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In vitro AND *In vivo* STUDIES ON ANTIOXIDANT POTENTIAL OF METHANOLIC EXTRACT OF POMEGRANATE FRUITS

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ABSTRACT

The present study was aimed to assess the antioxidant potential of methanolic extract of pomegranate (*Punica granatum)* by *in vitro* assays and *in vivo* model. Under *in vitro* conditions the antioxidant activity of methanolic extract of pomegranate fruit was 198.3, 192.2, 181.1 and 143.2 mg 100 g^{-1} by DPPH, nitric oxide, superoxide radical scavenging assays and ferric reducing antioxidant power assay, respectively. Under *in vivo* conditions, the levels of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase were increased and lipid peroxidation level decreased after supplementation of fresh fruits. The free radical scavenging and antioxidant activities may be ascribed to the presence of total polyphenols (148.6 mg 100 g^{-1}), total flavonoids (209.8 mg 100 g^{-1}) and ascorbic acid content (24.2 mg 100 g^{-1}) in pomegranate fruits. The results indicate that pomegranate fruit is a potential source of natural antioxidant.

Key words: Antioxidant enzyme, lipid peroxidation pomegranate, radical scavenging activity, reducing power

INTRODUCTION

Oxidative damage plays a role in the development of chronic age-related degenerative diseases. The dietary antioxidants oppose this and lower the risk of diseases (Atoui *et al.,* 2005). A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants scavenge free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS is a collective term used for a group of oxidants which are either free radicals or molecular species capable of generating free radicals. Intracellular generation of ROS mainly comprises of superoxide ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) radicals. Nitric oxide and superoxide radicals are converted to powerful oxidizing radicals like hydroxyl radical ($^{\bullet}OH$), alkoxy radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), etc. by complex transformation reactions. Some radical species are converted to molecular oxidants like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), etc. Sometimes these molecular oxidants act as source of ROS (Winterbourn, 2008). Antioxidants greatly reduce the damage by neutralizing free radicals before they may attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and nucleic acid.

Antioxidants can be classified into two major classes i.e., enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione reductase, polyphenol oxidase, etc., and non-enzymatic antioxidants includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, etc. (Mandal *et al.,* 2009). A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases. Though some synthetic antioxidant compounds *viz.*, butylated hydroxy-

toluene, butylated hydroxyanisole, tertiary butyl-hydroquinone, etc. are commonly used in processed foods; however, these compounds have toxic effects like liver damage mutagenesis. Flavonoids and other phenolics of plant origin have been reported as scavengers of free radicals. Therefore, search for natural antioxidant source is gaining more importance.

Pomegranate (*Punica granatum* L.) is a tropical and sub-tropical fruit, consumed either fresh or as beverage (Sumner *et al.,* 2005). Pomegranate is source of many phytochemicals mainly polyphenols which act as antioxidants with potent health benefits. Pomegranate juice contains high concentration of total polyphenols as compared to other fruit juices. The red colour of pomegranate is primarily associated with anthocyanin (cyanidin, delphinidin and pelargonidin) pigments and its peel is rich in polyphenols including ellagitannins such as punicalin, pedunculagin, punicalagin and ellagic acid (Kulkarni and Aradhya, 2005). Pomegranate fruit contains about 40% of an adult's recommended daily requirement of vitamin C and is high in polyphenolic compounds which have been suggested to be involved in the prevention of many diseases. Clinical studies suggest that pomegranate juice changes blood parameters such as low density lipoprotein, high density lipoprotein and cholesterol (Aviram *et al.,* 2004) and is useful against heart disease (Sumner *et al.,* 2005), Alzheimer's disease (Singh *et al.,* 2008) and cancer (Seeram *et al.,* 2007). The present study was aimed to assess the antioxidant potential of methanolic extract of pomegranate fruits through *in vitro* and *in vivo* experiments as well as by determining total phenolic, flavonoid and ascorbic acid contents so as to find relationship between antioxidant activity and phytochemical constituents.

