Protective effect of pomegranate peel ethanol extract against ferric nitrilotriacetate induced renal oxidative damage in rats

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Abstract

Pomegranate is an important source of bioactive compounds. The nephroprotective effect of pomegranate peel ethanol extract against ferric nitrilotriacetate (Fe-NTA)-induced renal oxidative stress was studied. The results showed that Fe-NTA enhances renal lipid peroxidation with reduction in renal glutathione content, antioxidant enzymes, viz., glutathione peroxidase, catalase, glutathione reductase and phase-II metabolizing enzyme, glutathione-S-transferase. It also enhances serum urea and creatinine. Treatment of rats orally with pomegranate peel extract (100 and 200 mg/kg/day, for seven days) resulted in significant decrease in lipid peroxidation and serum urea and creatinine levels. Renal glutathione content, glutathione-S-transferase and antioxidant enzymes were also recovered to a significant level (P<0.05). The obtained data demonstrate that pomegranate peel ethanol extract is a potent nephroprotective agent and suppresses Fe-NTA-induced renal oxidative damage in rats.

Keywords: Nephroprotection; Fe-NTA; pomegranate peel ethanol extract; oxidative stress, antioxidant enzymes, glutathione-S-transferase.

Suçanlarda Nar Kabuğu Etanol Özütünün Ferrik Nitrilotriasetat ile İndüklenen Renal Oksidatif Hasara Karşı Koruyucu Etkisi

Özet

Nar önemli bir biyoaktif bileşke kaynağıdır. Nar kabuğu etanol özütünün ferrik nitrilotriasetat (Fe-NTA) ile indüklenen renal oksidatif hasara karşı böbrek koruyucu etkisi çalışılmıştır. Sonuçlar Fe-NTA’nın renal glutatyon içeriğindeki antioksidan enzimler olan glutatyon peroksidaz, katalaz, glutatyon redüktaz ve faz-II metabolize eden enzim olan glutatyon-S-transferazdaki azalma ile renal lipit peroksidasyonunu artırığı gösterdi. Aynı zamanda, Fe-NTA serum üresini ve keratinini de artırır. Suçanlara nar kabuğu özütünün oral muamelesi (100 ve 200 mg/kg/gün, 7 gün boyunca), lipit peroksidasyonunun ve serum üre ve keratin değerlerinin önemli ölçüde düşmesi ile sonuçlandı. Renal glutatyon içeriği, glutatyon-S-transferaz ve antioksidan enzimler de önemli ölçüde tekrar geri kazanmıştı (P<0.05). Elde edilen bulgular, nar kabuğu etanol özütünün güçlü bir böbrek koruyucu ajan olduğunu ve suçanlarda Fe-NTA ile endüklenen oksidatif hasarı baskıladığı gösterir.

Anahtar Sözcükler: Böbrek koruyucu; Fe-NTA; nar kabuğu etanol özütü; oksidatif stres, antioksidan enzimler; glutatyon-S-transferaz
**Introduction**

Pomegranate (*Punica granatum* L., Punicaceae), is one of the oldest known drugs. It is mentioned in the Ebers papyrus of Egypt written in about 1550 BC (Ross, 1999). Dried fruit peel is used for diarrhea and to treat respiratory and urinary tract infections. Also, pomegranate fruit peel exerted diverse pharmacological functions as antioxidant activity (Yunfeng et al., 2006 and Thring et al., 2009), antifertility effect (Gujraj et al., 1960), cytotoxic activity (Sato, 1990 and Kulkarni et al., 2007), hepatoprotective activity (Murthy, 2002) and hypoglycemic activity (Dhawan et al., 1977 and Hontecillas et al., 2009). Also, pomegranate peel ethanol extract (500 mg/kg b.wt.) has ameliorative effect against chlorpyrifos-ethyl-induced oxidative stress in rats (Ahmed and Zaki, 2009). Pomegranate peel contains substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid (Naser et al., 1996).

