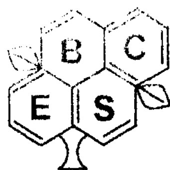

Journal

NEPERO PROTECTIVE EFFECT OF POMEGRANATE PEEL EXTRACT AGAINST FERRIC NITRILOTRIACETATE INDUCED RENAL OXIDATIVE DAMAGE IN RATS

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ABSTRACT

Ferric nitrilotriacetate (Fe-NTA) is a well-known renal carcinogen. The nephroprotective effect of pomegranate peel extract against Fe-NTA induced renal oxidative stress was studied. Fe-NTA enhances renal lipid peroxidation with reduction in renal glutathione content, antioxidant enzymes, viz., glutathione peroxidase, catalase and superoxide dismutase 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) resulted in significant decrease in lipid peroxidation, serum urea and creatinine. Renal glutathione content, glutathione-S-transferase and antioxidant enzymes were also recovered to significant level ($P<0.001$). The obtained data demonstrate that pomegranate peel extract is a potent nephroprotective agent and suppresses Fe-NTA-induced renal carcinogenesis and oxidative damage in rats.

Keywords: Chemoprotection; Ferric nitrilotriacetate; Pomegranate peel extract; oxidative stress

INTRODUCTION

Pomegranate (*Punica granatum* L., Punicaceae), is one of the oldest drug known. 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, antifertility effect (Gujral et al., 1960), cytotoxic activity (Sato, 1990), hepatoprotective activity (Murthy, 2002) and hypoglycemic activity (Dhawan et al., 1977). 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 injection 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 golmeruli 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, γ-glutamyl transpeptidase hydrolyses glutathione to cysteinylglycine that is rapidly degraded to cysteine and glycine by dipeptidase (Khan and Sultana, 2005). Cysteinylglycine and cysteine are the proposed thiol reductants that reduce Fe3+ -NTA to Fe2+ -NTA. The auto-oxidation of Fe2+ -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).

For the present study, we prepared the ethanolic extract (80%) of the pomegranate peel which exerted the highest antioxidant effect in vitro.

The objective of the present study was to determine the prophylactic treatment of rats with pomegranate peel extract on Fe-NTA induced renal oxidative response in rats.

MATERIALS AND METHODS

Plant Material

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

Plant extract

Powdered plant material (500 g) 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 diphenylpicrylhydrazyl (DPPH*) assay.

Chemicals

Ferric 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).

Animals

Albino male mice (30±6 g) were used in the present study. The mice were obtained from the animal house of the National Organisation 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). They were provided with a nutritionally adequate standard laboratory diet.

Preparation of Fe-NTA solution

The Fe-NTA solution was prepared as described by 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 I received only saline injection intraperitonealy at a

dose of 10ml/kg body weight. Group II received only a single intraperitoneal injection of Fe-NTA solution at a dose of nine mg Fe/Kg body weight. Group III received pretreatment with pomegranate peel extract by gavage once daily for seven days at a dose of 100mg/Kg body weight and group IV and V received pretreatment with pomegranate peel extract once daily for seven days at a dose of 200 mg/kg body weight. After the last treatment with pomegranate peel extract, the animals of group II, III and IV received a single intraperitoneal injection of Fe-NTA at a dose level of 9mg Fe/kg body weight. After 12h, the animals were killed by cervical decapitation. Blood was collected and the separated serum was used for the estimation of creatinine (Bartles et al., 1972) and urea (Patton and Crouch, 1977)

Post-mitochondrial supernatant and microsomal preparation (PMS)

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 10min at 4°C by cooling ultra centrifuge (model sigma 3k 30) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12000 rpm for 20 min at 4°C to obtained 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 pH7).

Biochemical determinations

Estimation of reduced glutathione in PMS

Reduced glutathione was determined by the method of Ellman (1959).

Estimation of Lipid peroxidation in micrososmes

The assay for microsomal lipid peroxidation was done following the method of Okhawa et al. (1979).

