An aqueous pomegranate seed extract ameliorates oxidative stress of human hepatoma HepG2 cells

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Abstract

BACKGROUND: Aqueous pomegranate seed extract (PSE), a by-product of the pomegranate juice industry, was recently identified as a potential antiglycative ingredient. Ellagic acid was proposed as the major polyphenol responsible for the antiglycative activity as exerted in *in vitro* models. However, there is no information on safety aspects of this extract in biological systems before its application as ingredient. The cytotoxicity of PSE (1–100 µg mL⁻¹) was evaluated by determining its effect on cell viability and redox status of cultured HepG2 cells. The protective effect of the PSE against oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BOOH) was also investigated.

RESULTS: No changes in cell integrity or intrinsic antioxidant status resulted from a direct treatment with aqueous PSE, even at high dosage. In addition, reactive oxygen species (ROS) induced by *t*-BOOH were reduced by 21% when cells were pretreated with 100 µg mL⁻¹ of aqueous PSE at 180 min. The range of concentrations investigated was effective in decreasing the ROS formation but not in a dose-dependent manner.

CONCLUSION: Aqueous pomegranate seed extract enhances human hepatoma cells integrity and resistance to cope with a stressful situation at concentration up to 100 µg mL⁻¹

INTRODUCTION

Punica granatum (pomegranates), belonging to the *Punicaceae* family, has a remarkable content in polyphenols, particularly hydrolysable tannins (ellagitannins and gallotannins) and anthocyanins (delphinidin, cyanidin and pelargonidin). Based on its high content of antioxidants, consumption of pomegranate as fruits or juice has been largely related to chemopreventive and anti-inflammatory effects.¹ Hence several pomegranate containing products have been marketed around the world in recent years. However, pomegranate fruits contain considerable amounts of seeds (about 20% w/w), which are an important byproduct in the elaboration of the pomegranate juice. Recently, our group identified a significant antiglycative activity of an aqueous pomegranate seeds extract (PSE). PSE showed *in vitro* inhibitory effect of the formation of advanced glycation end-products (AGEs) through two mechanisms: (1) preventing glycation and (2) scavenging methylglyoxal, a potent AGE precursor.²

AGEs have received great attention because of their association with the ageing process³ and related chronic diseases such as diabetes mellitus and its complications⁴ Alzheimer's disease⁵ or atherosclerosis.⁶ For this reason, many investigations have been driven by the search for inhibitors of AGE formation. Initial investigations focused on synthetic AGE inhibitors such as aminoguanidine,⁷ and other inhibitors (carnosine⁵ or pyridoxamine⁷). However, it has been demonstrated that aminoguanidine can exert serious side effects in animal models. In fact, aminoguanidine can inhibit diamine oxidase and nitric oxide synthase enzymes involved in vascular and inflammatory processes.⁸ In this sense, efforts have been devoted to the study of natural substances.⁷ Plant by-products have been considered as a good alternative to present compounds with antiglycative and antioxidant capacity. These phytochemicals are mostly phenolic compounds, being the most widely investigated ellagic acid, chlorogenic acid, gallic acid, ferulic acid and caffeic acid.^{9–12} Several studies have shown that pomegranate fruit has a positive effect on the prevention and treatment of the diseases mentioned previously. Again, the high content of phenolic compounds plays a pivotal role on the biological properties of the pomegranate, although each compound contributes in a different way.¹³ On the other hand, the fruit part, variety, extraction method or industrialisation process influence the content of these biochemical compounds.¹ In this regard,

research has been carried out with pomegranate juice or peel, while studies with seeds have been focused on the beneficial effects of certain polyphenols, polyunsaturated fatty acids, conjugated linolenic acid, punicic acid and tocopherols,¹⁴ all present in the seed oil. Nevertheless, there are few studies on the potential genotoxicity of whole pomegranate hydroalcoholic extracts tested both *in vitro* and *in vivo*.¹⁵ Basic toxicological information is required before further suggestion of its application as an ingredient in foods or pharmacological preparations.

The aim of this study was to investigate the toxicity, in terms of cell injury, and cellular redox status potential change of a human hepatoma cell line (HepG2) after treatment with different concentrations of PSE. The effect of PSE on cell viability and oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BOOH) was evaluated.

