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Original Article

Hepatoprotective Effect of Pomegranate *(Punica Granatum)* Fruit Juice and Seed Extracts against CCL_4-Induced Toxicity

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Abstract

Punica granatum is used as a medicinal plant, and its fruit concentrate has been used for the prevention and treatment of liver diseases in Iran. The effects of different concentrations of the hydroalcoholic, ethyl acetate and n-hexane extracts of the *Punica granatum* (fruit juice and seed) were investigated against CCl_4-induced cytotoxicity in HepG2 cells. Concentrations (1-10000 µg/ml) of the extracts were added to the cells, 1 h before the addition of 100 mM of CCl_4. After 24 h, the cells were evaluated for toxicity, TBARs level and GSH content. The hydroalcoholic extracts of fruit juice and seeds with concentrations of 100 to 1000 µg/ml protected the cells against CCl_4 induced cytotoxicity, but the ethyl acetate extract of fruit juice with higher concentration (1000 µg/ml) protected the cells against CCl_4 cytotoxicity and the n-hexane extracts were less effective. The ethyl acetate and n-hexane extracts of seeds with different concentrations did not have any significant protective effect. The *Punica granatum* extracts themselves were not toxic towards cells with concentrations up to 1 mg/ml. Therefore, the results of the present study are somehow consistent with traditional beliefs about hepatoprotective effects of *Punica granatum*.

Keywords: Carbon tetrachloride; Hepatoprotective; HepG2 cells; *Punica granatum*.
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1. Introduction

The reactive oxygen species (ROS) including superoxide anion radical, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$.) are implicated in oxidative damage to various cellular macromolecules [1]. Several synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are available, but they are quite unsafe and their toxicity is a problem of great concern [2].

Many natural compounds are now known to have a modulator role on physiological functions and biotransformation reactions

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Table 1. Extraction yield and total phenolic content of Pomegranate juice and seed by various solvents.

		Yield%	Phenolic content
Fruit juice	JEE	37.52%	342.6
	JHE	28.51%	268
	JNE	27.12%	244.4
Seed	SEE	19.87%	99.65
	SHE	22.38%	123.92
	SNE	17.40%	91.31

Note: Total phenolic content (gallic acid equivalent g/100 g dry extract). Abbreviations: JEE: Juice ethyl acetate extract; JHE: Juice hydro alcoholic extract; JNE: Juice n-hexane extract; SEE: Seed ethyl acetate extract; SHE: Seed hydro alcoholic extract; SNE: Seed n-hexane extract.

involved in the detoxification process, thereby affording protection from cytotoxic, genotoxic, and metabolic actions of environmental toxicants [3].

Punica granatum (PG) (Punicaceae), commonly known as pomegranate, is a shrub or a small tree, native to the Mediterranean region. A number of biological activities such as antitumour [4], antibacterial [5], antidiarrhoeal [6], antifungal [7], antiulcer [8] effects have been reported on various extracts/constituents of different parts of this plant. Also, PG is now gaining importance because of its potent antioxidant activity. PG fruit juice, peel extracts, seed oil and seed extracts have been found to possess a potent antioxidant activity [9-11]. Also, PG juice has been shown to modulate the expression of oxidation-sensitive genes in cultured endothelial cells and in atherosclerosis-prone areas of hypercholesterolemic mice [12].

The protective activity of pomegranate flower extract was demonstrated against ferric nitrilotriacetate (Fe-NTA) induced hepatoxicity in mice. Fe-NTA is known to generate ROS and induce oxidative stress in the liver and kidney. Also, hepatoprotective role and antioxidant capacity of pomegranate flowers infusion against trichloroacetic acid was studied in rats. This study revealed that constituents present in PG can create protection against carcinogenic chemical induced oxidative injuries [13].

