EFFECTS OF POMEGRANATE SEED METHANOLIC EXTRACT ON METHOTREXATE-INDUCED CHANGES IN RAT LIVER ANTIOXIDANT COMPOUNDS

Mehran Mesgari Abbasi, Reza Heidari, Rogayeh Amini afshar, Parvin Zakeri Milani and Neda Ghamarzad Shishavan

Drug Applied Research Center, Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran; Faculty of Science, Urmia University, Urmia, Iran; Liver and Gastrointestinal Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; and Department of Biochemistry and Diet Therapy, Nutrition Research Center, Faculty of Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran.

[Received April 26, 2015; Accepted August 20, 2015]

[Communicated by Prof. Rotimi Aluko]

ABSTRACT: For therapeutic targets, use of pomegranate - a fruit with high polyphenol contents therein which accounts for its great antioxidant and anti-inflammatory properties - is on the rise. Particularly, it might be so helpful in control of oxidative stress induced by drugs. Methotrexate (MTX) as one of the most important drugs that has been routinely prescribed to control numerous diseases is involved in oxidative stress induction. As a result, antioxidant administration during the treatment period appears to be necessary. The aim of this study was to investigate whether pomegranate seed extract (PSE) could ameliorate methotrexate-induced oxidative liver injury in rats. Thirty-two male Wistar rats were randomly divided into orally received normal saline as placebo control group, orally received 500 mg/kg PSE group, intramuscularly received 10 mg/kg Methotrexate, MTX and PSE received group. After the intervention, all rats were euthanatized and liver tissue samples were taken for subsequent analysis on liver antioxidant system. Low liver activity of Catalase (CAT) in MTX group increased significantly following PSE administration (P<0.004), Glutathione peroxidase (GPx) also showed a great but non-significant enhancement in co-administration of PSE and MTX group compared to MTX received group. Surprisingly Superoxide dismutases (SOD) and Total Antioxidant Capacity (TAC) which increased in MTX group tended to decrease in group received both PSE and MTX (p=0.149, 0.001 respectively). Although pomegranate methanolic extract increased CAT and GPx liver content, understanding its exact effects on antioxidant system is still murky and demands further researches on the current topic.

KEY WORDS: Liver, Methotrexate, Oxidative stress, Pomegranate seed extract

Corresponding Author: Neda Ghamarzad Shishavan, Department of Biochemistry and Diet Therapy, Nutrition Research Center, Faculty of Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran; Fax: +98 4133372653; E-mail: Ghamarzad.n@gmail.com

Abbreviations Used: MTX, Methotrexate; PSE, Pomegranate Seed Extract; SOD, Superoxide dismutases; GPx, Glutathione peroxidase; MDA, Malondialdehyde; CAT, Catalase; TAC, Total Antioxidant Capacity

INTRODUCTION

Pomegranate (Punica granatum L) which is rich in different sorts of phytochemicals especially polyphenols is considered as a functional food (Seeram et al., 2005; Akhtar et al., 2015). There are lines of evidence suggesting its anti-inflammatory and antioxidant effects in control of diseases such as diabetes mellitus and cardiovascular diseases (Akhtar et al., 2015; Jurenka, 2008). Therefore, the use of divergent parts of this natural food such as seed, peel, flowers, bark, buds and leaves have been recently on the rise as a promising factor in food industry and drug preparation (Akhtar et al., 2015).

Clinical use of several drugs is restricted because of their unwanted serious effects on human health. If a way is discovered to mitigate the adverse effects of drugs, there won’t be a reason to refuse the completion of treatment. Methotrexate (MTX), one of these drugs which is routinely prescribed in many
diseases such as autoimmune disorders and cancers, adversely influences several tissues particularly liver and causes hepatic fibrosis or cirrhosis during long term application (Chauhan and Cronstein, 2013). MTX, which acts as an anti-folate substance by virtue of its inhibitory effects on dihydrofolate reductase, suppresses DNA synthesis in many cells (Chauhan and Cronstein, 2013; Braun and Rau, 2009). In addition, it can disturb the balance between antioxidant and pro-oxidants in favor of pro-oxidants. All these result in an enhancement in oxidative stress and injuries in some tissues (Babiak et al., 1998). Thus, it appears that antioxidant administration might be an influential approach during treatment with MTX (Jahovic et al., 2003).

