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Effect of Pomegranate (*Punica granatum* L.) Juice and Methanolic Peel Extract on Testis of Male Rats

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Abstract.- Although only recently *Punica granatum* (pomegranate) has been acclaimed for its health benefits, this fruit has long been cultivated and consumed, as a fresh fruit or in the form of beverage, especially in the Mediterranean region. In the present study, the antioxidant effects of the methanolic extract of pomegranate peels (MEPP) and pomegranate juice (PJ) were evaluated in normal male rats. Also, there is no evidence about the positive and/or negative effect of pomegranate and/or its extracts on male fertility. In order to evaluate the beneficial effect of PJ and MEPP on lipid peroxidation and nitric oxide levels in testicular of rats. In addition, non-enzymatic and enzymatic antioxidant molecules as glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) were estimated. Also, testosterone, follicular stimulating hormone (FSH) and luteinizing hormone (LH) were also evaluated in the serum of rats. The results revealed that both MEPP and PJ have potent antioxidant activity by reducing lipid peroxidation and nitric oxide formation in testis tissues of rats. Those activities were extended to non-enzymatic and enzymatic antioxidant defense components such as GSH, CAT, SOD, GR, GST and GPx. Additionally, MEPP and PJ caused high elevation in male sex hormones as testosterone, follicular stimulating hormone and luteinizing hormone. The results obtained showed that MEPP and PJ may contain some biologically active components that may be active against oxidative stress, and this may be the basis for its traditional use for environmental toxins.

Key words: Pomegranate (*Punica granatum* L.), oxidants/antioxidants status, sex hormones, testis, rats.

INTRODUCTION

Sexual dysfunctions increase with ageing and etiological factors, including degenerative diseases, increase in injuries and stress associated with industrialized lifestyles. Reactive oxygen species (ROS) are highly reactive oxidizing agents belonging to the class of free radicals. The production of ROS in various organs including the testis is a normal physiological event; however, the alterations in their synthesis stimulate the oxidation and DNA damage of cells (Sikka, 1996). The plasma membrane of sperms contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa and it is associated with loss of motility and the defects of membrane integrity (Sanocka and Kurpisz, 2004; Henkel, 2005). Hence, the application of ROS scavengers is likely to improve sperm function (Sikka, 1996; Vernet *et al*., 2004).

Pomegranate (*Punica granatum* L.) has been used in the folk medicine of many cultures especially in the Middle East. Pomegranate is rich in antioxidant of polyphenolic class which includes tannins and anthocynins and flavonoids (de Nigris *et al*., 2007; Ricci *et al*., 2006). The content of soluble polyphenols in pomegranate juice varies within the limits of 0.2% to 0.1% including mainly tannins, ellagic tannins, anthocyanins, catechins, gallic and ellagic acids (Gil *et al*., 2000). There are many evidences that flavonoids interact with various biological system (Lairon and Amiot, 1999). It is widely reported that pomegranate exhibits antivirus, antioxidant, antidiabetic, antidiarrheal, anti cancer and antiproliferative activities (Faria *et al.*, 2006; Abdel Moneim, 2012, 2013).

The current study aims to evaluate the beneficial effect of juice and peel extract of pomegranate on testis and male fertility that may makes it one of the most important foods for the future.

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MATERIALS AND METHODS

Experimental animals

Adult male albino Wister rats weighing 120–150g 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-hours 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 juice preparation

The fresh pomegranate fruits, free of blemishes or obvious defects, purchased in August 2010 were dried and powdered before extraction. Ten kg of pomegranates were washed and manually peeled, without separating the seeds. Juice was obtained using a commercial blender (Braun, Germany), filtered with a Buchner funnel and immediately diluted with distilled water to volume of 1:3 and stored at -20ºC for no longer than 2 months (Abdel Moneim *et al*., 2011).

Pomegranate peel extracts preparation

An aqueous extract of the pomegranate peel prepared by mashing in a proportion of 1:2:2 (w peel/v water/v methanol) and left about 48 hours in refrigerator. After meshing, the resulting extract was filtered and then the alcohol extract was pooled. The solvent was evaporated under reduced pressure at 40°–50°C. It was stored at -20°C until used and designated as methanol extract of pomegranate peel (MEPP).

