Toxicology Reports 1 (2014) 945–954

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# *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

Wei Zhao, Jianke Li*, Xiaoye He, Ou Lv, Yujiang Cheng, Run Liu

*College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, Shaanxi 710062, PR China*

ARTICLE INFO

*Article history:*
Received 14 August 2014
Received in revised form 15 October 2014
Accepted 17 October 2014
Available online 29 October 2014

*Chemical compounds studied in this article:*
Gallic acid (PubChem CID: 370)
Punicalagin (PubChem CID: 44584733)
Ellagic acid (PubChem CID: 5281855)
Epigallocatechin gallate (PubChem CID: 65064)
Phlorizin (PubChem CID: 6072).

*Keywords:*
Pomegranate peel polyphenols (PPPs)
Plant polyphenols
Hepatic cell
Lipid-lowering effects
Molecular mechanisms

ABSTRACT

This study was aimed to compare the relative activities of the purified pomegranate peels polyphenols (PPPs) with some other plant polyphenols including punicalagin, ellagic acid, gallic acid, phlorizin, and epigallocatechin gallate (EGCG) on the lipid metabolism regulation, and the cholesterol efflux mechanisms of PPPs and punicalagin were also investigated. In this paper, a convenient and accurate *in vitro* HL7702 steatosis hepatic cell model was applied to evaluate the lipid-lowering effects of the tested polyphenols. The results showed that PPPs possessed the strongest lipid-lowering effects. Prevention group (treated with polyphenols when establishing of steatosis model) was more effective than treatment group (treated with polyphenols after establishment of steatosis model). Punicalagin displayed the strongest lipid-lowering effects among all the tested components of pomegranate peel polyphenols. Moreover, PPPs and punicalagin (10, 20, 40 μg/mL) significantly increased the mRNA expression of LXRα (Liver X receptor alpha) and its target genes-ABCA1 (ATP-binding cassette transporter A1) in a dose-dependent manner in HL7702 steatosis hepatic cells. The high mRNA expression of LXRα and ABCA1, next to lovastatin, was observed in cells treated with 40 μg/mL of PPPs. These *in vitro* findings suggested that PPPs might have great potential in the clinic treatment of hyperlipemia.


## 1. Introduction

Pomegranate (*Punica granatum* L.), a seeded or granular apple, is derived from the name *Pomum* (apple) *granatus* (grainy) [1,2]. It is one of the most investigated fruits in recent years [3]. Numerous studies show that pomegranate fruits possess significant anti-diabetic, anti-inflammatory, anti-oxidant, anti-tumor, and anti-obesity activities *in vivo* and *in vitro* [4–6]. Consumption of pomegranate is of great benefits to patients with metabolic syndrome, such as coronary heart disease, diabetes, and hyperlipidemia [7,8]. It has reported that consumption of pomegranate juice is able to decrease oxidative stress in serum and the macrophage uptake of oxidized low-density lipoprotein (ox-LDL) of diabetic patients [9].

Pomegranate fruits consist of carbohydrates, minerals, crude fibers, vitamin C, and considerable varieties of phenolic compounds, including anthocyanins (3-glucosides and 3, 5-diglucosides of delphinidin, cyanidin, and pelargonidin), ellagic acid (EA), gallic acid (GA), punicalin, punicalagin, pedunculagin, and different flavanols [5,10]. There are abundant phenolic compounds, punicalagin, EA

* Corresponding author. Tel.: +86 29 85310519; fax: +86 29 85310517.
*E-mail address:* jiankel@snnu.edu.cn (J. Li).

http://dx.doi.org/10.1016/j.toxrep.2014.10.013

and GA in the pomegranate peels, and they have known as natural antioxidants [11]. Pomegranate shows potent anti-atherosclerotic activity which could be attributed to the abundant contents of polyhenols. Punicalagin is the most abundant polyphenols, and it presents in two anomers: punicalagin A and B, [6,11–14]. Studies have shown that punicalagin has antioxidant, antifungal and antibacterial properties [2]. Ellagic acid has also been demonstrated to reduce white fat deposits and triglycerides accumulation in the body during regular intake of high-fat diets [15].

In addition, scientific researches have suggested that green tea and plant sterols possess the lipid-lowering effects, and lower the risk of heart diseases [16–18]. Epigallocatechin gallate (EGCG), the main component of tea polyphenols, has attracted much attention in recent years. EGCG exhibits significant lipid-lowering activity in mice [19,20]. However, there are many questions remaining to be answered, such as, which components are the main bioactive ingredients of PPPs? How they play this role? Moreover, are the lipid-lowering effects of the bioactive monomers of PPPs stronger than other common plant polyphenols? Further studies are required to compare the lipid-lowering and hepatoprotective effects between PPPs and its main components (including punicalagin, EA, GA), EGCG, and phlorizin.