Iron is the most abundant metal in the human body. Although iron is an essential nutritional element for all life forms, iron overload may lead to various diseases (De Freitas and Meneghini, 2001). The iron complex of the chelating agent nitrilotriacetic acid is nephrotoxic (Khan and Sultana, 2005). Intraperitoneal injection of Fe-NTA induces renal proximal tubular damage associated with oxidative damage that eventually leads to a high incidence of renal cell carcinoma in rodents after repeated administration (Okada and Midorikawa, 1982). Intraperitoneally injected of ferric nitritolriacetate (Fe-NTA) is absorbed into portal vein through mesothelium and passes into circulation via the liver (Umemura et al, 1990). The low molecular weight Fe-NTA is easily filtered through the glomeruli into the lumen of the renal proximal tubules where Fe\(^{3+}\)-NTA is reduced to Fe\(^{2+}\)-NTA by the glutathione degradation products cysteine or cysteinylglycine (Taso and Curthoys, 1980). In the brush border surface of the renal proximal convoluted tubules, \(\gamma\)-glutamyl transpeptidase hydrolyses glutathione to cysteinylglycine and glycine by dipeptidase (Khan and Sultana, 2005). Cysteinylglycine and cysteine are the proposed thiol reductants that reduce Fe\(^{3+}\)-NTA to Fe\(^{2+}\)-NTA. The auto-oxidation of Fe\(^{2+}\)-NTA generates superoxide radicals (O\(^2-\)) which subsequently potentiate the iron catalysed Haber-Weiss reaction to produce hydroxyl radical (OH), leading to induction of lipid peroxidation and oxidative DNA damage (Umemura et al, 1990 and Khan and Sultana, 2005).

For the present study, we prepared the ethanol extract (80%) of the pomegranate peel which exerted the highest antioxidant effect *in vitro*. The objective of the study was to determine the possible effect of prophylactic treatment with pomegranate peel extract on Fe-NTA induced renal oxidative damage in rats.

**Materials and methods**

**Plant material**

Pomegranate fruit peel purchased from local market was dried and powdered before extraction.

**Plant extract**

Powdered plant material (500g) was repeatedly extracted with 2000 ml solvents of increasing polarity starting with benzene, chloroform, ethyl acetate, ethanol (80%) and distilled water. The percolation time for each solvent was 24h. The extracts were filtered, concentrated and freeze dried. The residues yielded for each solvent were stored at 4°C. The ethanol extract (80%) was used for further study after preliminary *in vitro* tests viz. lipid peroxidation, deoxyribose and DPPH assays.

**Chemicals**

Ferrie nitrate, NTA disodium salt, reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin, 1,2-dithio-bis-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) were obtained from Sigma Chemical (St Louis, USA). All solvents used were HPLC grade (Merck, Darmstadt, Germany).

**Total phenolics**

Total phenolics in the pomegranate peel ethanol extract were determined according to Jayaparakashsa et al. (2001) using Folin-Ciocalteu reagent. Four hundred microlitres of sample were taken in test tubes; 1.0 ml of Folin–Ciocalteu reagent (diluted 10-fold with distilled water) and 0.8 ml of 7.5% sodium carbonate were added. The tubes were mixed and allowed to stand for 30 min.
and the absorption at 765 nm was measured against a blank, which contained 400 µl of ethanol in place of sample. The total phenolic content was expressed as gallic acid equivalents in mg/g of ethanol extract.

**Animals**

Albino male rats (170±30 g) were used in the present study. The rats were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR), Egypt. The animals were kept under standard laboratory conditions of light/dark cycle (12/12h) and temperature (25±2°C). The rats were allowed food and water ad libitum. They were provided with a nutritionally adequate standard laboratory diet.

**Animals’ diet**

The basal diet consists of casein 10%, cotton seed oil 4%, salt mixture 4%, vitamin mixture 1%, carbohydrates (sucrose, starch 1:1) 80.8% and choline chloride 0.2% (American Institute of Nutrition, 1980).

**Preparation of Fe-NTA solution**

The Fe-NTA solution was prepared as described in Deiana et al. (2001) and Khan and Sultana (2005), ferric nitrate and NTA disodium salt were dissolved in distilled water to form a 300 and 600 mM solution, respectively. The two solutions were combined in a volume ratio of 1:2 with magnetic stirring at room temperature and the pH was adjusted to 7.4 with sodium bicarbonate.

**Experimental design**

Thirty albino rats were randomly allocated to five groups of six rats each:

- **Group 1** received only saline injection intraperitoneally at a dose of 10 ml/kg body weight.
- **Group 2** received only a single intraperitoneal injection of Fe-NTA solution at a dose of nine mg Fe/kg body weight (Athar and Iqbal 1998).
- **Group 3** received pomegranate peel extract by gavage once daily for seven days at a dose of 100 mg body weight, p.o. (Parmar and Kar, 2008).
- **Group 4** received pomegranate peel extract once daily for seven days at a dose of 200 mg/kg body weight, p.o. (Parmar and Kar, 2008).

