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 ($\text{H}_2\text{O}_2$) and hydroxyl radicals (•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...
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 nitritolriacetate (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 CCl₄ induced damage in HepG2 cells. Also, its hepatoprotective effect was compared to the effect of silymarin which is known to be hepatoprotective against CCl₄- 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, methylthiazolyldiphenyl-tetrazolium bromide (MTT), DTNB, and Na₂HPO₄ 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 dryer.

<table>
<thead>
<tr>
<th>Fruit juice</th>
<th>JEE</th>
<th>37.52%</th>
<th>342.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JHE</td>
<td>28.51%</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>JNE</td>
<td>27.12%</td>
<td>244.4</td>
</tr>
<tr>
<td>Seed</td>
<td>SEE</td>
<td>19.87%</td>
<td>99.65</td>
</tr>
<tr>
<td></td>
<td>SHE</td>
<td>22.38%</td>
<td>123.92</td>
</tr>
<tr>
<td></td>
<td>SNE</td>
<td>17.40%</td>
<td>91.31</td>
</tr>
</tbody>
</table>

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.
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:

\[
\text{Extraction yield (\%)} = \left( \frac{\text{weight of dry extract}}{\text{weight of raw material}} \right) \times 100
\]

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% NaHCO₃ 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% CO₂-95% air mixture at 37 °C.

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 CCl₄ (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. Measurement of thiobarbituric acid-reactive substances

As a biomarker for lipid peroxidation, concentration of thiobarbituric acid-reactive agents was measured. HepG2 cells (3×10⁶ cells/flask) were preincubated in flasks for 24 h at 5% CO₂-95% air at 37 °C. 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. Measurement of reduced and oxidized glutathione

HepG2 cells (3×10⁶ cells/flask) were pre-incubated in flasks for 24 h at 5% CO₂-95% 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 Na₂HPO₄ (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 Na₂HPO₄ (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.

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 LC₅₀ 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.

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 CCl₄. Silymarin also caused a significant protection against CCl₄ cytotoxicity with a concentration of 20 µM (Table 3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0±16.15</td>
</tr>
<tr>
<td>CCl₄ (100 mM)</td>
<td>55.82±8.86</td>
</tr>
<tr>
<td>CCl₄ + Silymarin 1 µM</td>
<td>60.1±9.1</td>
</tr>
<tr>
<td>CCl₄ + Silymarin 5 µM</td>
<td>62.2±10.3</td>
</tr>
<tr>
<td>CCl₄ + Silymarin 10 µM</td>
<td>65.5±9.9</td>
</tr>
<tr>
<td>CCl₄ + Silymarin 20 µM</td>
<td>83.3±16.34**</td>
</tr>
</tbody>
</table>

Note: (Values are Means ± SD). **Significantly different from the CCl₄ group (p<0.01).

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 CCl₄ (100 mM) leads to the reduction of cytotoxicity. But the n-hexane extract with similar concentrations did not protect against CCl₄–induced cytotoxicity. Also, preincubation of cells with the hydro-alcoholic seed extract (100 to 1000 µg/ml) reduced toxicity of CCl₄, but the n-hexan and ethyl acetate extracts of varying concentrations did not have any protective effects on CCl₄ toxicity. Incubation of the cells with 100 mM CCl₄ 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
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 CCl₄ 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 CCl₄. GSH depletion and TBARs content of the cells was increased by CCl₄ 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 CCl₄.

The respective cytoprotective and hepatoprotective effects against CCl₄ could be by one or several mechanisms such as: inhibition of CYP450 responsible for the metabolism of CCl₄ 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,
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 antiviral 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.

**Acknowledgments**

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**References**


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