Assay for glutathione-S-transferase in PMS

Glutathione-S-transferase activity was assayed by the method of Habig et al. (1974).

Assay for glutathione peroxidase activity in PMS

Glutathione peroxidase activity was assayed by the method of Mohandas et al. (1984).

Assay for glutathione reductase activity in PMS

Glutathione reductase activity was determined by the method of Carlberg and Mannervik (1975).

Assay for catalase activity in PMS

Catalase activity was assayed by the method of Takahara et al. (1960).

Assay for glucose-6-phosphate dehydrogenase activity in PMS

The activity of glucose-6-phosphate dehydrogenase was determined by the method of Zaheer et al. (1965).

Estimation of protein

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 analysed by the least significant differences (LSD) at the level 5% of the probability procedure according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

1- 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 control 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 pretreatment with pomegranate peel extract on Fe-NTA-induced enhancement of serum creatinine and urea in rats

Treatment groups	Creatinine (μmol/L)	Urea (mmol/L)
- Saline (control)	36.2±5.3	8.9±2.1
- Fe-NTA (9 mgFe/kg b.w.)	89.4±7.2[a]	35.9±4.1[a]
- P. extract (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w)	64.3±8.4[b]	19.6±3.9[b]
- P. extract (200 mg/Kg b.w) + Fe-NTA (9 mgFe/Kg b.w.)	46.1±7.2[b]	11.5±2.9[b]
- P. extract (200 mg/kg b.w.)	38.2±4.1	8.5±2.3

Values are Mean±SEM (n=6 animals). [a] $p<0.05$, (Student's *t test*) significantly different from normal group. [b] $p<0.05$, significantly different from control group. P, pomegranate.

Table2: Effect of pretreatment with pomegranate peel extract on Fe-NTA-mediated depletion of renal glutathione content and decreased in the activities of glutathione metabolizing enzymes, glutathione-s-transferase and glutathione reductase.

Treated groups	Reduced glutathione (nmol GSH/g tissue)	Glutathione-s-transferase (nmol CDNB conjugated formed/min/mg protein)	Glutathione reductase (nomol NADPH oxidized/min/mg protein)
- Saline (control)	0.526±0.03	220.9±4.9	280.7±10.9
- Fe-NTA (9 mgFe/kg b.w.)	0.273±0.02[a]	99.8±9.2[a]	151.4±6.8[a]
- P. extract (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w)	0.488±0.03[b]	146.5±4.6[b]	212.6±15.8[b]
- P. extract (200 mg/Kg b.w) + Fe-NTA (g mgfe/Kg b.w.)	0.501±0.04[b]	172.7±8.7[b]	260.1±14.6[b]
- P. extract (200 mg/kg b.w.)	0.562±0.02	225.2±7.2	288.6±12.3

Values are Mean±SEM (n=6 animals). [a] $p<0.05$, (Student's *t test*) significantly different from normal group. [b] $p<0.05$, significantly different from control group. P, pomegranate.

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 by 49% respectively as compared to saline-treated control 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 pretreatment with pomegranate peel extract on Fe-NTA-induced reduction in the level of renal antioxidant enzymes and enhancement in the level of microsomal lipid peroxidation in rats

Treatment group	Catalase (nmol H_2O_2 consumed/min/ mg protein)	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glucose-6-phosphat dehydrogenase (nmol NADP reduced/min/mg protein)	Lipid peroxidation (nmol MDA* formed/mg protein)
- Saline (control)	172.3±11.3	250.[illegible]±9.9	50.3±3.9	4.9±0.34
- Fe-NTA (9 mg Fe/kg b.w.)	50.5±5.1^{a}	122.7±10.8^{a}	23.2±4.1^{a}	7.31±0.62^{a}
- P. extract (100 mg/kg b.w.) + Fe-NTA (9 mg Fe/kg b.w)	109.4±6.9^{b}	199.[illegible]±12.4^{b}	36.1±3.1^{b}	5.6±0.19^{b}
- P. extract (200 mg/Kg b.w) + Fe-NTA (9 mg Fe/Kg b.w.)	136.6±8.9^{b}	239.[illegible]±8.6^{b}	48.3±8.8^{b}	4.9±0.29^{b}
- P. extract (200 mg/kg b.w.)	189.2±17.3	272.[illegible]	56.5±4.9	3.8±0.31

2- Discussion:

Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted currently as one of the main sources of cancer 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., 1994). Nasr 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 anticarcinogenicity of peel extracts (Gil et al., 2000). So the observed nephropreventive activity of pomegranate peel extract in our study may be suggested due to the presence of these compounds.

