

Full Length Research Paper

Antioxidant activities of *Punica granatum* (pomegranate) peel extract on brain of rats

Ahmed E. Abdel Moneim

Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt.
E-mail: aest1977@hotmail.com. Tel: (+2) 01003499114.

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Methanol extract of *Punica granatum* (pomegranate) peel was screened for its antioxidant activity on brain of adult male Wister albino rats. The antioxidant activity was determined by measuring reduced glutathione, catalase, superoxide dismutase, glutathione reductase, glutathione-S-transferase, and glutathione peroxidase. In addition, hydrogen peroxide (H₂O₂), nitric oxide (NO) and lipid peroxidation (MDA) were also measured in brain homogenate. Pomegranate peel treatment resulted in marked increase in most antioxidant parameters with reduction in oxidant H₂O₂, NO and MDA. On the basis of the previous results it can be concluded that pomegranate methanol peel extract is a promising natural product, which could be useful for the prevention of neurodegenerative diseases caused by oxidative stress.

Key words: Pomegranate peel, antioxidant enzymes, brain, rats.

INTRODUCTION

Punica granatum L. (Punicaceae), commonly called pomegranate, is a plant used in folkloric medicine for the treatment of various diseases, such as ulcer, hepatic damage and snakebite (Ajaikumar et al., 2005). Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of pomegranate and the individual constituents responsible for them. Extracts of all parts of the fruit appear to have therapeutic properties (Lansky and Newman, 2007), and some studies report that the bark, roots, and leaves of the tree have medicinal benefit as well (Jurenka, 2008).

Studies in rats and mice confirm the antioxidant properties of a pomegranate by-product extract made from whole fruit minus the juice, showing a 19% reduction in oxidative stress in mouse peritoneal macrophages, a 42% decrease in cellular lipid peroxide content, and a 53% increase in reduced glutathione levels (Rosenblat et al., 2006). *In vitro* assay of a fermented pomegranate juice extract and a cold pressed seed oil extract found the antioxidant capacity of both are superior to red wine and similar to green tea extract (Schubert et al., 1999). A separate study in rats with CCl₄-induced liver damage demonstrated pretreatment with a pomegranate peel extract enhanced or maintained the free-radical scavenging activity of the hepatic enzymes catalase,

super oxide dismutase, and peroxidase, and resulted in 54% reduction of lipid peroxidation values compared to controls (Chidambara et al., 2002).

Little information has been published regarding the antioxidant activities of pomegranate peel extract on brain. The current study aimed to evaluate the beneficial effect of pomegranate peel on brain antioxidant activity that may make it one of the more important foods of the future.

MATERIALS AND METHODS

Experimental animals

Adult male albino Wistar rats weighing 120 to 150 g were obtained from The Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). Animals were kept in wire bottomed cages in a room under standard condition of illumination with a 12 h light-dark cycle at 25±1°C. They were provided with water and balanced diet *ad libitum*. The experiments were approved by the state authorities and followed Egyptian rules on animal protection.

Pomegranate peel extracts preparation

Pomegranate peels were manually separated, sun-dried and

ground to powder. The powder was extracted with methanol (100 ml) at 45°C for 1 h. The extract was filtered to remove the peel particles. The extracts were concentrated under vacuum at 40°C to get a concentrate, which was dried in a vacuum at 40 to 50°C and stored at 3 to 4°C until used and designated as methanol extract of pomegranate peel.

Test for tannins

The aqueous extract (1 ml) was mixed with 10 ml of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins or catechol tannins, respectively.