MATERIALS AND METHODS

The present study was conducted at Home Science College and Research Institute, TNAU, Madurai, Tamil Nadu (India). Fresh ripe pomegranate fruits were collected from local market, Madurai, Tamil Nadu. Fruits were washed with distilled water and edible portion (arils) separated. The fruit (50 g) was ground in a domestic blender and 1 g ground-blended fruit sample crushed using a pestle and mortar with 30 mL methanol. The homogenate was centrifuged at 4,500 rpm for 10 min. to obtain a clear supernatant liquid. The residue was re-extracted with methanol (15 mL) and centrifuged. The supernatants were pooled together, filtered through Whatman filter paper (No. 1) and volume made upto 100 mL (Gupta and Prakash, 2009). The extract was kept in a freezer till use. Ascorbic acid content was estimated by titration method and total polyphenols by spectrophotometric method (Sadasivam and Manickam, 2008). Total flavonoids were measured using aluminum chloride colorimetric assay (Marinova *et al*., 2005). The *in vitro* antioxidant activity was measured by using diphenyl- picryl-hydrazyl (DPPH) assay (Goupy *et al.,* 1999), nitric oxide scavenging activity (NOSA) (Garrat, 1964), superoxide scavenging activity (SOSA) (Winterbourn *et al*., 1975) and ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996) with some modifications. The IC_{50} value, defined as the concentration of extract causing 50% inhibition of absorbance, was calculated. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value reflects greater antioxidant activity of the sample. The antioxidant potential is inversely proportional to IC_{50} value, which was calculated from the linear regression of antioxidant activity versus extracts concentration.

Wistar strains of male albino rats each weighing in between 180-220 g were used for *in vivo* antioxidant assay. The experiments were approved by the Institutional of Animal Ethics Committee, India (KMCP/IAEC/115/2013-14). The animals were housed in large spacious cages and fed with commercial pellets with access to water *ad libitum.* The animals were well acclimatized to the standard environmental conditions of temperature (22±5°C), humidity (55±5%) and 12 h light/ dark cycles throughout the experiment. Diabetes mellitus was induced in Wistar rats by single intraperitoneal injection of freshly prepared solution of alloxan monohydrate (150 mg kg^{-1} body weight) in physiological saline after overnight fasting for 12 h. The development of hyperglycemia in rats was

confirmed by plasma glucose estimation 72 h post-alloxan injection. The rats with fasting plasma glucose level of >150 mg dL^{-1} were used for this experiment. A total of 18 rats (12 diabetic surviving rats and 6 normal rats) were used. Diabetes was induced in rats 3 days before the start of experiment. The rats were divided into three groups after induction of 150 mg kg^{-1} of alloxan mono-hydrate through intra-peritoneal diabetes. The groups were as: group - I = normal control (rats given 10 mL kg^{-1} normal saline only with normal diet), group - II = diabetic control (diabetic control received normal diet) and group-III = treatment group (diabetic rat received pomegranate fruit orally for 28 days).

After 28 days of treatment, body weight, blood glucose, plasma insulin and antioxidant enzymes levels were measured. Blood samples was collected in EDTA coating plasma tubes for the estimation of blood parameters. The liver was dissected out immediately, washed with ice-cold saline and 10% homogenates in phosphate buffer solution (pH 7.4) were prepared. Liver homogenate was used for the assay of lipid peroxidation (LPO) while some fraction of homogenate was centrifuged at 7,000 rpm for 10 min at 4°C using refrigerated centrifuge and the supernatants used for the assay of SOD, CAT and GPx. The analysis was performed in triplicate for each sample and data expressed as mean ± standard deviation after proper statistical analysis. Data was analyzed using Data Entry Module for AGRES Statistical Software (Version 3.01) developed by TNAU, Coimbatore. The data was subjected to statistical analysis by using factorial completely randomized design method (Gomez and Gomez, 1984). Correlations were worked out using Pearson's correlation coefficient (r) with help of Statistical Package for Social Science for Windows version 16.0. The IC_{50} values were calculated from linear regression analysis using Microsoft excel program.

RESULTS AND DISCUSSION

In vitro assay revealed that the antioxidant activity of methanolic extract of pomegranate fruits was 198.3; 192.2, 181.1 and 143.24 mg AAEAA 100 g^{-1} by DPPH, NOSA, SOSA and FRAP methods, respectively (Fig. 1). Akbarpour *et al.* (2009) found the total antioxidant activity within a range of 157.3-419.3 m mol 100 mL^{-1} in pomegranate juice as measured by FRAP assay. Tehranifar *et al.* (2010) reported antioxidant activity of various pomegranate cultivars within the range of 15.6 to 40.7%. DPPH-ABTS scavenging activities of pomegranate extracts were in the range of 0.467 to 0.857 TEAC mM 100 mL^{-1} juice (Elfalleh *et al.*, 2009).