After the last treatment with pomegranate peel extract, the animals of group 2, 3 and 4 received a single intraperitoneal injection of Fe-NTA at a dose level of 9mg Fe/kg body weight.

- **Group 5** received pomegranate peel extract orally once daily for seven days at a dose of 200 mg/kg body weight (Parmar and Kar, 2008). We used the high dose of pomegranate peel ethanol extract (200 mg/kg b.w. p.o.) to study its effect on kidney. All rats were sacrificed 12 h after the treated with Fe-NTA. Blood was collected and the separated serum was used for the estimation of creatinine (Bartless et al., 1972) and urea (Patton and Crouch, 1977).

**Post-mitochondrial supernatant and microsomal fraction preparation**

Kidneys were removed quickly and washed in cold isotonic saline. The kidneys were homogenized in 50 mM phosphate buffer (pH 7) using an electronic homogenizer to prepare 10% w/v homogenate. The homogenate was centrifuged at 3000 rpm for 10 min at 4°C by cooling ultracentrifuge (model Sigma 3K 30) to separate the nuclear debris. The aliquot so obtained was used at 12000 rpm for 20 min at 4°C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzymes (Khan and Sultana, 2005). A portion of the PMS was centrifuged for 60 min at 34000 rpm at 4°C. The pellet was washed with phosphate buffer (50 mM pH 7).

**Estimation of reduced glutathione (GSH) in PMS**

Reduced GSH in mitochondria was determined by measuring the rate of formation of chromophoric product in a reaction between 5,5'-dithiobis-2-(nitrobenzoic acid) (DTNB) and free sulphydryl groups, such as GSH, at 412 nm as described by Ellman (1959).

**Estimation of Lipid peroxidation (LPO) in microsomal fraction**

The measurement of microsomal fraction lipid peroxide by a colorimetric reaction with thiobarbituric acid was done as described by Ohkawa et al. (1979), and the determined lipid peroxide is referred to as malondialdehyde. Briefly, in a test tube, 2.5 ml of 20% trichloroacetic acid solution and 1ml of 0.67% thiobarbituric acid solution were added to the samples. The color of thiobarbituric acid pigment was developed in a
water bath at 100 °C for 30 min. After cooling with tap water to room temperature, 4ml n-butanol was added and shaken vigorously. After centrifugation, the color of butanol layer was measured at 535 nm.

**Assay for glutathione-S-transferase (GST) activity in PMS**

Glutathione-S-transferase activity was assayed by the method of Habig et al. (1974). The method is based on the rate of conjugate formation between GSH and 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance change was recorded at 340 nm and the enzyme activity calculated as nmol CDNB conjugates formed/min/mg protein.

**Assay for glutathione peroxidase (GPx) activity in PMS**

Glutathione peroxidase activity was assayed by the method of Mohandas et al. (1984). The change in absorbance was recorded spectrophotometrically at 340 nm. GPx activity was expressed as nmol NADPH oxidized/min/mg protein.

**Assay for glutathione reductase (GR) activity in PMS**

Glutathione reductase activity was determined by the method of Carlberg and Mannervik (1975). GR was assayed by following the oxidation of NADPH at 340 nm at 37°C. GR activity was expressed as nmol NADPH oxidized/min/mg protein.

**Assay for catalase (CAT) activity in PMS**

CAT activity measurement in PMS was measured by the method of Takahara et al. (1960). The reduction rate of H₂O₂ was followed at 240 nm for 30 s at room temperature. CAT activity was expressed in nmol H₂O₂ consumed/min/mg protein.

**Assay for glucose-6-phosphate dehydrogenase (GPD) activity in PMS**

The activity of glucose-6-phosphate dehydrogenase was determined according to the method of Zaheer et al. (1965). The changes in absorbance were recorded at 340 nm and enzyme activity was calculated as nmol NADP reduced/min/mg protein.

**Estimation of protein concentration**

The protein concentration in all samples was determined by the method of Lowry et al. (1951).

**Statistical analysis**

The results are expressed as Mean±SEM. The collected data were statistically analyzed by the least significant differences (LSD) at the level 5% of the probability procedure according to Snedecor and Cochran (1980).

**Results**

**Effect of pomegranate peel extract on renal toxicity markers**

The effect of pretreatment of rats with pomegranate peel extract on Fe-NTA-induced enhancement in the level of serum creatinine and urea are shown in Table (1). Fe-NTA treatment leads to about 147% and 303% enhancement in the values of creatinine and urea, respectively, as compared with saline-treated group. Prophylaxis with pomegranate peel extract at both doses resulted in 28-45% and 48-88% reduction in the values of serum creatinine and urea respectively as compared with Fe-NTA-treated group.