Reducing power

The Fe³⁺ reducing power of the extract was determined according to the method of Oyaizu (1986). The extract (2 ml) was mixed with 0.2 M phosphate buffer, pH 6.6 (2 ml) and 1% potassium ferricyanide (2 ml). The mixture was then incubated at 50°C for 20 min. Afterwards, the mixture was stopped by adding 10% trichloroacetic acid (2 ml) and then centrifuged at 3,000 rpm for 10 min. The upper layer of supernatant (2 ml) was mixed with distilled water (2 ml) and 0.1% FeCl₃ solution (0.5 ml). The absorbance was measured at 700 nm against a blank with a spectrophotometer, and ascorbic acid was used as a standard. Higher absorbance of the reaction mixture indicated greater reducing power. The percents of reducing power were presented as ascorbic acid equivalents using a calibration curve between the absorbance of the reaction and the percent of the reducing power ability of ascorbic acid:

$$OD = (0.0146 \times [\text{percent}]) + 0.0016, R^2 = 0.9999.$$

Determination of total flavonoids

For the assessment of flavonoids, colorimetric method introduced by Dewanto et al. (2002) was adapted. To determine the amount of flavonoids by the aforementioned method, 1.50 ml of the deionized water was added to 0.25 ml of the sample extract and then 90 µl of 5% sodium nitrite (NaNO₂). Six min later, after addition of 180 µl of 10% AlCl₃, mixture was allowed to stand for another 5 min before mixing 0.6 ml of 1 M NaOH. By adding deionized water and mixing well, final volume was made upto 3 ml. Using blank, absorbance was measured at 510 nm. Calibration curve was prepared using quercetin acid as standard for total flavonoids which was measured as mg quercetin equivalents (QE) per gram of the sample (mg/g).

Determination of total phenolics

To analyze the total phenolic content (TPC), Kim et al. (2003) method was followed to make the use of Folin Ciocalteu reagent. To 0.4 ml of the extract (prepared in methanol with a concentration of 1.0 mg/ml), 1.0 ml of (10%) Folin-Ciocalteu reagent was mixed and solution was allowed to stand at 25°C for 5 to 8 min before adding 0.8 ml of 7.5% sodium carbonate solution and using deionized water, final volume was made to 10.0 ml. After two hours, absorbance was measured at 765 nm. Calibration curve was prepared using gallic acid as standard for TPC which was measured as mg gallic acid equivalents (GAE) per gram of the sample (mg/g).

Experimental protocol

To study the effect of pomegranate peel, twelve adult male Wistar

albino rats were randomly divided into two groups, six rats of each. Group I served as control (CON) and received saline (0.2 ml saline/rat) by oral administration via epigastric tube. Group II received oral administration of 200 mg/kg methanol extract of pomegranate peel (Parmar and Kar, 2008) for 21 days and served as methanol extract of pomegranate peel (MEPP) group. The animals of all groups were cervically dislocated and blood samples were collected from retro-orbital plexus. Blood stranded for half an hour and then centrifuged at 500 g for 15 min at 4°C to separate serum and stored at -70°C until analysis. Brains of rats were carefully removed, weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose (Tsakiris et al., 2004) and centrifuged at 500 g for 10 min at 4°C. The supernatant was used for the various biochemical determinations.

Biochemical estimations

Lipid peroxidation

Lipid peroxidation in brain homogenate were determined according to the method of Ohkawa et al. (1979) by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, followed by heating in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as malondialdehyde (MDA) equivalents formed.

Nitrite/Nitrate

The assay of nitrite in brain homogenate was done according to the method of Berkels et al. (2004). In acid medium and in the presence of nitrite the formed nitrous acid diazotises sulphanilamide, which is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color which was measured at 540 nm.

Hydrogen peroxide assay

Hydrogen peroxide content in brain tissues of different groups were determined according to the method of Fossati et al. (1980). In briefly, In the presence of peroxidase, H₂O₂ of brain homogenate was reacted with 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore determined spectrophotometrically at 510 nm.

Estimation of reduced glutathione

The reduced glutathione (GSH) of brain was determined by the methods of Ellman (1959). The method based on the reduction of Ellman's reagent (5,5'-dithiobis, 2-nitrobenzoic acid) with GSH to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance were measured at 405 nm.