Most of researches in this field found that the impaired antioxidant system especially in liver and other tissues such as colon cells following MTX administration was improved after antioxidants consumption such as resveratrol (Dalaklioglu et al., 2013; Tunali-AkKay et al., 2010), curcumin (Hemeida and Mohez, 2008), lipoic acid (Tabassum et al., 2010), pomegranate peel extract (Waly et al., 2012), and pomegranate juice supplementation (Shadmanfard et al., 2013; Al-Olayan et al., 2014).

Recent development in antioxidant property of pomegranate fruit has led to a renewed interest in its efficacy in the improvement of drug-induced oxidative stress or oxidative stress-mediated diseases. However, far too little attention has been paid to the use this fruit in such conditions. Thus, this paper determined whether pomegranate seed extract could decrease oxidative stress following MTX therapy in rats.

**MATERIALS AND METHODS**

**Extraction**

The pomegranate fruits (Post-Ghermez variety, 5-64-WS) (Kazemialamuti et al., 2012) were obtained from the suburbs of Shiraz (Fars, Iran). All the fresh pomegranates were washed and peeled manually and the seeds were separated. The seeds were air dried in an oven (40°C) for 24 h, and then turned into a coarse powder. Afterwards, 500 g of the powder was extracted by methanol (Merck, Germany) in a ratio of 1:10 w/v at 25 ± 2°C. The mixture was then filtered (0.45 µm) after 24 h and the solvent was completely removed by a rotary vacuum evaporator (Hidolf, Germany) at 40°C and the pomegranate seed extract (PSE) stored in -70°C freezer until use (Basiri, 2013).

**Animals**

In this experimental study, 32 male Wistar rats, weighing 200 ± 20 g, were used. The animals were housed in polycarbonate standard cages in a temperature-controlled room (22 ± 2°C) and 12/12 hours of light/dark cycles for one week, before and during the experiments. Next, the animals were provided with a standard rat pellet diet and clean drinking water ad libitum.

Animal protocols were approved by the Research Ethics Committee of Tabriz University of Medical Sciences (ethical approval code: S4/110/60, date: February 24, 2014) and performed according to the Helsinki’s humanity research declaration. All the ethical and humanity considerations as well as euthanasia of the animals were considered and performed.

**Procedures**

The sample size was determined according to the previous similar animal studies (Al-Olayan et al., 2014). Using the Randlist software, the rats were randomly divided into four groups (eights animals in each group), as follows: Group I served as the placebo control and orally (gavage) received a normal saline, daily for 18 days. Group II served as the pomegranate group and orally received 500 mg/kg PSE, daily for 18 days. Group III served as the Methotrexate group and Intramuscularly (IM) received 10 mg/kg Methotrexate, daily for three days beginning from the tenth day. Group IV served as the treatment group and orally received 500 mg/kg PSE, daily for 18 days and also received 10 mg/kg IM Methotrexate, daily for three days beginning from the tenth day.

At the end of the intervention period, the animals were euthanatized and tissue samples were taken and 100 µg of the tissue homogenized in 1 mL ice cold phosphate buffer solution (PBS) (pH, 7.4) using a glass tissue homogenizer. Then, the homogenized samples were centrifuged for 10 min at 10000 g and 4°C temperature. The supernatants were removed and stored in -70°C freezer until use.

**Biochemical Analyses**

The supernatant superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant capacity (TAC) were determined using commercial assay kits (Biorex diagnostics, UK) with the automated Abbott, Alcyon 300 biochemistry analyzer (USA). The instrument was calibrated and validated using related calibrators before testing the samples. Catalase activities of samples were assayed by Cayman kit (USA). Equivalent activities of supernatants were analyzed by barbituric acid method (Jiang et al., 2012). All results were divided and normalized with supernatants protein contents, which were assessed by commercial kit (Parsazmun, Iran).

