Effects of Pomegranate Seed and Peel Methanolic Extracts on Methotrexate-Induced Hepatotoxicity in Rats

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A B S T R A C T

Background: Hepatotoxicity—the most important side effect of the Methotrexate (MTX)—seems to relate to the generation of reactive oxygen species. Pomegranate has high anti-oxidant capacities. We studied if MTX-induced hepatotoxicity can be protected by pomegranate peel and seed methanolic extracts (PPE and PSE) in rats.

Methods: Forty-eight Wistar rats were divided on the basis of: orally received normal saline as control, orally received 500 mg/kg PSE, orally received 500 mg/kg PPE, intramuscularly (IM) received 10 mg/kg MTX, MTX- and PSE-received, and MTX- and PPE-received groups. After the intervention, blood and liver samples were obtained.

Results: The results showed considerable antioxidant activity (510.7 ± 2.5 μg/ml) and total phenolic content (147.2 ± 0.2 mg GAE/g extract) of PSE and PPE, respectively. The ALT value reached the levels of the control group after treatment with PSE in PSE + MTX group. The serum level of ALT showed a significant increase in PPE+MTX group in comparison with MTX group. The results indicated that the PSE and PPE did not have considerable effect on ALP levels alone or together with MTX. Our results showed that PSE and PPE decreased the pathological changes induced by methotrexate.

Conclusion: The present study demonstrated that PPE and PSE that have notable total flavonoid and phenolic contents and also antioxidant activity, can protect the liver against histo-pathological and some enzymatic changes induced by MTX in rats.

Introduction

Liver is the main organ of metabolism and has multiple functions. It plays an important role in maintaining the homeostasis of the body system. The liver is the principal detoxifying organ that detoxifies metabolic waste products and toxic chemicals and derived from drugs, foods, pathogens, and xenobiotics. The liver aerobic metabolism leads to the production of pro-oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species under normal conditions that are balanced by antioxidants, with a similar rate. ROS have an important biological role at the physiological level and are considered vital cellular mediators in numerous signalling and metabolic pathways. In parenchymal cells, the mitochondrion, microsomes, and peroxisomes can produce ROS. After a cytotoxic drug administration, ROS excesses compared to anti-oxidants will disturb the homeostasis, resulting in oxidative stress. The parenchymal cells are primary cells subjected to oxidative stress, resulting in injury to the liver. Some innate immune cell populations, including Kupffer, natural killer, and dendritic cells contribute to numerous liver pathologies. Oxidative stress can induce the production of a variety of cytokines like tumour necrosis factor—a in Kupffer cells—that may increase inflammation and apoptosis. Evaluations of hepatic enzymes including alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) may indicate the hepato-cellular damage. They are specific and sensitive clinical and preclinical hepatotoxicity biomarkers. They may thus be appropriate determinant variables in monitoring the progression of a disease and treatment efficiency. Methotrexate (MTX)-a folic acid antagonist is widely prescribed as a cytotoxic agent in many diseases, such as malignancies, as well as in the inflammation and autoimmune diseases. MTX can suppress DNA synthesis and influence several tissues, particularly the liver. Hepatotoxicity—the most important potential major side effect of the drug—seems to relate to the generation of reactive oxygen species (ROS). MTX disturbs the balance between pro-oxidants and antioxidants, resulting in oxidative stress, followed by
some tissues injuries. Natural antioxidant administration might be an effective approach for preventing the adverse effect during treatment with MTX.

The pomegranate (Punica granatum L.) is a fruit that possesses considerable phytochemical contents. This fruit has been extensively referenced in medical folklore and used for centuries to treat disorders such as parasitic and microbial infections, ulcers, stomach ache, and diarrhoea. The fruit is composed of two parts: the aril (52 % w/w), which is the edible part, and the peel, which is the non-edible part. The edible part constitutes 22 % seeds and 78 % juice. Both edible and non-edible parts have been used in folk medicine, traditionally. Various phytochemicals has been demonstrated in these two parts, including polyphenolic compounds, such as anthocyanins and tannins. Previous studies demonstrated that pomegranate peel extracts (PPE) have significant anti-oxidative, anti-inflammatory, anti-microbial, hepatoprotection, antiatherogenic, anti-diarrheal, and antimutagenic properties. The main benefits of pomegranate are attributed to the unique composition of polyphenols, and it exhibits high anti-inflammatory, anti- proliferative, anti-hypertensive, anti-invasive, anti-metastatic, and apoptotic properties and antioxidant capacities.