**Effect of pomegranate peel extract on glutathione metabolism**

Table (2) shows the effect of pretreatment of rats with pomegranate peel extracts on Fe-NTA-mediated renal glutathione content and on the activities of its metabolizing enzymes, viz, glutathione-S-transferase and glutathione reductase. Treatment with Fe-NTA alone resulted in the depletion of renal glutathione and reduction in the activities of glutathione-S-transferase and glutathione reductase by 48%, 55% and 46% respectively, as compared with saline-treated group. However, pretreatment of animals with pomegranate peel extract at 100 and 200 mg/kg body weight resulted in the recovery by 79-83%, 46-73% and 40-72% respectively, as compared with Fe-NTA-treated group.
Table 1. Effect of pomegranate peel ethanol extract on Fe-NTA-induced enhancement of serum creatinine and urea in rats. Values Mean±SEM (n=6 animals). a p<0.05, (Student’s t test) significantly different from saline treated group. b p<0.05, significantly different from Fe-NTA-treated group. P.E., pomegranate Extract.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Creatinine mmol/L</th>
<th>Urea mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>36.2±5.3</td>
<td>8.9±2.1</td>
</tr>
<tr>
<td>Fe-NTA (9 mgFe/kg b.w.)</td>
<td>89.4±7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.9±4.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P.E. (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w.)</td>
<td>64.3±8.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.6±3.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P.E. (200 mg/kg b.w.) + Fe-NTA (9 mgFe/kg b.w.)</td>
<td>46.1±7.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. E. (200 mg/kg b.w.)</td>
<td>38.2±4.1</td>
<td>8.5±2.3</td>
</tr>
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</table>

Table 2. Effect of pomegranate peel ethanol extract on Fe-NTA-mediated depletion of renal glutathione (GSH) content and decreased in the activities of glutathione-S-transferase (GST) and glutathione reductase (GR). Values are Mean±SEM (n=6 animals). a p<0.05, (Student’s t test) significantly different from saline treated group. b p<0.05, significantly different from Fe-NTA-treated group. P.E., pomegranate Extract.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>GSH (nmol GSH/g tissue)</th>
<th>GST (nmol CDNB conjugated formed/min/mg protein)</th>
<th>GR (nmol NADPH oxidized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.526±0.03</td>
<td>220.9±4.9</td>
<td>280.7±10.9</td>
</tr>
<tr>
<td>Fe-NTA (9 mgFe/kg b.w.)</td>
<td>0.273±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.8±9.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151.4±6.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P.E. (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w.)</td>
<td>0.488±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>146.5±4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>212.6±15.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P.E.(200 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w.)</td>
<td>0.501±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>172.7±8.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>260.1±14.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P.E. (200 mg/kg b.w.)</td>
<td>0.562±0.02</td>
<td>225.21±7.2</td>
<td>288.6±12.3</td>
</tr>
</tbody>
</table>

Effect of pomegranate peel extract on renal antioxidant enzymes and lipid peroxidation

The effect of prophylactic treatment with pomegranate peel extract on Fe-NTA-induced reduction in the activities of renal antioxidant enzymes and enhancement in microsomal lipid peroxidation is shown in Table 3. Fe-NTA alone treatment caused reduction in the activities of renal antioxidant enzymes such as catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase by 71%, 51% and 54% and enhancement in lipid peroxidation level by 49% respectively as compared to saline-treated group. Treatment with pomegranate peel extract at two doses 100 and 200 mg/kg body weight caused the recovery of the above enzymes by 117-170%, 62-95% and 55-108%, and reduction in lipid peroxidation by 23-33% as compared with Fe-NTA-treated group.
Table 3. Effect of pomegranate peel ethanol extract on Fe-NTA-induced reduction in the activity of renal antioxidant enzymes (CAT, GPx and GPD) and enhancement in the level of microsomal lipid peroxidation (LPO) in rats. Values are Mean±SEM (n=6 animals). a p<0.05, (Student’s t test) significantly different from saline treated group. b p<0.05, significantly different from Fe-NTA-treated group. P.E., pomegranate Extract.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>CAT (nmol H_2O_2 consumed/min/mg protein)</th>
<th>GPx (nmol NADPH oxidized/min/mg protein)</th>
<th>GPD (nmol NADP reduced/min/mg protein)</th>
<th>LPO (nmol MDA formed/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>172.3±11.3</td>
<td>250.5±9.9</td>
<td>50.3±3.9</td>
<td>4.9±0.34</td>
</tr>
<tr>
<td>Fe-NTA (9 mgFe/kg b.w.)</td>
<td>50.5±5.1a</td>
<td>122.7±10.8a</td>
<td>23.2±4.1a</td>
<td>7.31±0.62a</td>
</tr>
<tr>
<td>P.E. (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w)</td>
<td>109.4±6.9b</td>
<td>199.3±12.4b</td>
<td>36.1±3.1b</td>
<td>5.6±0.19b</td>
</tr>
<tr>
<td>P.E. (200 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w.)</td>
<td>136.6±8.9b</td>
<td>239.5±8.6b</td>
<td>48.3±8.8b</td>
<td>4.9±0.29b</td>
</tr>
<tr>
<td>P.E. (200 mg/kg b.w.)</td>
<td>189.2±17.2</td>
<td>272.3±17.2</td>
<td>56.5±4.9</td>
<td>3.8±0.31</td>
</tr>
</tbody>
</table>

