022,21:47–76. DOI: <https://doi.org/10.17179/excli2021-4453>.
6. Lagace TA. PCSK9 and LDLR degradation: regulatory mechanisms in circulation and in cells. *Curr Opin Lipidol* 2014,25:387–93. DOI: <https://doi.org/10.1097/MOL.0000000000000114>.
7. Tadevosyan A, Hakobjanyan A, Tovmasyan A, Asatryan A, Roosta HR, Daryadar M. Hypoglycemic and hypolipidemic activity of moringa grown in hydroponics and soil in Ararat

- Valley. *Funct Foods Heal Dis* 2023,13:398–408.
DOI: <https://doi.org/10.31989/ffhd.v13i8.1158>.
8. Thomford NE, Senthebane DA, Rowe A, Munro D, Seele P, Maroyi A, Dzobo K. Natural Products for Drug Discovery in the 21st Century: Innovations for Novel Drug Discovery. *Int J Mol Sci* 2018,19. DOI: <https://doi.org/10.3390/ijms19061578>.
 9. Rauf A, Akram M, Anwar H, Daniyal M, Munir N, Bawazeer S, Bawazeer S, et al. Therapeutic potential of herbal medicine for the management of hyperlipidemia: latest updates. *Environ Sci Pollut Res* 2022,29:40281–301.
DOI: <https://doi.org/10.1007/s11356-022-19733-7>.
 10. Zibaeenezhad MJ, Elyaspour Z. Effects of Nut Consumption on Cardiovascular Risk Factors and Coronary Heart Diseases. *Funct Foods Heal Dis* 2022,12:639–65.
DOI: <https://doi.org/10.31989/ffhd.v12i11.1022>.
 11. Natale F, Molinari R, Covino S, Piccinocchi G, Salvetti A, Monda E, Limongelli G, et al. Effectiveness in the short-term of a novel nutraceutical for the management of hypercholesterolemia: an observational multicenter primary care experience. *Funct Foods Heal Dis* 2022,12:627–38.
DOI: <https://doi.org/10.31989/ffhd.v12i11.1023>.
 12. Zarfeshany A, Asgary S, Javanmard SH. Potent health effects of pomegranate. *Adv Biomed Res* 2014,3:100.
DOI: <https://doi.org/10.4103/2277-9175.129371>.
 13. Hou C, Zhang W, Li J, Du L, Lv O, Zhao S, Li J. Beneficial Effects of Pomegranate on Lipid Metabolism in Metabolic Disorders. *Mol Nutr & Food Res* 2019,63:1800773. DOI: <https://doi.org/10.1002/mnfr.201800773>.
 14. Colombo E, Sangiovanni E, Dell'agli M. A review on the anti-inflammatory activity of pomegranate in the gastrointestinal tract. *Evid Based Complement Alternat Med* 2013,2013:247145.
DOI: <https://doi.org/10.1155/2013/247145>.
 15. Ranjha MMAN, Shafique B, Wang L, Irfan S, Safdar MN, Murtaza MA, Nadeem M, et al. A comprehensive review on phytochemistry, bioactivity and medicinal value of bioactive compounds of pomegranate (*Punica granatum*). *Adv Tradit Med* 2023,23:37–57.
DOI: <https://doi.org/10.1007/s13596-021-00566-7>.
 16. Giménez-Bastida JA, Ávila-Gálvez MÁ, Espín JC, González-Sarrías A. Evidence for health properties of pomegranate juices and extracts beyond nutrition: A critical systematic review of human studies. *Trends Food Sci Technol* 2021,114:410–23.
DOI: <https://doi.org/10.1016/j.tifs.2021.06.014>.
 17. Valero-Mendoza AG, Meléndez-Rentería NP, Chávez-González ML, Flores-Gallegos AC, Wong-Paz JE, Govea-Salas M, Zugasti-Cruz A, et al. The whole pomegranate (*Punica granatum* L), biological properties and important findings: A review. *Food Chem Adv* 2023,2:100153.
DOI: <https://doi.org/10.1016/j.focha.2022.100153>.
 18. Mo Y, Ma J, Gao W, Zhang L, Li J, Li J, Zang J. Pomegranate Peel as a Source of Bioactive Compounds: A Mini Review on Their Physiological Functions. *Front Nutr* 2022,9.