Russo et al. (2018) found thirty-five phenolic compounds belonging to different classes in different pomegranate parts by high-performance liquid chromatography coupled with photodiode array and mass spectrometry detection. Twenty-eight of them were in four different phenolic compound classes including anthocyanins, phenolic acids, hydrolyzable tannins, and flavonoids. The health benefits of pomegranate consumption in preventing metabolic and non-metabolic diseases have been widely investigated in clinical and experimental studies.

To the best of our knowledge this is the first experimental study that investigates MTX-induced hepatic damage in rats and the possible protective effects of pomegranate peel and seed methanolic extracts (PPE and PSE) against it, using histological and biochemical parameters.

Material and Methods

Extraction

The pomegranate fruits (Punica granatum L., post-Ghermez variety, 5-64-WS) were purchased from the suburbs of Tabriz (East Azarbaijan, Iran). The fruits were carefully washed and peeled manually. The seeds and peels were separated and dried at 40 °C in an oven for 48 h, and then turned into a coarse powder by a blender. Then, 500 g of pomegranate seed powder was extracted by methanol (Merck, Germany) in a ratio of 1:10 w/v at 25 °C for 24 h. The mixture was then filtered through 0.45 µ pore size filters, and the solvent was completely removed by a rotary vacuum evaporator (Hidolf, Germany) at 40 °C. The procedure was also done with pomegranate peels. The difference was only the extraction of peels by methanol at 25°C for 96 h instead of 24 h for seeds. The PSE and PPE were stored in a deep freezer (-80°C) until use.

Animals

Forty-eight male Wistar rats (200 ± 20 g) were obtained from Pasteur Institute (Karaj, Iran). The animals were maintained under standard conditions of a temperature-controlled room (22 ± 2 °C), 50–70% humidity, and 12/12 h light/dark cycle, and were provided water and food ad libitum. Before experimentation, they were adapted for one week to the conditions described above. All studies were approved by the Research Ethics Committee of Tabriz University of Medical Sciences (code: 5-4-110-60) and were done in accordance with the approved procedures.

Procedures

The rats were randomly divided into the following 6 groups (eight animals in each group): the placebo control group (orally received a normal saline, daily for 18 days), PSE group (orally received 500 mg/kg PSE, daily for 18 days), PPE group (orally received 500 mg/kg PPE, daily for 18 days), MTX group (IM received 10 mg/kg MTX, daily for three days beginning from the tenth day), PSE + MTX (A6770, Sigma-Aldrich, UK) group (orally received 500 mg/kg PSE, daily for 18 days and also IM received 10 mg/kg MTX, daily for three days beginning from the tenth day), PPE + MTX group (orally received 500 mg/kg PPE, daily for 18 days and also IM received 10 mg/kg MTX, daily for three days beginning from the tenth day).

At the end of the procedure, blood samples were obtained by cardiac puncture (under anaesthetic condition). The samples were centrifuged at 2000 g (4°C, 10 min). The blood serum samples were placed in -80°C freezer until use. All the rats were sacrificed and liver tissues were excised. Tissue samples were fixed in 10 % neutral buffered formalin during 24 hours and embedded in paraffin.

Biochemical analyses

The ALT, AST, ALP, and gamma glutamyl-transferase (GGT) of blood samples were assayed by commercial kits (Pars Azmun, Karaj, Iran). After calibrating and validating them, the automated Abbott biochemistry analyser (Alcyon 300, USA) was used for the analysis. The enzymes’ activities were expressed as IU/L.