The methanolic extract of pomegranate fruits contained total polyphenols (148.6 mg 100 g^{-1}), total flavonoids (209.8 mg 100 g^{-1}) and ascorbic acid (24.21 mg 100 g^{-1}) [Fig. 2]. Ozgen *et al*. (2008) have reported phenolic content of 1245-2076 mg GAE L^{-1} in 6 pomegranate varieties grown in Mediterranean region of Turkey. Less total phenolics in present study could partially be attributed to the differences in solvents used for extracting fruits, geographic sources of fruits and varieties used.

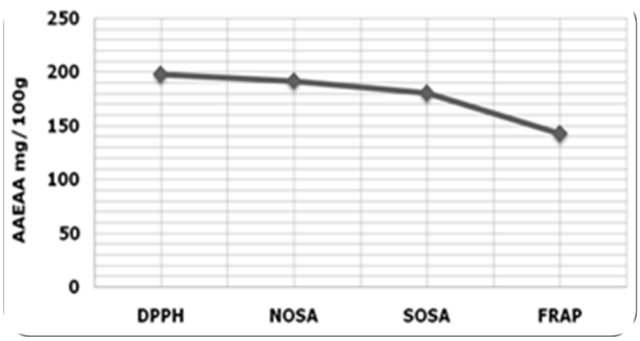

Fig. 1: Antioxidant activity of methanolic extract from pomegranate fruits by different *in vitro* antioxidant assays

Polyphenols are secondary metabolites and derivatives of pentose phosphate, shikimate and phenyl-propanoid pathways in plants (Balasundram *et al.*, 2006). They are one of the most occurring phytochemicals in plants including fruits pericarp. In addition to their contribution to and sensory characteristics in fruits and vegetables, phenolics play an important role in providing protection against *in vivo* and *in vitro* oxidation. Ardekani *et al.* (2011) found the flavonoid content in pulp extracts of Persian pome-

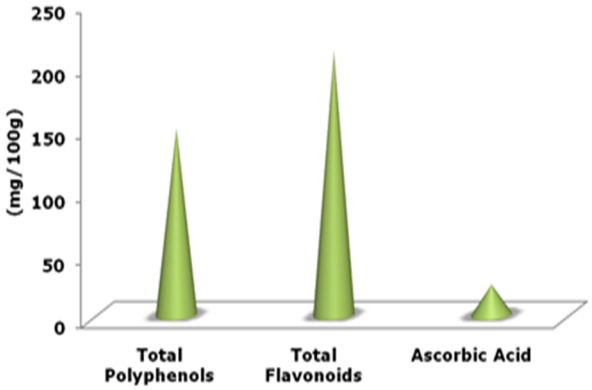

Fig. 2: Total polyphenols, flavonoid and ascorbic acid content of in methanolic extracts from pomegranate fruits

granate cultivars within the range of 0.84-2.14 mg CE g^{-1}. Tehranifar *et al.* (2010) reported ascorbic acid content in 20 Iranian pomegranate cultivars within the range of 9.9 to 20.9 mg 100 g^{-1}.

DPPH free radical scavenging activity has widely been used to detect antiradical activity antiradical activity of different samples due to its sensitivity to lower concentrations of active principles from natural sources. The stable radical, DPPH, has maximum absorbance at 517 nm and could readily be scavenged by antioxidants. Higher free radical scavenging activities of samples was indicated by lower absorbance at 517 nm. The free radical scavenging activity of methanolic extract of pomegranate fruits was detected by DPPH assay and compared with standard ascorbic acid (Table 1). The IC_{50} values for methanolic extract of pomegranate fruits and ascorbic acid were 400 and 6.7 µg mL^{-1}, respectively. Khomdram and Shantibaladevi (2010) observed the IC_{50}value of 398 µg mL^{-1} *in* pomegranate. Reddy *et al.* (2007) have reported total crude tannins in pomegranate fruit and purified constituents (ellagic acid and punicalagins) possess antioxidant activity and strongly inhibit ROS generation with IC_{50} of 0.33-11 µg mL^{-1}. Depending on the polyphenolic composition, DPPH scavenging activity varied from 74 to 4485 µM g^{-1} in pomegranate fruit products (Madrigal-Carballo, 2009). Antioxidant activity (DPPH, FRAP, NOSA and SOSA) was found significantly correlated (p<0.01) with antioxidant components (Table 2). Highly significant positive correlation indicates that free radical scavenging and ferric reducing activities are mainly attributed to the antioxidants involved. More likely they appear responsible for scavenging most of the free radicals in pomegranate fruits. This revealed that ascorbic acid, total polyphenols and total flavonoids are major contributors to antioxidant activity in pomegranate. The highly positive correlation between antioxidant activity and ascorbic acid has previously been reported by Amararatne *et al.* (2012) in pomegranate fruit juice.