Discussion

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in metabolism of aerobic cells. If not eliminated, ROS can attack important biological molecules, such as lipids, proteins, DNA, enzymes, and RNA. Thus, ROS are involved in a number of degenerative diseases such as cancer, cirrhosis, diabetes, and Alzheimer’s Disease (Halliwell and Gutteridge 1986).

Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted currently as one of the main sources of chemo preventive drug discovery and development (Aruoma, 2003). It has been observed that many plant polyphenols, such as ellagic acid, catechins, and chlorogenic, caffeic and ferulic acids act as potent antioxidant, antimutagenic and anticarcinogenic agents (Ayrton et al., 1992 and Bu-Abbas et al., 1993). Also, carob polyphenols has nephroprotective effect against cisplatin (Ahmed, 2010). Nacr et al. (1996) have reported that pomegranate peel contains ellagic acid, ellagitannins and gallic acids. The presence of these polyphenols in the pomegranate peel may be responsible for antioxidant and anticarcinogenic effect of peel extracts (Gil et al., 2000). Also, in this study the ethanol extract had 210.6±7.3 mg/g total phenolics, gallic acid equivalents. Hence, it can be suggested that the observed nephroprotective activity of pomegranate peel ethanol extract in our study due to the presence of these compounds.

The renal antioxidant status, such as GR, CAT, GPx activities and GSH concentration are significantly decreased in the Fe-NTA-treated group of animals compared to saline treated group. The decline of antioxidant status partially explains the mechanism of nephrotoxicity induced by Fe-NTA.

Pomegranate peel ethanol extract ameliorated Fe-NTA-induced inhibition of the activity of antioxidant enzymes, viz., GR, CAT, GPx, activities and phase-II metabolism enzyme GST. Pomegranate peel extract has established antioxidant properties that might have counteracted the oxidant effects of Fe-NTA. Many environmental toxicants such as pesticides require metabolism to their fully toxic forms. They are
often metabolized to proximate toxicants by phase I enzymes, e.g., cytochrome P450 which catalyze oxidative reactions. The oxidized metabolites of potentially toxic xenobiotics are then detoxified by Phase II metabolizing enzymes into the forms that are relatively inert and more easily excreted (Talalay et al., 1995).

GSH depletion increases the sensitivity of organ to oxidative and chemical injury. Studies with a number of models show that the metabolism of xenobiotics often produced GSH depletion (Mitchell et al., 1973 and Ahmed and Zaki, 2009). The depletion of GSH, also, seems to be the prime factor that permits lipid peroxidation in the Fe-NTA treated group. Pretreatment of pomegranate peel extract reduced the depletion of GSH levels and provided protection to the kidney. The protection of GSH is by forming the substrate for GPx activity that can react directly with various aldehydes produced from the peroxidation of membrane lipid. Pomegranate peel extract pretreatment also reduced the elevated levels of serum urea and creatinine that are marker parameters of kidney toxicity.

In conclusion, we can say that, the high antioxidant and nephroprotective effect of the pomegranate peel extract appeared to be attributed to its high phenolics content. The mechanism of action of pomegranate peel extract may be through induction of various antioxidant and phase II enzymes, and scavenging reactive oxygen species. Thus our data suggest that pomegranate peel ethanol extract is a potent nephroprotective agent. Further work is required for the isolation and characterization of individual phenolic compounds present in peel ethanol extract and to determine the mechanisms involved in the nephroprotective effect of pomegranate peel extract.

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