MATERIALS AND METHODS

Materials

Pomegranate seeds were provided by TIKTA (Ankara, Turkey). Human hepatoma HepG2 cells were supplied by Dr Paloma Martín-Sanz (Instituto de Bioquímica, CSIC, Madrid, Spain). Dubelcco's modified Eagle's medium (DMEM-F12) and fetal bovine serum (FBS) were purchased from Biowhitaker Europe (Innogenetics, Madrid, Spain). Gentamicin, penicillin, streptomycin, *t*-BOOH, crystal violet, nicotine adenine dinucleotide reduced form (NADH) and Tris were from Sigma Chemical (Madrid, Spain). Sodium dodecyl sulfate salt, dimethyl sulfoxide (DMSO) and pyruvate were obtained from Panreac Quimica (Barcelona, Spain). The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA) and cell culture dishes were acquired from Falcon (Cajal, Madrid, Spain). All other chemicals and reagents were of analytical grade.

Preparation of seed extracts

A portion (500 mg) of ground pomegranate seeds previously characterised² was extracted in water (25 mL, twice) by means of agitation for 10 min at 50°C. Supernatants were pooled, centrifuged (1400×g for 10 min at 4°C) and lyophilised. The extract thus obtained is rich in phenolic compounds, mostly gallic and ellagic acids, as reported elsewhere.²

Cell culture and treatment with pomegranate seed extracts

HepG2 cells were grown in DMEM-F12 medium, supplemented with 2.5% FBS and 50 mg L⁻¹ of gentamicin, penicillin and streptomycin antibiotics. Cells were incubated in humidified atmosphere of 5% CO₂ at 37°C. Under these conditions, two sets of experiments were designed for this study: (1) experiments of direct treatment of cells with PSE for 20 h to test for an effect of the tested compounds dissolved in FBS-free medium, since FBS might interfere in the performance of the assays and alter the results;¹⁶ and (2) experiments of pretreatment of cells with PSE 20 h [cytotoxicity and reactive oxygen species (ROS) experiments] before submitting cells to an oxidative stress induced by *t*-BOOH (200 µmol L⁻¹, 3 h) to test for a protective effect.

Cell viability assay

The viability of HepG2 cells was determined using the crystal violet assay.¹⁷ Cells were plated in 96-well plates with a density of 104 cells per well. Then, these were grown for 20 h with different concentrations of tested extracts (1–100 µg mL⁻¹), washed with PBS and incubated with 50 µL of crystal violet (0.2% in 2% ethanol) for 20 min. Plates were rinsed with water and dried twice. After adding 100 µL of 1% sodium dodecyl sulfate, the absorbance was measured at 570 nm using a SynergyTM HT-multimode microplate spectrophotometer (Biotek Instruments, Inc, Winooski, VT, USA). Results are expressed as percentage of cell viability regarding viable untreated cells.

Cytotoxicity assay

Cell cytotoxicity was determined following the lactate dehydrogenase leakage (LDH) method.¹⁸ Cells were plated at a density of 1.5×10⁶ cells per plate in 60-mm diameter plates and pretreated with different concentrations of pomegranate extract (1–100 µg mL⁻¹) for 20 h in FBS-free medium. After that, culture medium was collected and the cells scraped in phosphate-buffered saline (PBS). Cells were sonicated to breakdown the cell membrane and release the total amount of LDH. Then, they were centrifuged (1000×g, 15 min) and the supernatant was collected. LDH was determined in culture medium and cell lysates, mixing with 5 mmol L⁻¹ pyruvate, 0.35 mmol L⁻¹ NADH and 84 mmol L⁻¹ Tris. LDH leakage was estimated as the ratio between the LDH activity in the culture medium and total LDH cell

content. In parallel, to evaluate the protection of PSE against a cytotoxic agent HepG2 cells were submitted to the procedure described above but, in this case, *t*-BOOH (200 μ mol L⁻¹) was added 3 h before the measurement.