Histopathological evaluation

Paraffin-embedded liver tissue samples were cut into 5-micrometer thick sections. The sections stained with haematoxylin and eosin (H & E) stain. A light microscope (BH-2; Olympus, Tokyo, Japan) was used for the examination. The liver damage scores were semi-quantitatively stated as follows: necrosis, inflammatory cells infiltration, fatty change, and focal haemorrhage and congestion. The histopathologic score of each parameter of each tissue sample was separately calculated. The sum of the scores was also given to each criteria. For each
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criterion, the scores were absent (0), slight (1), moderate (2), and severe (3). The maximum score was 12.15

**Analyses of PSE and PPE**
The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay method was used for assessing the antioxidant properties of the PSE and PPE. In brief, the extracts bleaching of purple colored methanol solution of DPPH was measured by spectrophotometric assay. Dilutions series were prepared to obtain different sample concentrations. 50 µl of each concentration of methanolic extract were added to 5ml of 0.004% methanolic solution of DPPH. After incubation of solutions at room temperature for 30 min, bleaching of DPPH was measured at 517 nm against a blank. Inhibition of DPPH was calculated as RC₅₀ (50 % reduction capacity), extrapolated from dose-response curve. RC₅₀ of the samples were expressed as mg/ml. The RC₅₀ of quercetin as the control material was 0.004 mg/ml.14 Total phenolic equivalent were determined using Folin-Ciocalteu reagent. In brief, Folin-Ciocalteau reagent was diluted 1:1 with distilled water. 200 µl of the sample was mixed with 200 µl of Folin-Ciocalteau reagent (diluted) and allowed to stand at room temperature for 5 min. Two ml sodium bicarbonate solution (7% w/v) was added to the mixture. After 90 min at room temperature, absorbance was measured at 700 nm. Total phenolics were quantified by calibration curve obtained from measuring the absorbance of a known concentration of gallic acid standard. The concentrations are expressed as milligrams of gallic acid equivalents (GAE) per ml of sample.16,17 Total flavonoids were determined using the AlCl₃ spectro-photometric method. Sample solutions were prepared in 80% methanol. To prepare AlCl₃ reagent, 400 mg crystalline sodium acetate and 133 mg crystalline aluminium chloride were dissolved in 100 ml of 80% methanol. For flavonoid estimation, 1 ml of AlCl₃ reagent and 400 µl of water were added to 2 ml of sample. Absorbance was recorded at 430 nm against blank without AlCl₃ reagent. Stock solution of quercetin (1 mg/ml) was prepared in 80% methanol and various dilutions of quercetin (5-25 µg/ml) were prepared in methanol and a standard curve was plotted. The amount of flavonoids was calculated as quercetin equivalent from the calibration curve of quercetin.17

**Statistical analyses**
Statistical analyses were performed using SPSS (version 13) for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was performed with Q-Q chart for surveying the normality. One-way analysis of variance (ANOVA) was used to compare parameters between the groups for normally distributed data, followed by multiple comparisons with the Tukey post-hoc test. The data were expressed as mean ± standard deviation (SD). Statistical analyses of the histopathologic evaluation of the groups were carried out by the Kruscal-Wallis test followed by Mann-Whitney test. P-values less than 0.05 were considered statistically significant.

**Results**

**Analysis of PSE and PPE**
Antioxidant activity, total phenolic, and flavonoid compounds of PSE and PPE were assayed and the results were extracted into Table 1.

<table>
<thead>
<tr>
<th>Sample (n = 3)</th>
<th>Antioxidant activity (RC₅₀; µg/ml)</th>
<th>Total phenolic content (mg GAE/g extract)</th>
<th>Total Flavonoid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSE</td>
<td>510.7 ± 2.5</td>
<td>41.1 ± 0.2</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>PPE</td>
<td>27 ± 0.3</td>
<td>147.2 ± 0.2</td>
<td>1.17 ± 0.04</td>
</tr>
</tbody>
</table>

Results are the means ± 1SD.