**Analyses of PSE**

The antioxidant properties of the PSE were assessed by the DPPH method of Delazz et al. (2006) and 50% reduction capacity (RC50) of the samples were expressed as mg/mL. The RC50 of quercetin as the control material was 0.004 mg/mL. Total phenolic equivalent was determined using Folin-Ciocalteu reagent and estimated using a standard curve prepared using gallic acid and expressed as mg of gallic acid equivalents (GAE) per gram of extract (El-LateefGharib and Teixeira da Silva, 2013). Total flavonoids were assessed using spectrophotometric method as described by Vadop et al. (2012).

**Statistical Analysis**

Statistical analyses were performed using SPSS (version 11.5) for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was performed with Q-Q chart for surveying the normality. In addition, results of Levene
test showed equality of variances of the groups. For normally distributed data, One-way analysis of variance (ANOVA) was used to compare different parameters between the groups, followed by multiple comparisons with the Tukey post-hoc test and the data were expressed as mean ± standard deviation (SD). For non-normally distributed data Wilcoxon test and median ± Interquartile Range were used. P-value less than 0.05 were considered statistically significant.

**RESULTS**

**Composition of PSE**

Analysis of PSE composition shows its antioxidant activity, total phenolic and flavonoid compounds in the pomegranate seed methanolic extract as follows: 0.526±0.002 (RC50; µg/ml), 37.2±0.1(mg GAE/g extract) and 0.35±0.01(%).

**Induction of oxidative stress following MTX administration**

Based on our findings, liver activities of CAT and GPx as important antioxidant enzymes decreased in MTX-treated group. Additionally, MTX administration augmented MDA activity of liver which is a pro-oxidant and considered as a byproduct of peroxidation. All these outputs are consistent with oxidative stress-induced by MTX. On the other hand, some striking results also found which implies an enhancement in some other antioxidants. SOD and TAC as cardinal component of antioxidant defense system, surprisingly increased following MTX group.

**TABLE 1. Effects of pomegranate methanolic extract and methotrexate on oxidative stress in liver of rats.** MTX, Methotrexate; PSE, Pomegranate Seed Extract; GPx (u/mg protein), Glutathione peroxidase; SOD (u/mg protein), Superoxide dismutase; CAT (nmol/min/mg protein), Catalase; MDA (nmol/mg protein), Malondialdehyde; TAC (nmol Trolox equivalent/mg protein) Total Antioxidant Capacity. Results are the means ± SD. §Significantly different at (p < 0.05) when compared with control group; *Significantly different at (p < 0.05) when compared with PSE combined MTX -treated rats; †Significantly different at (p < 0.05) when compared with PSE received rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>TAC</th>
<th>SOD</th>
<th>GPx</th>
<th>MDA</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.197 ± 0.005</td>
<td>5.180 ± 1.007</td>
<td>0.427 ± 0.075</td>
<td>0.533 ± 0.028</td>
<td>25.615 ± 1.397</td>
</tr>
<tr>
<td>PSE</td>
<td>0.195 ± 0.005</td>
<td>7.350 ± 1.709</td>
<td>0.360 ± 0.138</td>
<td>0.538 ± 0.125</td>
<td>21.355 ± 4.149</td>
</tr>
<tr>
<td>MTX</td>
<td>0.216 ± 0.005††</td>
<td>8.004 ± 2.641</td>
<td>0.260 ± 0.168</td>
<td>1.698 ± 0.400†</td>
<td>18.828 ± 2.323†</td>
</tr>
<tr>
<td>MTX + PSE</td>
<td>0.198 ± 0.007</td>
<td>6.340 ± 0.500</td>
<td>0.270 ± 0.142</td>
<td>2.126 ± 0.461</td>
<td>30.125 ± 6.846</td>
</tr>
</tbody>
</table>