In the Persian folk medicine, the concentrate of PG fruit juice has been considered to be hepatoprotective and blood purifier. However, to our knowledge no report is available on such effects of the juice and seed extracts. The present study was undertaken to investigate the hepatoprotective activity of different extracts of fruit and seed demonstrates any against CCl_4 induced damage in HepG2 cells. Also, its hepatoprotective effect was compared to the effect of silymarin which is known to be hepatoprotective against CCl_4- or acetaminophen-induced liver damages.

2. Materials and methods

2.1. Cells and chemicals

Human liver cancer cell line HepG2 was obtained from Pasteur Institute, Tehran, Iran. RPMI-1640 and FBS were from GibCo, United States. Trypon blue, methylthiazoly-diphenyl-tetrazolium bromide (MTT), DTNB, and Na_2HPO_4 were from Merck, Germany, and thiobarbituric acid was from Sigma Chemical Company, Germany. All other used chemicals of high quality were available in the market.

2.2. Preparation of fruit juice and seed extracts

Fresh *Punica granatum* (pomegranate) fruit was purchased during November 2010, from local market in Shiraz, Fars, Iran, and was authenticated by Prof. Khosravi at the Botany Department, Shiraz University, where voucher specimens were maintained.

The fruits were manually peeled and then the juice was prepared by manual press machine and then concentrated by a rotary

Table 2. Cytotoxicity of pomegranate juice and seed extracts on HepG2 cells.

	% Viability			
Concentration	**10 µg/ml**	**100 µg/ml**	**1000 µg/ml**	**10000 µg/ml**
Control	100±8.8	100±7.7	100±7.9	100±8.9
JEE	97.2±5.6	95.3±6.7	93.3±7.5	40.1±6.3***
JHE	99.3±4.4	98.8±4.9	97.7±6.1	39.9±5.5***
JNE	96.5±5.3	95.8±5.6	94.3±6.5	35.5±5.8***
SEE	95.2±6.4	94.3±6.7	93.3±7.5	25.1±5.3***
SHE	95.3±3.4	95.8±3.9	93.7±4.1	29.9±4.5***
SNE	96.5±4.3	95.8±5.6	94.3±4.5	31.5±4.8***

Note: (Values are Means ± SD). Abbreviations: JEE: Juice ethyl acetate extract; JHE: Juice hydro alcoholic extract; JNE: Juice n-hexane extract; SEE: Seed ethyl acetate extract; SHE: Seed hydro alcoholic extract; SNE: Seed n-hexane extract. ***Significantly different from the control group (p<0.001).

evaporator under reduced pressure at 55 °C till the residue remained at a constant weight. The remaining seed was washed carefully, air-dried and ground into fine powder by laboratory mill. The materials were stored at refrigerator till later use.

Concentrated juice and dry seed (100 g) were extracted using 3 solvents: methanol (70%; v/v), ethyl acetate and n-hexane. The solvent to material ratio was 2:1 by refluxing method. The time of the refluxing procedure was 2 h. The supernatant was collected by centrifugation at 2000 g for 10 min, and then concentrated by a rotary evaporator under reduced pressure at 55 °C till the residue remained at a constant weight. The yield of each extract was calculated as follows:

Extraction yield (%) = (weight of dry extract/weight of raw material) × 100

Total phenol contents of the extracts were determined using Folin–Ciocalteu method [14] and gallic acid was used as a standard.

2.3. In vitro studies

Human hepatoma cell lines (HepG2) were cultured and maintained in RPMI-1640, pH 7.3, containing 0.37% $NaHCO_3$ supplemented with 10% FCS (fetal bovine serum albumin), 1% penicillin and streptomycin (100 IU/ml penicillin and 100 IU/ml streptomycin) in a humidified 5% CO_2-95% air mixture at 37 ºC.