Levels of the brain anti-oxidant enzymes

Catalase activity was assayed by the method of Aebi (1984). Catalase reacts with a known quantity of H₂O₂. The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase (HRP), the remaining H₂O₂ reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color

Table 1. Quantitative analysis of tannins and their type in the methanol extract of pomegranate peel.

Parameters	Amount
Gallic tannins	+++
Catechol tannins	-

+: present; -: not present.

intensity inversely proportional to the amount of catalase in the original sample.

Brain superoxide dismutase (SOD) activity was assayed by the method of Nishikimi et al. (1972). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

Glutathione-S-transferase (GST) activity in brain was assayed by the method of Habig et al. (1974). The total GST activity is estimated by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample.

Brain glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine (1967). The assay is an indirect measure of the activity of GPx. Oxidized glutathione (GSSG), produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase. The reaction was initiated by the addition hydrogen peroxide to the reaction and the oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm.

Glutathione reductase activity of brain was assayed by the method of Factor et al. (1998). Glutathione reductase catalyzes the reduction of glutathione in the presence of NADPH, which is oxidized to NADPH⁺. The decrease in absorbance at 340 nm is measured.

Statistical analysis

The obtained data were presented as means \pm standard error. Statistical analysis was performed using an unpaired Student's *t*-test using a statistical package program (SPSS version 17.0).

RESULTS

Tannins, reducing power, total phenolic and flavonoids contents on pomegranate peel extract

The MEPP was given positive tests for gallic tannins while the extract given negative result for catechol tannins as shown in Table 1. The reducing power (RP) and the total phenolic (TPC) and flavonoids (TFC) contents in pomegranate peel methanol extracts are shown in Table 2. The direct correlation between antioxidant activity and reducing power of certain plant extracts were reported. The presence of reductants (antioxidants) in the extracts would result in the reduction of iron (III) to iron (II). MEPP showed 20.8% of activity at 1 g sample/ml. The result indicated that MEPP contained electron donors and possessed the ability to reduce iron

(III) to iron (II).

It was well-known that plant phenolics and flavonoids are highly effective free radical scavengers and antioxidants. *P. granatum* contained high amounts of phenolic and flavonoids compounds. It was shown that MEPP contained phenolic and flavonoids compounds at 124.34 mg GAE/g and 59.44 mg QE/g, respectively. This result indicated that the potent antioxidant activity of MEPP may be related to the phenolic and flavonoids compounds in the extract.

Potential role of pomegranate peel on oxidant molecules

In MEPP treated animals H₂O₂, MDA and NO as markers for oxidative stress were determined in brain and serum (Table 3) where H₂O₂ reduced significantly in both serum and brain (-12.3% and -15.6% *p* < 0.005), respectively, while MDA (-10.9%) and NO (-23.6%) significantly reduced in brain homogenates only.

Beneficial effect of pomegranate peel on antioxidant molecules

Reduced glutathione in brain and serum changed non-significantly (Table 4) due to MEPP treatment, while SOD and CAT increased significantly in brain homogenates of rats by 258.9 and 22.0%, respectively.

The activity of antioxidant enzymes (GR, GPx and GST) were measured in brain homogenates of rats (Table 5). MEPP administration caused significant increase in GR and GST activity (61.8 and 11.1%, respectively) with non-significant change in the activity of brain GPx.

DISCUSSION

It has been reported that oxidizing biological material leads to a rapid burst of ROS, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl ([•]OH) generated primarily because of the ionizing of water molecules (Agrawal et al., 2001), which then interact with biological target molecules, causing lipid peroxidation and DNA damage, and subsequently resulting in cell killing and

Table 2. Reducing power (RP), total phenolic content (TPC) and total flavonoids content (TFC) in the methanol extract of pomegranate peel.

Parameters	Contents
RP (%/ g sample)	20.80 ± 1.56 ^a
TPC (mg GAE/g sample)	124.34 ± 5.42 ^a
TFC (mg QE/ g sample)	59.44 ± 3.71 ^a

^a Values expressed are the mean of three replications.