In the MTX group, the levels of ALT and ALP activities significantly decreased (p = 0.009 and p = 0.001 respectively), while the reduction of GGT was not significant (p = 0.076) compared with the samples of rats in the control group.

![Figure 1](image-url)  
*Figure 1.* Effects of pomegranate seed extract (PSE) and pomegranate peel extract (PPE) on blood serum (a) aspartate transaminase (AST) and (b) alanine transaminase (ALT) contents in rats following MTX treatment. The values are expressed as means ± 1SD. * and ** significantly different when compared with the control group (p < 0.05 and p < 0.01, respectively).
Figure 2. Effects of pomegranate seed extract (PSE) and pomegranate peel extract (PPE) on blood serum (a) alkaline phosphatase (ALP) and (b) gamma glutamyl-transferase (GGT) contents in rats following MTX treatment. The values are expressed as means ± 1SD. ** significantly different when compared with the control group (p < 0.01).

When rats were given PSE + MTX and PPE + MTX, their ALT levels significantly increased in comparison with the MTX group, and reached the level of the control group (Figure 1 and 2). The AST levels of the samples of the PSE + MTX and PPE + MTX treated groups rose to significantly different levels (p = 0.049 and p = 0.034) in comparison with the control group, while the ALP levels did not show considerable differences in comparison with MTX group. The GGT levels returned to the level of the control group in the samples of the PSE + MTX and PPE + MTX groups (Figure 1 and 2).

Results of histopathologic examinations
The histopathological changes of the liver samples were expressed in Table 2. The liver parenchyma of control, PSE-, and PPE-treated rats showed a normal architecture. There were also mild fatty changes and congestion in some tissue samples of the groups. In MTX-treated rats, the liver histopathological examination revealed significant degenerative changes when compared with the control group (p < 0.05) (Table 2). Mild to severe hepatocytes necrosis, mononuclear cells infiltration, vessels congestion, and focal haemorrhage were detectable in the sections (Figure 3). The liver tissue sections of MTX + PSE and MTX + PPE treated rats, despite the presence of mild histopathological changes (Figure 3), showed a better morphology and histopathologic index when compared with the MTX group (Table 2).

Figure 3. Photomicrograph of rat liver tissue that shows (a) hepatocytes necrosis associated with vessels congestion (asterisk) and focal hemorrhage (arrows) caused by methotrexate, (b) single necrosis of hepatocytes (short arrows) associated with mononuclear cells infiltration (arrow head) and bile stasis (long arrows) caused by methotrexate and pomegranate peel extract, and (c) presence of mononuclear cells in portal area (arrow) associated with vessels and sinusoidal congestion (asterisk) caused by methotrexate and pomegranate seed extract (H&E).
phenols at is in the opposite. One total induced increase of ALT, AST, and ALP enzymes.

our results; in one of the studies, the serum ALT levels that PPE treatment enhances the antioxidant defence induced oxidative stress with their function, and also leads to enzymes leakage. This leads to damage of hepatic synthesis. This leads to damage of hepatic sinusoids that are the main sites of the enzyme action. Serum after MTX injections. An explanation of the significant decreases in concentrati and serum albumin. MTX treatment decreased ALT, ALP, serum total protein a significant decrease in ALT and ALP values after MTX. In agreement with our findings, Shibayama et al. have described statistically significant effects on ALP levels alone or together with MTX. Our results showed that PSE + MTX and also PPE + MTX treatment caused significant increases in serum AST values in comparison with control and MTX groups. MTX also decreased GGT of the blood serum, but it was not statistically significant.

In agreement with our findings, Shibayama et al. showed a significant decrease in ALT and ALP values after MTX treatment (150 mg/kg, IP). Rofe et al. have reported that MTX treatment decreased ALT, ALP, serum total protein and serum albumin. Likewise, MTX treatment decreased ALT and ALP in the present study. In another report, Al-Motabagani et al. describe statistically significant decreases in concentration of ALP in blood serum after MTX injections. An explanation of the reduction may be the considerable damage of the endothelial cells lining the portal tracts blood vessels and sinusoids that are the main sites of the enzyme action.