Cells were seeded in 96-well microplates (30000 cells/well/90 µl) and routinely cultured in a humidified incubator for 24 h. The cells were then treated with different concentrations from 10 µg/ml to 10 mg/ml of the fruit juice and seed extracts (10 µl/well.), 1 h before CCl_4 (100 mM) exposure. A control group (RPMI-1640 without fruit juice and seed extract) and a positive control group (silymarin 20 µg/ml) were also included. After 24 h of incubation, 10 µl MTT solution was added to every well, cells were re-incubated for an additional 4 h. The cell culture media and MTT solution were removed and the cells remained in the bottom of the wells. Then, 100 µl of DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of the converted dye was measured at a wavelength of 570 nm. Six wells were used for each concentration of fruit juice and seed extracts, and three independent experiments were performed for each extract [15].

2.4. Measurment of thiobarbituric acid-reactive substances

As a biomarker for lipid peroxidation, concentration of thiobarbituric acid-reactive agents was measured. HepG2 cells (3×10^6 cells/flask) were preincubated in flasks for 24 h at 5% CO_2-95% air at 37 ºC. The control cultures were prepared by adding only RPMI-1640. After incubation with or without extract, the culture medium was removed. Having been rinsed with 0.5 ml free PBS twice, cells were collected by trypsinization. After determining the viability of the detached cells, 250 µl of 70% (w/v) trichloroacetic acid containing 1 ml of 0.8% (w/v) thiobarbituric

acid with 750 ml deionized water was added to the cells and shaken with vortex. The suspensions were transferred into glass tubes and boiled for 30 min. After cooling to room temperature and centrifugation for 10 min at 5000 rpm, the absorbance of the supernatant was determined at 532 nm [16].

2.5. Measurment of reduced and oxidized glutathione

HepG2 cells (3×10^6 cells/flask) were pre-incubated in flasks for 24 h at 5% CO_2-95% air at 37 ºC. Cells were rinsed with PBS and were collected by trypsinization, after determining the viability of detached cells, 200 µl of 20% trichloroacetic acid with 1800 µl of PBS were added to the cell suspension. Having shaken with vortex and centrifuging, we divided the supernatant into two even parts (each 1 ml). To measure the reduced glutathione (GSH), two ml of Na_2HPO_4 (0.3 M) and 0.5 ml of DTNB (0.01 M) were added to 1 ml of the supernatant and was shaken with vortex. The absorbance was then measured at 412 nm.

For oxidized glutathione (GSSG), 1 ml of 5% sodium borohydrid was added to 1 ml of the supernatant, and incubated for 1 h at 45 ºC, then 0.5 ml of Na_2HPO_4 (0.3 M) was added to each tube. After neutralization with HCl (2.7 N), 0.5 ml of DTNB (0.01 M) was added and shaken with vortex. The absorbance was then measured at 412 nm [17].

2.6. Statistical analysis

All of the values in at least three performed experiments refer to Mean±SD. Statistically significant differences found between control and experimental groups using one way ANOVA test. The GraphPad Instat and Microsoft Office Excel were used for Statistical analysis. The minimal level of significance was chosen $p<0.05$.

3. Results

Carbon tetrachloride was toxic towards HepG2 cells in a dose-dependent manner. It caused loss of cell viability for 24 h with and LC_{50} of about 100 mM (data not shown). As shown in Table 1, the yields of juice and seed extracts were sequentially ethyl acetate > hydro-alcoholic > n-hexane. The results presented in Table 2 indicate that the incubation of HepG2 cells for 24 h with concentrations of 100 to 1000 µg/ml of each of the extracts did not have any toxicity on the cells, but higher concentrations were toxic.

Table 3. Cytopreotective activity of silymarin against CCl_4 induced toxicity.

Treatment	% Viability
Control	100.0±16.15
CCl_4 (100 mM)	55.82±8.86
CCl_4 + Silymarin 1 µM	60.1±9.1
CCl_4 + Silymarin 5 µM	62.2±10.3
CCl_4 + Silymarin 10 µM	65.5±9.9
CCl_4 + Siyimarin 20 µM	83.3±16.34**

Note: (Values are Means ± SD). **Significantly different from the CCl_4 group ($p<0.01$).