Pomegranate peel extracts ameliorated Fe-NTA-induced inhibition of the activity of antioxidant enzymes, viz., glutathione peroxidase, glutathione reductase, catalase, glucose-6-phosphate dehydrogenase and phase-II metabolism enzyme glutathione-s-transferase. 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). It has been shown that most of the chemo-preventive agents results in the induction of glutathione-s-transferase and quinone reductase activity and results in the degradation of electrophilic metabolities (DeFlora and Ramel, 1988).

There was also dose-dependent decrease in the Fe-NTA mediated susceptibility of renal microsomal membrane for iron-ascorbate induced lipid peroxidation through decreased production of free radicals as shown by ameliorated malondialdehyde levels. The decreased level of reduced glutathione following Fe-NTA administration due to decrease reduction of oxidized glutathione and increased activity of γ-glutumyl transpeptidase cause accumulation of peroxides thereby leading to oxidative damage (Khan and Sultana, 2005). Increased γ-glutamyl-transpeptidase activity fixes degradation of GSH, which may lead to higher accumulation of cysteinyl-glycine and cysteine. High levels of both cysteine and cysteinyl-glcyine have been suggested to enhance reduction of Fe-NTA to its ferrous complex, which in turn enhances peroxidative damage to membrane or tissue. 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 nephropreventive 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 extract is a potent nephropreventive agent. Further work is required for the isolation and characterization of individual phenolic compounds present in peel extract and to determine the mechanisms involved in the chemopreventive effect of pomegranate peel extract.

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التاثير الواقى للكلى لمستخلص قشر الرمان ضد مركب نيترواستيات الحديديك التى تسبب تلف تأكسدى فى فئران التجارب

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يعتبر الرمان مصدر هام للمركبات الفعالة بيولوجيا. وقد اشتملت الدراسة على التاثير الواقى للمستخلص الأيثانولى لقشر الرمان ضد مركب نيترواستيات الحديديك الذى يسبب تلف لكلى فئران التجارب. وتشير النتائج ان مركب نيترواستيات الحديديك قد تسبب فى زيادة مستوى اكسدة الليبيدات فى الكلى مع حدوث إنخفاض واضح ومعنوى فى مستوى الجلوتاثيون وانزيمات الأكسدة (جلوتاثيون بيروكسيديز، الكتاليز وجلوكوز-6- فوسفات ديهيدروجينيز) وكذلك انزيم جلوتاثيون-س- ترانسفيريز.

بالأضافة الى ذلك فان هذا المركب قد تسبب فى ارتفاع مستوى اليوريا والكرياتينين فى سيرم الدم لهذه الفئران. وعند معاملة هذه الفئران بالمستخلص الأيثانولى لقشر الرمان عن طريق الفم بتركيزين هما 100، 200 مجم/كجم/يوم فقد ظهر تحسن ملحوظ ومعنوى فى هذه الفئران حيث انخفض مستوى اكسدة الليبيدات فى الكلى وكذلك مستوى اليوريا والكرياتنين فى السيرم. وايضا حدث تحسن فى مستوى انزيمات الأكسدة الثلاثه وانزيم جلوتاثيون-س- ترانسفيريز حيث كان مستواهم قريب من المستوى الطبيعى.

وتشير النتائج ان المستخلص الأيثانولى لقشر الرمان يعتبر مضاد للأكسدة وواقى للكلى ضد مركب نيترواستيات الحديديك الذى يسبب تلف تأكسدى فى كلى الفئران.