**FIGURE 1. Effects of PSE and MTX on liver SOD activity.** Values are expressed as means ± SD. MTX: Methotrexate; PSE: Pomegranate Seed Extract; SOD: Superoxide dismutases

**FIGURE 2. Effects of PSE and MTX on liver MDA activity.** Values are expressed as means ± SD. §Significantly different at (p < 0.05) when compared with control group. *Significantly different at (p < 0.05) when compared with PSE combined MTX -treated rats. †Significantly different at (p < 0.05) when compared with PSE received rats. MTX: Methotrexate; PSE: Pomegranate Seed Extract; MDA: Malondialdehyde

**Effects of pomegranate seed extract on liver activities of antioxidant enzymes**

Pomegranate extract increased the liver activity of SOD in favor of antioxidant system as compared to control group. However, in comparison to control group a reduction in levels of CAT, GPx and TAC were observed.

---

**Composition of PSE**

Analysis of PSE composition shows its antioxidant activity, total phenolic and flavonoid compounds in the pomegranate seed methanolic extract as follows: 0.526±0.002 (RC50; µg/ml), 37.2±0.1(mg GAE/g extract) and 0.35±0.01(%).
Changes in liver activities of antioxidant enzymes following MTX combined pomegranate seed extract

Administration of pomegranate seed extract in MTX received group caused an enhancement in CAT, GPx and MDA and a reduction in SOD and TAC levels compared to MTX group. The changes in all measured parameters are represented in Table 1 that shows the significant differences between groups. Figures 1 to 6 separately displays the liver activity of each parameter including SOD, MDA, Catalase, GPx and TAC respectively in the four intervention groups.

DISCUSSION

Composition of the pomegranate seed methanolic extract used in our study was shown to have higher contents of polyphenol, flavonoid and antioxidant activity when compared with previous literature reports (Jing et al., 2012; Tehranifar et al., 2010 and Cuccioloni et al., 2009). On the other hand, a previous investigation reported higher contents of total phenolic compounds in pomegranate seed (Nuncio-Jáuregui et al., 2015). One explanation for these differences in the extract composition is related to various pomegranate cultivars and maturity which led to the variation in the biosynthesis of phenolic metabolites (Turfan et al., 2011). Another explanation is due to the different solvents used for extraction (Saad et al., 2012). According to a previous study, methanol extract produces the maximum antioxidant activity among methanol, acetone or water used for antioxidant extraction from pomegranate (Negi and Jayaprakasha, 2006). Additionally, it has been suggested that mean annual precipitation may influence the polyphenol contents owing to elimination of water-soluble polyphenols by washing (Saad et al., 2012). Incidentally, the high extent of pomegranate varieties in Iran depending on its genotype diversity, which is classified on the basis of different geographical region, fruit characteristics as morphology, taste, size, color, soluble solids/acidity ratio, and time of ripening, leads to differences in the extract composition (Kazemialamuti et al., 2012).

With regards to the changes in antioxidant enzymes during the current study, some findings are supporting our hypothesis.
on the efficacy of pomegranate extract in amelioration of oxidative stress induced by methotrexate. CAT and GPx are the evident examples of these parameters which increased following treatment with MTX combined pomegranate compared to MTX received group. Surprisingly, administration of pomegranate extract to MTX treated rats increased levels of SOD, TAC and MDA.

There is a large body of evidence supporting the function of MTX in oxidative stress induction which represents a side effect of this drug through attenuation of antioxidant enzymes in different tissues. Liver as the most important target cells of MTX, might be more influenced and it can result in abnormality of liver enzymes, hepatotoxicity and pneumonitis (Chan and Cronstein, 2013; Hashkes et al., 2014).