Assessment of quality of pomegranate juice and peel extract quality

Pomegranate juice and peel extract quality were assessed by measuring the total phenolic content and evaluating the alterations after 2 and 3 days of exposure to the same conditions as the juice supplied to the animals. The total polyphenol contents of the pomegranate juice and peel extract were 74.8 and 124.3 µg gallic acid equivalent/ml juice, respectively, determined following the Folin-Ciocalteu method (Kim *et al*., 2003). This parameter was not changed for the evaluated period.

Experimental protocol

To study the effect of pomegranate peel and juice, eighteen adult male albino rats were randomly divided into three 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 for 21 days. Group II received oral administration of 200 mg/kg methanol extract of pomegranate peel (Abdel Moneim *et al*., 2011) for 21 days and served as methanol extract of pomegranate peel (MEPP) group. Group III received oral administration of 3 ml/kg pomegranate juice for 21 days and served as pomegranate juice (PJ) group. The animals of all groups were cervically dislocated and blood samples were collected. Blood standed for half an hour and then centrifuged at 500 x g for 15 min at 4°C to separate serum, the serum was stored at -70°C until analysis. Pieces of testis were weighed and homogenized immediately to give 50% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose. The homogenate was centrifuged at 500 x g for 10 min at 4°C. The supernatant (10%) was used for the various biochemical determinations.

Testis index

At the end of the experimental period, each rat was weighed. The left testis was then removed and weighed. Finally, the testis index was calculated by dividing left testis weight by body weight and then multiplying it by 100 (left testis weight/body weight×100).

Determination of malondialdehyde and nitric oxide

Malondialdehyde (MDA) and nitric oxide (NO) were assayed colorimetrically in testes homogenate according to the method of Ohkawa *et al*. (1979) and Berkels *et al*. (2004), respectively. Where MDA determined by using 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67% and were then heated in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as MDA formed. NO was determined in acid medium and in the presence of nitrite the formed nitrous acid diazotise

sulphanilamide is coupled with N-(1–naphthyl) ethylenediamine. The resulting azo dye has a bright reddish – purple color which can be measured at 540 nm.

Estimation of non-enzymatic and enzymatic antioxidant components

The testicular glutathione (GSH) level was determined by the methods of Ellman (1959). The method based on the reduction of Ellman's reagent (5,5` dithiobis (2-nitrobenzoic acid), DTNB) with GSH to produce a yellow compound . The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. In addition, the activity of testicular antioxidant as catalase (CAT), catalase reacts with a known quantity of H_2O_2 according to the method of Aebi (1984). The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase (HRP), remaining H_2O_2 reacts with 3,5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the activity of catalase in the original sample. 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. Also, the activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) activities were determined by the methods of Paglia and Valentine (1967), Habig *et al*. (1974) and Factor *et al*. (1998), respectively.

Estimation of serum testosterone, follicle stimulating hormone and luteinizing hormone

Quantitative measurement of serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were carried out adopting ELISA technique using kits specific for rats purchased from BioVendor (Gunma, Japan) according to the protocol provided with each kit.

Statistical analysis

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

RESULTS AND DISCUSSION

Pomegranate is an important source of tannins punicalagin, anthocyanins and punicalin (Afaq *et al*., 2005), gallic and ellagic acids (Lansky and Newman, 2007) and also contains vitamin C (Turk *et al*., 2008). The antioxidant and free radical scavenging activity of pomegranate phenolic compounds (Rosenblat *et al*., 2006) and vitamin C (Sonmez *et al*., 2005) have been reported.

None of MEPP and PJ had statistically significant effect on body weights of the male rats during three weeks when compared to the control group. However, testis weights were increased significantly during this period by 54.22% and 46.99%, respectively. Moreover, administration of PJ caused significant increase in testis index at $P<0.05$ when compared to MEPP group (Fig. 1).

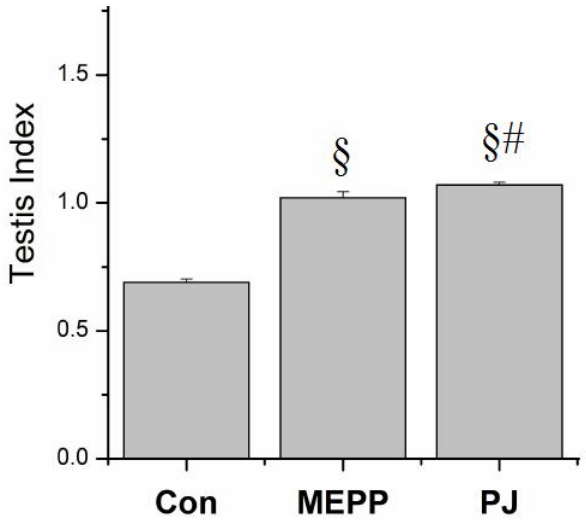

Fig. 1. Effect of methanolic extract of pomegranate peel (MEPP) and pomegranate juice (PJ) on testis index.
§: Significant change at $p < 0.05$ with respect to control group. #: Significant change at $p < 0.05$ with respect to pomegranate juice group.