Hepatotoxicity is resulted from binding of MTX to the dihydrofolate reductase enzyme that prevents conversion of folic acid to folinic acid, its active form, thereby blocking of the essential amino acids, nucleic acids, and proteins synthesis. This leads to damage of hepatic parenchymal cells plasma membranes and organelles that interferes with their function, and also leads to enzymes leakage. Kumar et al. investigated the protective effects of pomegranate peel methanol extract on mercuric chloride induced oxidative stress in a rat model. They concluded that PPE treatment enhances the antioxidant defence status against the toxicity induced by mercuric chloride. The results of some previous studies are the opposite of our results; in one of the studies, the serum ALT levels of the psoriasis patients increased significantly after MTX treatment. Results of another study revealed MTX-induced increase of ALT, AST, and ALP enzymes. In the study of Patel et al., the values of AST, ALT, and ALP were increased. In histopathologic findings of our study demonstrated histopathological changes after MTX treatment and relative protective effects of PPE and PSE against the changes. Our findings are in accordance with previous findings.

Results of PPE and PSE analysis showed notable total phenolic and total flavonoid contents and also antioxidant activity (Table 1). Poly-phenols and flavonoids have antioxidant activity in vitro and in vivo and are the major classes of phytochemicals in pomegranate. The antioxidant activity of dietary polyphenols include reactive species scavenging, enzyme modulation to interference with cell signalling, and oxidative stability. The pomegranate is a major source for soluble polyphenols such as ellagic and gallic acids, querectin and punicalagin. Our results indicate more phenolic compounds in PPE in compared with PSE that is in the accordance with findings of Russo et al. and Zhai et al. (2018). Russo et al. observed differences in phenolic compound profiles among the different pomegranate parts and also varieties. In their study, pomegranate peel samples showed a high concentration of phenolic compounds with respect to pulp and juice samples for each variety. In the present study the antioxidant activity of PSE was more than that of PPE, that is in the opposite of the results of Derakhshan et al. Their results were 45 to 58% and 26–54% for peel and seed of pomegranate, respectively. Mansure et al. reported the highest value of polyphenols in methanolic peel extract 230.4 mg GAE/g, which is higher than our study (147.2 ± 0.2 mg GAE/g). Although it must be considered that variation in the total phenolic contents of pomegranate can be influenced by solvent used for extraction.

From the above observations, it appears that PSE and PPE have considerable effects on hepatic enzymes and histopathological changes induced by MTX, which may be caused by their phenolic and antioxidant contents. Previous studies have demonstrated that polyphenols possess powerful antioxidant properties, which represent the most likely mechanism responsible for the pomegranate’s protective benefits. The antioxidant capacity of pomegranates has been shown to be 3 times higher than that of red wine or green tea infusion.

**Discussion**

In the present study, MTX significantly decreased ALT contents of the blood serum. The ALT value reached the levels of the control group after treatment with PSE in PSE + MTX group. The serum level of ALT also showed a significant increase in PPE + MTX group in comparison with the MTX group. MTX significantly decreased ALP content of the blood serum. The ALP values of MTX, PSE + MTX, and PPE + MTX were not significantly different. The results indicate that the PSE and PPE do not have considerable effects on ALP levels alone or together with MTX. Our results showed that PSE + MTX and also PPE + MTX treatment caused significant increases in serum AST values in comparison with control and MTX groups. MTX also decreased GGT of the blood serum, but it was not statistically significant.