Table 1: Free radical scavenging activity of fruit extract of pomegranate

Test material	Concentration used (µg mL^{-1})	Inhibition (%)	IC_{50} (µg mL^{-1})
Fruit extract	100	12.56	400
	200	25.48	
	300	37.62	
	400	50.21	
	500	62.91	
Ascorbic acid (standard control)	1	6.36	6.7
	2	15	
	4	30.9	
	6	43.63	
	8	60.45	
	10	67.27	

Table 2: Pearson's correlation coefficient (r) among antioxidants in pomegranate fruits

Attributes	Ascorbic acid	Total polyphenols	Total flavonoids
DPPH $_{AAEAA}$	0.636**	0.831**	0.632*
FRAP $_{AAEAA}$	0.895**	0.991**	0.721*
NOSA $_{AAEAA}$	0.888**	0.992**	0.715*
SOSA $_{AAEAA}$	0.876**	0.994**	0.705*

*Correlation is significant at the 0.05 level (p<0.05);
**Correlation is significant at the 0.01 level (p<0.01);
AAEAA-ascorbic acid equivalent antioxidant activity

The oral administration of pomegranate fruit for 28 days reduced blood sugar from 180.55±6.30 to 120.30±4.53 mg 100 mL^{-1} (Table 3). A significant increase in plasma insulin was noted when alloxan induced diabetic rats. Osman *et al.* (2012) found that oral administration of pomegranate juice (5 mL kg^{-1} b.w.) significantly decreased glucose level (227.8-164.4 mg dL^{-1}) and increased insulin level (2.10-3.54 µU mL^{-1}) as

Table 3: Effect of pomegranate fruits on antioxidant enzymes, blood glucose and plasma insulin in diabetic rats

Parameters	Normal control (G1)	Diabetic control (G2)	Treatment group (G3)
SOD (U mg^{-1} protein)	132.25 ± 2.40	68.20 ± 1.65*[a]	110.70 ± 3.70*[b]
CAT (U mg^{-1} protein)	290.40 ± 2.40	190.75 ± 2.70*[a]	255.82 ± 3.70*[b]
GPx (U mg^{-1} protein)	1.10 ± 0.05	0.40 ± 0.02*[a]	0.70 ± 0.02*[b]
MDA (U mg^{-1} protein)	3.90 ± 0.17	7.40 ± 0.12 *[a]	4.95 ± 0.0 *[b]
Blood glucose (mg 100 mL^{-1})	88.70 ± 3.25	180.55 ± 6.30**[a]	120.30 ± 4.53**[b]
Plasma insulin (µU mL^{-1})	38.60 ± 2.90	14.70 ± 1.72**[a]	28.40 ± 2.45**[b]

*[a]values are significantly different from normal control (G1) at $p < 0.01$.
*[b] values are significantly different from diabetic control (G2) at $p < 0.01$.

compared to diabetic rats. In liver homogenates significant decreases in SOD, CAT and GPx levels and increase in LPO levels were observed in rats treated with alloxan 150 mg kg^{-1} (G2) as compared to normal control. The oral administration of pomegranate fruit for 28 days increased the levels of SOD, CAT and GPx and decreased the levels of LPO significantly (p <0.01) in G3. The results are at par with Bhaskar and Kumar (2012) who found that oral administration of pomegranate flower extract significantly increased SOD, CAT and GPx levels in diabetic rats.

Conclusion: In this study the methanolic extract of pomegranate fruit was assessed with various antioxidant systems. The results revealed that pomegranate fruits possessed abundant phenolic and flavonoid contents and exhibited good antioxidant activities as compared to standard ascorbic acid. Further, the pomegranate fruits exhibited potential antioxidant effects in diabetic rats. The *in vitro* and in *vivo* antioxidant potential of pomegranate may be attributed to the presence of active compounds such as polyphenols, flavonoids and ascorbic acid.

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