The MDA and NO results of all treated groups are shown in Table I. MEPP and PJ administration caused significant decreases in testicular MDA and NO levels when compared to the control group ($P<0.05$). Additionally, no significant changes were observed in MDA and NO between MEPP and PJ groups.

Table I.- Malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), catalase (CA) and superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) content in testes of rats treated with methanol extract of pomegranate peel and pomegranate juice.

Parameters	Control group (n=6)	MEPP group (n=6)	PJ group (n=6)
Testicular MDA	548.64±15.96	296.61±4.08§	300.48±11.97§
Testicular NO	97.61±2.46	122.36±3.45§	117.92±4.38§
Testicular GSH (mmol/g)	18.08±0.65	21.39±0.63§#	19.94±1.11
Testicular CAT (U/g)	0.76±0.17	1.14±0.03§	1.10±0.02§
Testicular SOD (U/g)	0.43±0.01	0.60±0.01§#	0.53±0.01§
Testicular GR (µmol/g)	7.23±3.88	7.60±3.62#	6.34±2.26§
Testicular GST (µmol/h/g)	0.31±0.02	0.33±0.01	0.28±0.01
Testicular GPx (U/g)	1144.32±66.07	1281.24±31.63§#	1155.04±92.86

Values are means±SE (n=6). §: Significant change at $p < 0.05$ with respect to control group. #: Significant change at $p < 0.05$ with respect to pomegranate juice group.

Spermatozoa are especially susceptible to peroxidative damage because of the high concentration of polyunsaturated fatty acids which are involved in regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and eventually in membrane fusion and low antioxidant capacity. Obviously, peroxidation of sperm lipids destroys the structure of the lipid matrix in the membranes of spermatozoa and it is associated with the rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects and even it completely inhibits spermatogenesis in extreme cases (Turk *et al*., 2008).

All these activities may be related to diverse phenolic compounds present in pomegranate juice, including punicalagin isomers, ellagic acid derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides). These compounds are known for their properties in scavenging free radicals and inhibiting lipid oxidation in vitro (Li *et al.*, 2006).

Data in Table I demonstrate the potential effect of pomegranate as an antioxidant agent. MEPP was caused significant increase at $P<0.05$ in testisticular GSH, CAT and SOD as compared to control group. While, PJ treatment caused significant increase ($P<0.05$) only in CAT and SOD activity as compared to control values. Moreover, significant increase in GSH and SOD of MEPP group was observed when compared with PJ group.

The activities of GR, GPx and GST in all groups are shown in Table I. MEPP was caused significant increase at $P<0.05$ in GPx activity while PJ caused significant decrease in GR activity as compared with those of control values. In addition to those observations, there were also significant increase ($P<0.05$) between MEPP and PJ at GR and GPx activities.

Phytochemical analysis in my pervious study indicated that pomegranate peel extract gave positive tests for tannins, flavonoids and phenolics which posses potent antioxidant properties (Abdel Moneim, 2011). These results are in accordance with those reported by Qnais *et al*. (2007) whom confirmed that the extract gave positive results for tannins and flavonoid.

Supplementation with PJ and MPPE markedly enhanced the activities of SOD and CAT enzymes and reduced the elevated levels of MDA and NO. These improvements in oxidative stress parameters indicating that pomegranate can counteracte the oxidative stress through its antioxidant properties. Consistent with the results obtained from the study of Turk *et al*. (2008) suggested that pomegranate improved testesticular antioxidant enzymes activities and decreased testesticular MDA and NO levels in testis of rats.