Table 2. Histopathologic evaluation of liver sections in control and treated groups (n = 8).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>PSE</th>
<th>PPE</th>
<th>MTX</th>
<th>SPE + MTX</th>
<th>PPE + MTX</th>
<th>Kruskal-Wallis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degeneration (Mean rank)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.00 ± 0.63</td>
<td>0.80 ± 0.83</td>
<td>0.67 ± 0.51</td>
<td>0.003</td>
</tr>
<tr>
<td>(9.5)</td>
<td>(9.5)</td>
<td>(9.5)</td>
<td>(9.5)</td>
<td>(23.1)</td>
<td>(19.5)</td>
<td>(21.5)</td>
<td></td>
</tr>
<tr>
<td>Infiltration (Mean rank)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.67 ± 0.51</td>
<td>1.00 ± 0.63</td>
<td>1.00 ± 1.00</td>
<td>0.000</td>
</tr>
<tr>
<td>(8.5)</td>
<td>(8.5)</td>
<td>(8.5)</td>
<td>(8.5)</td>
<td>(25.0)</td>
<td>(20.1)</td>
<td>(20)</td>
<td></td>
</tr>
<tr>
<td>Congestion (Mean rank)</td>
<td>0.50 ± 0.57</td>
<td>0.20 ± 0.44</td>
<td>0.40 ± 0.54</td>
<td>1.67 ± 0.51</td>
<td>0.83 ± 0.40</td>
<td>1.00 ± 1.00</td>
<td>0.011</td>
</tr>
<tr>
<td>(12.5)</td>
<td>(8.6)</td>
<td>(11.2)</td>
<td>(25.6)</td>
<td>(16.8)</td>
<td>(18.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemorrhage (Mean rank)</td>
<td>0.75 ± 0.50</td>
<td>0.40 ± 0.54</td>
<td>0.40 ± 0.54</td>
<td>1.67 ± 0.51</td>
<td>1.33 ± 0.81</td>
<td>0.80 ± 0.83</td>
<td>0.008</td>
</tr>
<tr>
<td>(13.8)</td>
<td>(9.5)</td>
<td>(9.5)</td>
<td>(24.6)</td>
<td>(20.8)</td>
<td>(14.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total score (Mean rank)</td>
<td>1.75 ± 1.25</td>
<td>0.60 ± 0.54</td>
<td>0.80 ± 0.83</td>
<td>6.00 ± 1.67</td>
<td>3.83 ± 1.47</td>
<td>3.60 ± 1.67</td>
<td>0.001</td>
</tr>
<tr>
<td>(11.9)</td>
<td>(6.3)</td>
<td>(7.3)</td>
<td>(26.7)</td>
<td>(20.5)</td>
<td>(19.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(PSE: pomegranate seed extract; PPE: pomegranate peel extract; MTX: methotrexate). Scoring was done as follows for each microscopic field: none (0), mild (1), moderate (2), and severe (3). Results are presented as mean ± SD. Significantly different when compared with the control group (p < 0.05).
is also rich in antioxidants of the polyphenolic compounds, which include flavonoids such as ellagitannins, gallotannins, ellagic acids, gallic acids, catechins, anthocyanins, ferulic acids, and quercetins. These polyphenols exhibit numerous biological activities, such as eliminating free radicals, inhibiting oxidation, and reducing the risks of cardio-vascular diseases. It has been suggested that ellagitannins could be responsible for the promised antioxidant and anti-mutagenic activities of PPE. PPE exhibited strong antioxidant activities. Previous studies suggest that pomegranates can reduce oxidative stress, quench free radicals, and support the synthesis and activity of nitric oxide. The chemopreventive action of pomegranates was attributed to the modulation of cellular signalling processes, including activator protein-1 (AP-1) DNA binding and nuclear factor-kappa-B.

Conclusion
The present study demonstrated that MTX can induce liver histo-pathologic and enzymatic changes in rats. PPE and PSE, which have considerable antioxidant activity and total phenolic and flavonoid contents, can protect the liver against histo-pathological and some enzymatic changes (ALT and GGT) induced by MTX in rats. More studies are required to investigate mechanisms of hepatic changes induced by MTX, the protective effect of PPE and PSE, some unexpected results, and controversies between previous studies.

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Conflict of interests
The authors claim that there is no conflict of interest.

References

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