Table 3. Hydrogen peroxide content (H₂O₂) malondialdehyde (MDA) and nitrite/nitrate (NO) in brain and serum of rats treated with methanol extract of pomegranate peel.

Parameter groups	Brain H ₂ O ₂ (mM/g tissue)	Serum H ₂ O ₂ (mM/L)	Brain MDA (nmol/g tissue)	Serum MDA (nmol/g tissue)	Brain NO (µmol/ g tissue)	Serum NO (µmol/ g tissue)
Control group	0.89 ± 0.011	0.51 ± 0.004	578.45 ± 31.54	32.37 ± 1.50	110.05 ± 8.54	47.33 ± 4.03
MEPP group	0.78 ± 0.009*	0.43 ± 0.003*	515.49 ± 22.98*	32.58 ± 4.68	84.11 ± 5.26*	52.67 ± 5.85

Values are means ± SE (n=6). *: significant change at $p < 0.05$ with respect to control group.

Table 4. Reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) levels in brain and serum of rats treated with methanol extract of pomegranate peel.

Parameter groups	Brain GSH (mmol/g tissue)	Serum GSH (mmol/g tissue)	Brain SOD (U/g tissue)	Brain CAT (U/g tissue)
Control group	18.73 ± 1.19	1.55 ± 0.34	0.56 ± 0.01	0.82 ± 0.11
MEPP group	20.40 ± 1.20	1.71 ± 0.27	2.01 ± 0.02*	1.00 ± 0.07*

Values are means ± SE (n=6). *: significant change at $p < 0.05$ with respect to control group.

Table 5. Glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) on brain of rats treated with methanol extract of pomegranate peel.

Parameter groups	Brain GR (µmol/ g tissue)	Brain GPx (U/g tissue)	Brain GST (µmol/h/ g tissue)
Control group	82.39 ± 12.90	1891.30 ± 103.79	0.36 ± 0.11
MEPP group	133.30 ± 16.96*	1945.34 ± 95.28	0.40 ± 0.14*

Values are means ± SE (n=6). *: significant change at $p < 0.05$ with respect to control group.

mutations (Nair et al., 2001).

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). Ben 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 methanol extract of pomegranate peel had 210.6±7.3 mg/g total phenolics,

gallic acid equivalents. Hence, it can be suggested that the observed antioxidant activity of pomegranate peel methanol extract in the present study due to the presence of these compounds. In this study, the antioxidant activity of pomegranate peel was evaluated on brain of rats. The present data demonstrate that MEPP reduced lipid peroxidation and nitric oxide in both serum and brain tissue homogenate. The ability of MEPP to reduce the oxidant molecules, it seems likely by scavenging the reactive oxygen radicals. Our results are in agreement with the studies which have demonstrated that *P. granatum* peel extract decreased lipid peroxidation in hepatic, cardiac, and renal tissues (Parmar and Kar, 2008) and had a facilitatory effect on the scavenging ability of superoxide anion and hydrogen peroxide.

Previously, Toklu et al. (2007) have shown that chronic pomegranate peel extract supplementation alleviated oxidative injury of the liver and improved the hepatic structure and function in rats exposed to bile duct ligation. Another study in rats with carbon tetrachloride-induced liver damage demonstrated that pretreatment with pomegranate peel extract resulted in the reduction of lipid peroxidation, while the free-radical scavenging activity of catalase, superoxide dismutase, and peroxidase were significantly enhanced (Chidambara et al., 2002). In the present study, the GSH level that was increased due to MEPP treatment, suggesting that it may be an important factor in protecting the tissue against oxidative injury. Since GSH and the activities of glutathione reductase and glutathione peroxidase, which are critical constituents of GSH-redox cycle, provide major protection in oxidative injury by participating in the cellular system of defense against oxidative damage, they play a critical role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation (Toklu et al., 2009).

In conclusion, pomegranate peel is beneficial for neuronal tissue as well as it has an antioxidant properties and nutritive value.

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