In this study, the lipid-lowering effects of the six kinds of polyphenols were investigated *in vitro* simultaneously. In order to better understand the lipid-lowering effects of pomegranate peel polyphenols cholesterol, efflux mechanisms were also investigated. LXRα and its target gene-ABCA1 were known to play an important role in the cholesterol efflux pathway [21–23]. However, the effects and putative mechanisms of pomegranate peel polyphenols on these two genes remains poorly understood. The present study investigated the cholesterol efflux by evaluating LXRα and its target gene-ABCA1, and provided reference to the effective treatment and prevention of non-alcoholic fatty liver diseases and to the decrease in the risk rates of hyperlipidemia and cardiovascular diseases.

## 2. Materials and methods

### 2.1. Materials and chemicals

HL7702 human hepatic cells were purchased from China Center for Type Culture Collection (CCTCC). Punicalagin, ellagic acid (EA), gallic acid (GA), phlorizin, epigallocatechin gallate (EGCG), and lovastatin were obtained from Sigma–Aldrich (St. Louis, MO, USA), and the purities of all the standards were not less than 98%. Fetal calf serum was purchased from Hangzhou Sijiqing Company. Oil red O dye, insulin, penicillin–streptomycin solution, trypsin, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were provided by Sigma Chemical Company (Shanghai, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total triglycerides (TG), and total cholesterol (TC) testing cassette were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

### 2.2. Preparation of purified pomegranate peel extracts

Ripe pomegranates were obtained from Lintong, Shaanxi province of China. The peel was separated and cut into pieces, then dried, milled into fine powders and kept in a dessicator overnight before extraction for polyphenols. The extraction was performed using ethanol ultrasonic-assisted extraction method. Briefly, 1 g of sample was weighed, extraction solution was then obtained with ultrasonic power of 120 W, ethanol concentration of 60% (v/v), temperature at 60 °C, solid–liquid ratio of 1–20, as well as ultrasonic extraction time of 30 min. Thereafter, the extraction solution was concentrated by rotary evaporator at 40 °C, followed by vacuum freeze-drying to obtain crude extracts of pomegranate peel polyphenols. The crude extracts were dissolved in water, extracted by equal volume of petroleum ether and the aqueous phase was collected. The aqueous phase was then extracted by equal volume of chloroform for three times, and aqueous phase was collected with pH adjusted to 6.5 by 1 mol $L^{-1}$ NaOH. The aqueous phase was then extracted by two times volume of ethyl acetate for three times, and the ethyl acetate phase was collected. Thereafter, the pH of the aqueous phase was adjusted to 2.0 by 1 mol $L^{-1}$ HCl, then extracted by two times volume of ethyl acetate for three times. Finally, the six times of extracted acetate phase were merged together, the solvent was removed using a vacuum rotary evaporator at 40 °C, followed by vacuum drying to obtain the purified polyphenols obtained from pomegranate peel extracts.

### 2.3. HPLC analysis of the purified polyphenols extracts

The identification of phenolic compounds in the pomegranate peel polyphenols purified extracts was performed by HPLC. The analysis was carried out using a ZORBAX SB-C18 chromatographic column (4.6 mm i.d. ×250 mm, 5 μm, Agilent, American) on a 1525 Waters HPLC system equipped with an UV detector (Waters, USA). A gradient elution was performed by varying the proportion of solvent A (water containing 1% glacial acetic acid) and solvent B (methanol). The g–radient program was as follows: 0–70 min from 5% to 44% methanol; 70–80 min with 44% methanol. The flow rate of the mobile phase was 1 mL/min; the UV detection wavelength was 280 nm, the sample injection volume was 20 μL, and the column temperature was 30 °C.

### 2.4. Culture of HL7702 hepatic cells

Cells were kept at 37 °C, 95% air, 5% $CO_2$ in RPMI-1640 supplemented with 10% (v/v) FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Every 2 days, medium was refreshed, at 75–80% confluence; cells were split to a new 75 $cm^2$ flask.

### 2.5. Building of steatosis hepatic cell model

Cells were seeded in 6-well tissue culture plates (Jetbiofil, Guangzhou) at a cell density of $2 \times 10^5$ in 1000 μL/well and then incubated for 12 h with 10% (v/v)