Determination of reactive oxygen species

The dichlorofluorescein (DCFH) assay was used to quantify cellular ROS as a marker of the extent of cellular oxidative stress.¹⁹ Cells were plated at a cell density of 2×10^5 cells per well in 24-well plates and changed to FBS-free medium the day after. In direct treatment experiments, 10 μ L of DCFH (10 mmol L⁻¹ in DMSO) were added to the wells and incubated at 37°C for 30 min. Then, cells were washed twice with PBS and 0.5 mL of PSE dissolved in FBS-free medium (1–100 μ g mL⁻¹) were incorporated into the wells. Plates were immediately measured (time 0) using a microplate reader (SynergyTM HT-multimode microplate spectrophotometer) at an excitation/emission wavelength of 485/530 nm. This method is based on the DCFH probe oxidation, by intracellular oxidants or *t*-BOOH, to DCF which emit fluorescence. ROS were determined by quantifying fluorescence at different times (0, 60, 120 and 180 min). In protection experiments, cells were pre-incubated with 500 μ L of the PSE (1–100 μ g mL⁻¹) for 20 h. After that, 10 μ L of DCFH (10 mmol L⁻¹ in DMSO) were added to the wells and incubated at 37°C for 30 min. Then, cells were washed twice with PBS and 500 μ L of *t*-BOOH in FBS-free medium added. Control samples without *t*-BOOH were used as negative control. Results are expressed as fluorescence units.

Statistical analysis

Statistical analysis of data was performed using the Statgraphics Centurion XV Statistical program (Herndon, VA, USA). Data were tested for homogeneity of variances by using Levene's test. Analysis of variance was performed using ANOVA procedures followed by a Bonferroni test or Tamhane test when the variances were homogeneous or non-homogeneous, respectively. Differences were considered significant at $P < 0.05$. Data from three independent assays were expressed as the mean value $\pm$ SD.

RESULTS AND DISCUSSION

After our previous study on the antiglycative and antioxidant capacity of PSE,² a question concerning safety issues of the PSE arose. Therefore, potential toxicity of PSE in terms of cell viability and effects on the general oxidative status was assessed in a human cell line. Since liver is the major location for xenobiotic metabolism, research on xenobiotics should be focused first on liver cells. HepG2 cells are a transformed cell line, which is well differentiated and characterised. In addition, HepG2 is a reliable cell model that responds to different test conditions.¹⁸ Several studies have employed this cell line to evaluate the toxicity or protective effects of phenolic compounds against oxidative stress induced by *t*-BOOH.^{19–21}

After selecting HepG2 cells as an appropriate model, the crystal violet assay was used to determine potential effects of PSE on HepG2 viability. Cells were pretreated with different concentrations of PSE (1, 10 and 100 μ g mL⁻¹) for 20 h. As shown in Table 1, reduction of cell viability determined as the relative density of adherent cells was not observed. Cell viability remained unchanged even at high concentrations of PSE (100 μ g mL⁻¹), which represents a high dose of this natural ingredient. LDH leakage from the cell cytosol to the culture medium was used as an indicator of cytotoxicity, and generally as an indicator of cell integrity. LDH leakage is estimated from the ratio between the LDH activity in the culture medium and the whole cell content. A range of PSE doses between 1 and 100 μ g mL⁻¹ was tested for a period of 20 h for their potential deleterious effect on HepG2 cells, as depicted in Fig. 1 (empty bar). Treatment with PSE significantly decreased LDH leakage as compared with untreated control cells. However, a dose-dependent effect was not observed at the concentrations tested (1, 10 and 100 μ g mL⁻¹) since results only ranged from 9.7% to 9.2% LDH activity in the culture medium after 20 h for PSE concentrations from 1 to 100 μ g mL⁻¹. These results confirm the lack of cytotoxicity of PSE on HepG2 cells, even at high concentrations.

The protective effect of PSE against a cytotoxic inductor agent such as *t*-BOOH was also evaluated. It is known that decomposition of *t*-BOOH accelerates lipid peroxidation chain reactions, depletion of cell glutathione, and induction of cell toxicity by damage to DNA and apoptosis.¹⁸ Cells were pretreated with different concentrations of PSE (1, 10 and 100 μ g mL⁻¹) for 20 h and then treated with *t*-BOOH. A batch of cells not submitted to PSE was treated with 200 μ mol L⁻¹ *t*-BOOH for 3 h as a positive control for severe cell damage conditions. As depicted in Fig. 1 (solid bar), PSE efficiently prevented LDH leakage at concentrations of 10 and 100 μ g mL⁻¹ as compared with the control cells without

pretreatment with PSE. A 50.6% of LDH leakage was obtained in control cells whereas in cells pretreated with PSE toxicity was significantly decreased to 37.3 and 34.9% of LDH leakage at the concentrations of 10 and 100 $\mu\text{g mL}^{-1}$, respectively. However, this protection was not statistically significant at the concentration of 1 $\mu\text{g mL}^{-1}$.