MTX induces oxidative damage through impairing antioxidant /antioxidant balance; not only by free radical generation and lipid peroxidation, but also by inhibition of antioxidant enzymes (Tabassum et al., 2010). According to previous papers that revealed an inhibition of (NADP)-dependent dehydrogenases in presence of MTX, this conclusion can be drawn that MTX decreases the availability of NADPH in cells and consequently impairs the function of NADP-dependent enzymes such as glutathione reductase which is responsible for maintaining glutathione in the reduced form (Caetano et al., 1997; Babiak et al., 1998). Therefore, MTX leads to a decrease in cellular reduced form of glutathione (GSH), as a fundamental antioxidant and sensitzes the cells to reactive oxygen species (ROS) (Babiak et al., 1998). Given the vast majority of evidence, polyphenols have broad pharmacological properties and might be considered as great factors in control of inflammation and oxidative stress in many diseases (Zhong et al., 2014).

Antioxidant activity of methanolic extract of pomegranate was found in aspirin- and ethanol-induced gastric ulceration rats through MDA and hydro-peroxide reduction in addition to SOD, GPx and glutathione reductase (Ajaiikumar et al., 2005). Incidentally, on the basis of the research conducted by Rozenberg et al. (2006), pomegranate juice decreased the generation of reactive oxygen and nitrogen species in macrophage cell-line which is involved in lipid per-oxidation and progression of coronary atheroma.

Besides these experiments, an enhancement in serum total antioxidant capacity was also shown following pomegranate juice administration in group of 15-17 year old girls (Fazli et al., 2009).

In another study, pomegranate extract in healthy human volunteers increased serum total antioxidant capacity, though there was no significant reduction in reactive oxygen species (Mertens-Talcott et al., 2006).

Pomegranate juice effectively delays plasma lipid peroxidation through inhibition of chain reactions producing free radicals or via binding to copper and inhibition of its binding to lipoproteins (de Nigris et al., 2006; Zarban et al., 2007). In addition, Tapias et al. (2014) proposed a beneficial function of pomegranate juice in control of initial oxidative stress. While, its pro-oxidant property appeared as administered in a substantial ongoing oxidative stress - in rats with Parkinson disease (PD)- (Tapias et al., 2014).

One explanation for the discrepancy of results in prior studies is the use of mixture of polyphenols. Pomegranate seed extract contains several different polyphenols as follows: polyphenolic acid including ellagic acid and gallic acid, flavonoids like hydrolysable tannins and anthocyanins. Likewise, polyphenols have both anti and pro-oxidant properties depending on the combination and dosage. Polyphenols may overwhelm antioxidant defense in a dose-dependent manner. It has been suggested that epigallocatechin gallate (EGCG), ester of epigallocatechin and gallic acid, contributes in hydroxyl radical generation through reducing Fe²⁺ to Fe³⁺ (Fenton reaction) (Tapias et al., 2014). Therefore, the contradictory results compared to the findings of earlier research may be not only due to administration of different doses of methotrexate to induce oxidative stress, but also owing to different doses of pomegranate extract used in trials. Above all, use of different solvents for extraction influencing the antioxidant property of extract causes divergent outcomes as well. Additionally, intervention period for both MTX and PSE administrations may not be sufficient to determine their exact effects on antioxidant system. Given these parameters which make the interpretation of current findings difficult, further studies are absolutely required with different doses of drug and extract in longer intervention periods. To the best of our knowledge, this is the first study to investigate the effects of PSE administration on liver antioxidant changes induced by MTX. One of the strengths of this work is determination of polyphenolic and flavonoid compounds and also antioxidant activity in pomegranate seed methanolic extract.

In conclusion, contrary to our expectation, pomegranate extract failed to mitigate and control oxidative stress-induced by MTX. However, pomegranate administration led to enhancement in CAT and GPx activities. Therefore, PSE is still a relevant bioactive product because of its functional role in control of diseases and additional research is needed to assess its exact effects on antioxidant system in different tissues.

ACKNOWLEDGEMENTS
We are grateful to Student Research Committee and researches affaire of Tabriz University of Medical Sciences, Iran, for providing partial financial support to conduct this study.

REFERENCES


features. Food and Chemical Toxicology 174C:417-425.