The protective effect of the fruit juice extract was dose-dependent and concentrations higher than 1 mg/ml did not exhibit any further protective effect against CCl_4. Silymarin also caused a significant protection against CCl_4 cytotoxicity with a concentration of 20 µM (Table 3).

The results presented in Table 4 indicated that the preincubation of HepG2 cells with 100 to 1000 µg/ml of the ethyl acetate or 1000 µg/ml hydro-alcoholic extracts of PG juice 1 h before CCl_4 (100 mM) leads to the reduction of cytotoxicity. But the n-hexane extract with similar concentrations did not protect against CCl_4–induced cytotoxicity. Also, preincubation of cells with the hydro-alcoholic seed extract (100 to 1000 µg/ml) reduced toxicity of CCl_4, but the n-hexan and ethyl acetate extracts of varying concentrations did not have any protective effects on CCl_4 toxicity. Incubation of the cells with 100 mM CCl_4 for 24 h decreased the GSH content of the cells and increased GSSG and TBARs levels (Table 5). Incubation of the cells with hydro-alcoholic (1000 µg/ml) and ethyl acetate (100

to 1000 µg/ml) of fruit juice extracts significantly prevented GSH reduction induced by CCl_4, and prevented increase of TBARs levels induced by CCl_4 (Table 5). However, the seed extracts did not significantly TBARs or GSH content affected by CCl_4.

Table 4. Cytopreotective activity of pomegranate juice and seed extracts against CCl_4 induced toxicity.

Treatment	% Viability
Control	100.1±16.159
CCl_4(100 mM)	48.08±8.86***
+ Silymarin 20 µM	93.13±16.34+++
+ JEE 10 µg/ml	53.51±3.62
+ JEE 100 µg/ml	68.33±8.5++
+ JEE 1000 µg/ml	69.61±9.24++
+ JHE 10 µg/ml	53.11±5.34
+ JHE 100 µg/ml	55.63±7.66
+ JHE 1000 µg/ml	61.83±3.08+
+ JNE 10 µg/ml	49.89±5.22
+ JNE 100 µg/ml	49.63±7.66
+ JNE 1000 µg/ml	50.83±12.08
+ SEE 10 µg/ml	49.37±8.42
+ SEE 100 µg/ml	52.66±6.11
+ SEE 1000 µg/ml	53.12±5.09
+ SHE 10 µg/ml	50.18±3.22
+ SHE 100 µg/ml	59.72±4.79+
+ SHE 1000 µg/ml	66.79±7.86++
+ SNE 10 µg/ml	49.60±6.12
+ SNE 100 µg/ml	53.63±7.66
+ SNE 1000 µg/ml	55.83±11.08

Note: Values are Means ± SD. Abbreviations: JEE: Juice ethyl acetate extract; JHE: Juice hydro alcoholic extract; JNE: Juice n-hexane extract; SEE: Seed ethyl acetate extract; SHE: Seed hydro alcoholic extract; SNE: Seed n-hexane extract. ***Significantly different from the control group (p<0.001). +++Significantly different from the CCl_4 –treated group (p<0.001). ++ Significantly different from the CCl_4 –treated group (p<0.01). +Significantly different from the CCl_4 –treated group (p<0.05).

4. Discussion

Carbon tetrachloride-induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effects of drugs or medicinal plants' extracts, by *in vivo* and *in vitro* techniques [18, 19]. CCl_4 is believed to be metabolized by microsomal CYP450 in the liver to a highly reactive trichloromethyl free radical ($^{\bullet}CCl_3$) which can start a chain of reactive free radical formation resulting in peroxidation of lipids and damage to the proteins and components of the cells which can result in cell lyses [20, 21].

The protective and antioxidant properties of *Punica granatum* flowers infusion against trichloroacetic acid was evaluated in rats, and it was shown that the plant beverage supplement impart protection against carcinogenic chemical induced liver injury and oxidative stress [13].