Previous studies of Noda *et al*. (2002), Chidambara Murthy *et al*. (2002) and Singh et al. (2001) confirmed the potent antioxidant activity of pomegranate peel. Moreover, Li *et al*. (2006) found that pomegranate peel had the highest antioxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits. Also, the results of Qu *et al*. (2010) reported that the peel in particular possesses relatively higher antioxidant activity than seeds and

pulp and therefore might be rich sources of natural antioxidants.

Glutathione reductase is responsible for recycling GSSG formed during oxidation events reducing it back to GSH (Abdel Moneim *et al*., 2011). After pomegranate juice intake, a decrease in GR activity was observed. The reduction of this enzyme's activity could be a result of the decreased total glutathione levels. Less glutathione levels will indubitably require less GR activity. It has also been described that certain polyphenols, namely tannic acid and coumarins (Perez-Vicente *et al*., 2002), are able to reduce GR activity (Zhang *et al*., 1997) and the presence of these or related polyphenols, such as tannic acid, in pomegranate (Adams *et al*., 2006, Perez-Vicente *et al*., 2002) may account for the GR inhibition observed.

Testosterone (T) is the main male gonadal hormone produced by the interstitial cells of the Leydig in the testis. T also helps in maintaining body shape and increasing muscle mass and strength. The increase in testosterone should enhance androgen-dependent parameters such as mating behavior and maintenance of spermatogenesis. FSH stimulates spermatogenesis in the Sertoli cells and LH (ICSH) stimulates synthesis and release of testosterone in the Leydig cells (Chauhan and Dixit, 2010). Testosterone may also facilitate male sexual behavior by increasing dopamine release in the medial preoptic area and potentiating nitrergic neurotransmission (Sharma *et al*., 2011).

The effect of pomegranate on male sex hormones is showed in Figure 2. The administration of MEPP caused significant increase in T, FSH and LH levels at $P<0.05$. In addition, PJ administration caused significant increase in T and FSH levels as compared to control group. Moreover, there were significant increase between MEPP and PJ at T and FSH levels.

An increase in stress hormones, such as cortisol, leading to a subsequent decrease in another hormone called gonadotropin releasing hormone (GnRH). GnRH is made in a part of the brain called the hypothalamus and it plays a role in the production of key hormones (LH and FSH) that can affect the quality and quantity of sperm. This stress/fertility link has been fairly well established in years past (Collodel *et al*., 2008). Chronic exposure to stress increases hypothalamicpituitary-adrenal axis activity and concomitantly reduces hypothalamic-pituitary-gonadal axis activity. A study conducted on male rats showed that the sexual behaviour might be the most vulnerable aspect of male reproduction to acute and chronic stress due to the antagonistic relationship between testosterone and corticosteroids (Retana-Marquez *et al*., 2003; Jahan *et al*., 2011). The increase in sex hormones in the present study due to pomegranate juice and peel extract administration can be in part due to the ability of pomegranate to reduce cortisol level as seen by Hong *et al*. (2008).

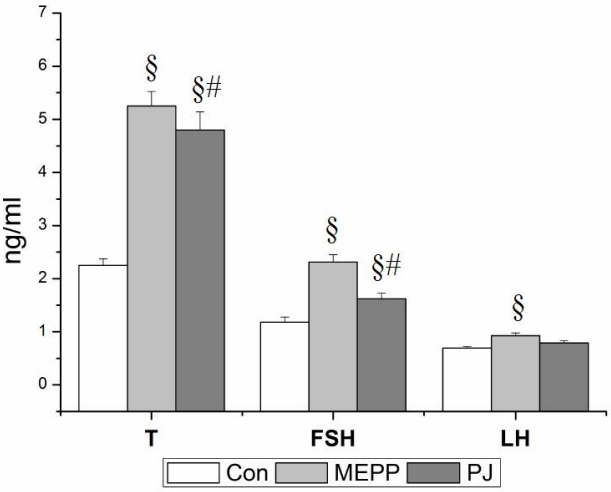

Fig. 2. Effect of methanolic extract of pomegranate peel (MEPP) and pomegranate juice (PJ) consumption on testosterone (T), follicle stimulating hormone (FSH) and luteinizing hormone (LH). Data expressed as ng/ml serum.

In conclusion, pomegranate juice and peel methanolic extract have potent effect on non-enzymatic and enzymatic antioxidant defense components against peroxidative and nitric oxide damage in healthy rats. Hence, it can be said that there is a positive effect of MEPP and PJ consumption on male fertility.

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Competing interests

The authors declared that they have no competing interests.

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