The DCFH assay was used to determine intracellular ROS generation by measuring the fluorescence emitted by DCF after oxidation of DCFH. ROS generation is an important marker of the cell status since it causes damage to cellular components impairing or altering their metabolic functions. Figure 2 shows that PSE was effective in decreasing the formation of intracellular ROS, but its effectiveness varies with the time of exposure and the concentration of the extract. It is important to notice that direct application of PSE on HepG2 cells did not promote intracellular ROS formation at any concentration or time tested. The rate of ROS formation after 60 min of exposure was significantly reduced to 87.1, 86.4, and 84.9% for 1, 10 and 100 $\mu\text{g mL}^{-1}$, respectively. Nevertheless, effective protection was not observed for the highest concentration of PSE tested at the longest time of exposure. On the other hand, PSE concentration at 1 and 10 $\mu\text{g mL}^{-1}$ for 180 min significantly reduced the generation of intracellular ROS. These results confirm that PSE, even at the highest rate of dosage, did not induce an oxidative damage into the cell model. In a next assay, cells were treated with *t*-BOOH as a chemical compound that induces oxidative stress. *t*-BOOH can decompose to other alkoxyl and peroxy radicals in a reaction that generates ROS.¹⁸ Figure 3 shows that PSE significantly decreased the ROS formation for all the concentrations tested as compared with untreated *t*-BOOH control. Although a dose-response effect was not observed, the formation of ROS was significantly decreased with the time of exposure (79.1, 76.9 and 79.3% were achieved for 1, 10 and 100 $\mu\text{g mL}^{-1}$ at 180 min, as compared with 100% of *t*-BOOH). These results confirm the protective effect of PSE against an oxidative insult on HepG2 cell.

Various studies have shown that pomegranate is a fruit with anti-ageing, anti-atherosclerosis and anti-inflammatory effects, with potential protective effect against other cardiovascular diseases and neurodegeneration.^{22–24} These effects are likely due to its content of bioactive compounds such as phenolic acids, flavonoids, punicalagin and ellagitannins.^{23,25} Extraction of the former bioactive compounds is strongly related to the extraction solvent used. Thus a methanolic extract of pomegranate peel presents higher antioxidant activity and phenolic content than does an aqueous extract and other parts of pomegranate, such as seed or leaf.²⁶ In the present study, the seed coat was chosen as a byproduct and water was used for extraction as a cost effective and environmentally compatible approach²⁷ that was safer than if nonpolar solvents were used. Under these conditions, the hydrophilic compounds with antioxidant and antiglycative activities, such as gallic acid, ellagic acid and other phenolic compounds, were efficiently extracted.^{2,28} Although methanol or ethanol have been largely used as extraction solvents pomegranate,^{15,28,29} aqueous extracts of tropical plants (roselle, spring onion, sweet potato, wild lime, or tapioca among other plants) have been proposed as a source of functional ingredients.³⁰ A number of researchers have stressed the need to study the possible toxicity of non-edible parts of pomegranate since few studies have shown a certain toxicity of whole fruit extracts both in *in vivo* and *in vitro* assays. Sánchez-Lamar *et al.*¹⁵ reported that a hydroalcoholic pomegranate fruit extract could be genotoxic at various expression levels. According to Vidal *et al.*²⁹ amounts over 0.2 mg of pomegranate hydroalcoholic extract produced embryotoxicity. In addition, other authors also suggested that roots and barks from pomegranate presented toxic effects probably due to their alkaloids, tannins or flavonoids.¹⁵ Nevertheless, these toxic effects have been observed at higher concentrations than those that are effective as antivirals or are intended for the treatment of respiratory diseases. On the contrary, studies performed with aqueous extracts have not shown toxic effects, although there is some controversy. Settheetham and Ishida³¹ reported that aqueous pomegranate peel extract induced apoptosis in human cells in *in vitro* assays. In the present study the safety of the PSE was evaluated employing HepG2 as a cellular model. Cells were pretreated with a range of PSE concentrations (1 to 100 $\mu\text{g mL}^{-1}$) and it was observed that extracts did not decrease cell viability or produce a cytotoxic effect. Moreover, treatment with PSE showed a significant protection of cells against *t*-BOOH toxicity. Aqueous extracts would be easier to obtain in a juice or seed infusion compared with non-aqueous extracts so it has more advantages for the consumer.