However, the hepatoprotective activity of *Punica granatum* has not been intensively studied, and it can serve a further research area. In the present study the ability of different extracts of pomegranate fruit juice and seed to prevent the CCl_4 toxicity in HepG2 cells was evaluated. The results showed that hydro-alcoholic and ethyl acetate extracts of fruit juice and seed extracts of pomegranate reduced cytotoxicity of CCl_4. GSH depletion and TBARs content of the cells was increased by CCl_4 and only hydro-alcoholic and ethyl acetate extracts of fruit juice were effective, indicating that the water soluble compounds of fruit were effective in preventing the toxicity induced by CCl_4.

The respective cytoprotective and hepato-protective effects against CCl_4 could be by one or several mechanisms such as: inhibition of CYP450 responsible for the metabolism of CCl_4 to reactive free radicals; antioxidant effects; scavenging free radicals responsible for cell damage; or induction or regeneration of the liver cells.

Antioxidant activities of pomegranate fruit, fruit juice, peel extracts, seed oil and seed extracts have been found to possess a potent antioxidant activity [22, 23]. Previous studies suggest that pomegranate flowers, too, boost an enormous antioxidant activity. Antioxidant potential of pomegranate juice and extracts is attributed to their high polyphenolics content including ellagic acid and ellagitannins [24]. Other researches have shown that pomegranate peel extract has markedly higher antioxidant capacity than the pulp extract. Among methanol, ethyl acetate and n-hexane,

Table 5. Effects of pomegranate juice and seed extracts on GSH, GSSG and TBARS levels of HepG2 cells.

	GSH ($\mu mol/3\times10^3 cell$)	GSSG ($\mu mol/3\times10^3 cell$)	TBARs ($ng/5\times10^5$ cell)
Control	24.96±2.66	3.83±0.103	0.889±0.043
CCl_4 (100 mM)	11.52±3.38***	19.02±3.47*	1.87±0.032***
+ JEE (100 µg/ml)	21.10±3.81	11.17±4.71	0.638±0.047+++
+ JEE (1000 µg/ml)	28.65±2.52+++	10.26±1.79+	0.736±0.064+++
+ JEH (1000 µg/ml)	15.43±2.81	18.87±3.65	0.651±0.151+++
+ JEN (1000 µg/ml)	13.30±3.1	17.70±2.6	1.731±0.120
+ SEE (1000 µg/ml)	14.54±2.98	18.87±3.10	1.636±0.064
+ SHE (1000 µg/ml)	12.98±2.24	17.99±2.74	0.0641±0.535
+ SEN (1000 µg/ml)	13.32±3.12	17.99±3.1	1.499±0.075

Note: Values are Means±SD. Abbreviations: JEE: Juice ethyl acetate extract; JHE: Juice hydro alcoholic extract; JNE: Juice n-hexane extract; SEE: Seed ethyl acetate extract; SHE: Seed hydro alcoholic extract; SNE: Seed n-hexane extract. ***Significantly different from the control group (p<0.001). +++Significantly different from the CCl_4 –treated group (p<0.001). *Significantly different from the control group (p<0.05).

used for extraction of antioxidants from pomegranate peel and seed, ethyl acetate yields to maximum antioxidant activity [25]. Pomegranate fruit and peel extracts [11, 26] have also been reported to inhibit lipid peroxidation and both seem to possess more potent antiperoxidative activity than pomegranate flower extract.

No toxic effects of *Punica granatum* in mice treated with aqueous extracts of pomegranate similar to those used in folk medicine was observed [27]. Vidal [28] reported that toxic effects of *Punica granatum* fruit extract occurred at higher doses than those effective in the models where the anti-viral activity has been studied or than those doses used in Cuban folk medicine.

In conclusion, the present study demonstrates that the alcoholic and ethyl acetate extracts of pomegranate possess a potent free radical scavenging, antioxidant and hepatoprotective activities. The results suggest that regular intake of pomegranate juice possessing varied biological activities could be potentially useful for the prevention of chronic degenerative liver diseases.

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