DOI: <https://doi.org/10.3389/fnut.2022.887113>.
 19. Akhtar S, Ismail T, Fraternali D, Sestili P. Pomegranate peel and peel extracts: Chemistry and food features. *Food Chem* 2015,174:417–25.
DOI: <https://doi.org/10.1016/j.foodchem.2014.11.035>.
 20. Marra F, Petrovicova B, Canino F, Maffia A, Mallamaci C, Muscolo A. Pomegranate Wastes Are Rich in Bioactive Compounds with Potential Benefit on Human Health. *Molecules* 2022,27.
DOI: <https://doi.org/10.3390/molecules27175555>.
 21. Chaves FM, Pavan ICB, da Silva LGS, de Freitas LB, Rostagno MA, Antunes AEC, Bezerra RMN, et al. Pomegranate Juice and Peel Extracts are Able to Inhibit Proliferation, Migration, and Colony Formation of Prostate Cancer Cell Lines and Modulate the Akt/mTOR/S6K Signaling Pathway. *Plant Foods Hum Nutr* 2020,75:54–62.
DOI: <https://doi.org/10.1007/s11130-019-00776-0>.
 22. Hikal WM, Said-Al Ahl HAH, Tkachenko KG, Mahmoud AA, Bratovcic A, Hodžić S, Atanassova M. An Overview of Pomegranate Peel: A Waste Treasure for Antiviral Activity. *Trop J Nat Prod Res* 2022,6:15–9.
DOI: <https://doi.org/10.26538/tinpr/v6i1.3>.
 23. Nicolson GL, Veljkovic V, Glisic S, Perovic V, Veljkovic N. Pomegranate (*Punica granatum*): a natural source for the development of therapeutic compositions of food supplements with anticancer activities based on electron acceptor molecular characteristics. *Funct Foods Heal Dis* 2016,6:769–87.
DOI: <https://doi.org/10.31989/ffhd.v6i12.289>.
 24. Asgary S, Karimi R, Joshi T, Kilpatrick KL, Moradi S, Samimi Z, Mohammadi E, et al. Effect of pomegranate juice on vascular adhesion factors: A systematic review and meta-analysis. *Phytomedicine* 2021,80:153359.
DOI: <https://doi.org/10.1016/j.phymed.2020.153359>.
 25. Sadeghipour A, Eidi M, Ilchizadeh KavGANI A, Ghahramani R, Shahabzadeh S, Anissian A. Lipid Lowering Effect of *Punica*

- granatum L. Peel in High Lipid Diet Fed Male Rats. Evid Based Complement Alternat Med 2014,2014:432650.
DOI: <https://doi.org/10.1155/2014/432650>.
26. Levy E, Ouadda ABD, Spahis S, Sane AT, Garofalo C, Grenier É, Emonnot L, et al. PCSK9 plays a significant role in cholesterol homeostasis and lipid transport in intestinal epithelial cells. *Atherosclerosis* 2013,227:297–306.
DOI: <https://doi.org/10.1016/j.atherosclerosis.2013.01.023>.
27. Duan Y, Gong K, Xu S, Zhang F, Meng X, Han J. Regulation of cholesterol homeostasis in health and diseases: from mechanisms to targeted therapeutics. *Signal Transduct Target Ther* 2022,7:265.
DOI: <https://doi.org/10.1038/s41392-022-01125-5>.
28. Goedeke L, Fernández-Hernando C. Regulation of cholesterol homeostasis. *Cell Mol Life Sci* 2012,69:915–30.
DOI: <https://doi.org/10.1007/s00018-011-0857-5>.
29. Devaraj S, Semaan JR, Jialal I. *Biochemistry, Apolipoprotein B.*, Treasure Island (FL): 2023.
30. Liu S, Yu T, Qiao Y, Gu S, Chai X. Research on Hepatocyte Regulation of PCSK9-LDLR and Its Related Drug Targets. *Chin J Integr Med* 2023.
DOI: <https://doi.org/10.1007/s11655-023-3545-z>.
31. Kuzmich N, Andresyuk E, Porozov Y, Tarasov V, Samsonov M, Preferanskaya N, Veselov V, et al. PCSK9 as a Target for Development of a New Generation of Hypolipidemic Drugs. *Molecules* 2022,27.
DOI: <https://doi.org/10.3390/molecules27020434>.