Hiraganahalli *et al.*³² stated that pomegranate peel extract exerts a significant liver protective effect against *t*-BOOH toxicity and this might be correlated with a radical scavenging effect. Likewise, other studies have shown a hepatoprotective capacity of pomegranate peel extract against CCl₄, a cytotoxic agent studied as a liver toxicant. Studies *in vivo* have shown a protective effect of pomegranate in liver cells by recovery the normal hepatic architecture.²⁸ In the present study, intracellular ROS production was reduced after undergoing oxidative injury with *t*-BOOH. The literature

points out that pomegranate's phenolic content may be related to its antioxidant capacity, which is higher in peels and flowers than in leaves and seeds.³³ Nevertheless, at high concentrations (100 µg mL⁻¹) the effect was significantly reduced regarding the time of exposure compared with lower concentrations of 1 and 10 µg mL⁻¹. This behaviour could be explained by dual antioxidant and pro-oxidant activities of phenolic compounds such as gallic or ellagic acid,³⁴ which are the most abundant phenolic compounds in PSE.^{2,35} Recent studies have reported several biological functions of ellagic acid, highlighting its anti-inflammatory, antioxidant and antimutagenic activities. In addition, ellagic acid can also exert a hepatoprotective effect against CCl₄ both *in vitro* and *in vivo*, contributing to the recovery of cellular antioxidants.³⁶ In a similar way, gallic acid may exhibit antimutagenicity and a significant antioxidant effect. Yeh *et al.*^{37,38} revealed that this phenolic acid is a potent inducer of phenolsulfotransferase which shows a cytoprotector effect against oxidative injury. However, gallic and ellagic acids may behave as pro-oxidants like other phenolic compounds, although the mechanism of action has not been elucidated yet.³⁴ Gallic acid has shown a slight metal chelating and high electron donating capacities, which could explain its pro-oxidant effects at high concentrations. Its ability to induce cytotoxicity and apoptosis in certain cell lines strengthens this dual activity.¹¹ On the other hand, the possibility that phenolic compounds may have antagonistic or synergistic effects with themselves or with other compounds can influence this duality.²

CONCLUSION

The studied aqueous pomegranate seed extract with antiglycative properties did not exert toxicity on HepG2 cell as a human hepatic cell model even at high exposure levels. Hepatic cells should metabolise PSE without detrimental effects on cell viability. Although literature describes that delphinidin, cyanidin and pelargonidin present in hydroalcoholic pomegranate extracts could exert cytotoxic effects,³⁹ the aqueous extract investigated in this study showed a remarkable positive effect on the prevention and reduction of the oxidative stress of HepG2 cells. The protective effect was particularly accentuated at the lowest PSE concentrations assayed. Although *in vivo* studies are needed in a further step, this investigation points out that aqueous pomegranate extract with antiglycative properties can be safely used, in terms of cell toxicity and protection against oxidative stress, as an ingredient in foods and pharmacological preparations.

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TABLES AND FIGURES

Table 1. Effect of pomegranate seed extract on HepG2 cells viability after treatment for 20 h

Sample	Cell viability (%)
Control	100.00±6.86
Seed extract ($\mu\text{g mL}^{-1}$)	
1	04.92±10.05
10	105.85±11.21
100	103.22±11.21

Results are expressed as mean $\pm$ SD ($n=4$).

Significant differences were not observed ($P<0.05$).

Figure 1. Lactate dehydrogenase (LDH) leakage after treatment with pomegranate seed extract (PSE; 1, 10 and 100 $\mu\text{g mL}^{-1}$) for 20 h (white bars) and after *t*-BOOH exposure for 3 h (black bars). Data show the mean $\pm$ SD ($n = 5$). Different lower-case (white bars) and upper-case (black bars) letters indicate significant differences ($P < 0.05$).

Figure 2. Intracellular generation of reactive oxygen species after treatment with pomegranate seed extract (PSE; 1, 10 and 100 $\mu\text{g mL}^{-1}$) at 0, 60, 120 and 180 min. Results are expressed as mean $\pm$ SD ($n = 4$). Different letters denote significant differences ($P < 0.05$) within the same incubation time. f.u.: fluorescence units.

Figure3. Protective effect of pomegranate seed extract (PSE) pretreatment (1, 10 and 100 µg mL⁻¹) for 20 h against oxidative stress induced by *tert*butyl hydroperoxide (*t*-BOOH) after 0, 60, 120 and 180 min of exposure. Data show the mean ± SD ($n = 4$). Different letters denote significant differences ($P < 0.05$). f.u.: fluorescence units.