Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Suppl 2, 2013 ISSN - 0974-2441

Research Article

ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITY OF FLAVANOID EXTRACT FROM FRUIT PEEL OF PUNICA GRANATUM

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Received: 2 March 2013, Revised and Accepted: 20 March 2013

ABSTRACT

Antihemolytic and antioxidant activities of pomegranate fruit peel were investigated employing different *in vitro* assay systems. The phytochemical screening reveals the presence of flavanoids in the crude extract. The total amount of phenolic compounds in flavanoid extract was determined as catechol equivalents. The extract showed good Fe^{2+} chelating ability, EC_{50} was 643 µg/ ml. The extract also exhibited high antioxidant activity (88%) against hemoglobin induced linoleic acid system and also capable of scavenging hydrogen peroxide in a concentration dependent manner. In addition, the extracts also showed higher antihemolytic activity with increase in concentration. So we can conclude that the *in vitro* study emphasized PPE (Pomegranate peel extract) effective antioxidant and scavenging activities which may be due to its phenolics and flavanoids contents.

Keywords: Antioxidant activity, Antihemolytic activity, *Punica granatum*

INTRODUCTION

Antioxidant provides protection against numerous chronic diseases, including cancer, coronary heart disease, and Alzheimer's diseases and also involves oxidative damage to cellular components. Reactive oxygen species (ROS), contribute to cellular aging, mutagenesis, carcinogenesis & coronary heart diseases likely through destabilization of membranes, DNA and protein damage[1]. Minimizing oxidative damage is one of the most important approaches in prevention of these age-related diseases and health problems, since antioxidants play a role in suppression of ROS formation, the inhibition of enzymes or chelating of elements involved in free radical production. Antioxidative compounds obtained from natural sources such as grains, oilseeds, beans, leaf waxes, bark, roots, spices, fruits and vegetables, have been investigated[2] .

Oxidation of erythrocytes has been used as a model system for oxidative damage of biomembranes. It has been found that most of ROS attack erythrocyte membranes causing oxidation of the lipids and proteins, and they are also involved in some changes in hemoglobin structure resulting in hemolysis of red blood cells[3]. Free radicals mediated damage of erythrocytes may be inhibited in vivo and in vitro by some antioxidants, especially by vitamin C, vitamin E[4]. Similar antioxidant effects of some food components, mostly hydroxy- and polyhydroxy-organic compounds from vegetables, fruits and some herbs have been also observed[5].

Plants are rich sources of natural antioxidants, the best known are tocopherols, carotenoids, vitamin C, flavonoids and different other phenolic compounds .Recently, among natural antioxidants, dietary flavanoids are considered to be more powerful antioxidants and received increasing attention as potential protectors against a variety of human diseases, in particular cardiovascular disease and cancer[6]. Flavanoids are found higher in plant fruit peel, and are proposed as food supplements. Flavanoids are known to be highly effective antioxidants by scavenging oxygen radicals. Moreover, the protective effects of flavanoids in biological systems are attributed to their capacity to scavenge free radicals, chelates metal catalysts, activate antioxidant enzymes, reduce alpha tocopherol radicals and inhibit oxidases.

The pomegranate (*Punica granatum)* belonging to punicaceae family is widely distributed all over the world and have highly distinctive nutritional value. In the ayurveda system of medicine, the pomegranate has extensively been used as a source of traditional remedies for thousands of years. The rind of the fruit and the bark of the pomegranate tree are used as a traditional remedy against diarrhea, dysentery and intestinal parasites. Pomegranate juice is also used as eye drops as it is believed to slow the development of cataracts. The aim of the present study is to investigate the antihemolytic and antioxidant potential of pomegranate fruit peel.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Pomegranate peel (*Punica granatum*) was collected from local juice centre at K. K. Nagar Chennai. The plant was identified with the help of available literature and authenticated by Dr. S. Sankaranarayanan, Head of the department, Department of Medicinal Botany, Sri Sairam Siddha Medical College, Tambaram, Chennai. The Pomegranate peel were washed with tap water followed by distilled water, dried in shade for 10 days prior to study and then stored in airtight glass jars, until in use.

PREPARATION OF POMEGRANATE PEEL EXTRACT (PPE)

The shade dried fruit peel was ground to fine powder and sieved. Exactly 20g of the finely grounded fruit peel were soaked in 70% methanol at room temperature for 24 hrs. The extract was filtered using Whattmann filter paper No.1 and then concentrated in vacuum at 40°C- 50°C (overnight) using a rotary evaporator. The residue was mixed with various solvents such as petroleum ether and ethyl acetate. Then the ethyl acetate extract was condensed and redissolved in 70% methanol and used for further studies[7].

PHYTOCHEMICAL SCREENING

TEST FOR FLAVANOIDS

The preliminary phytochemical studies[8] were conducted in methanolic crude extracts of pomegranate peel to find out the presence of flavonoids using standard analytical procedures.[9]

ESTIMATION OF FLAVANOIDS

A 1ml aliquot of filtered methanol extract is placed into a 25 ml measuring flask. To this sample,1ml of 2% aluminium chloride and 0.5 ml 0f 33% acetic acid was added, after which the flask is filled with 90% methanol to the mark and the content is thoroughly stirred. The obtained solution is allowed to stand for 30 minutes and the absorbance was measured at 414 nm using a UV-Visible Spectrophotometer. The content of flavonoids is determined by comparison with the optical density of a reference sample solution (RSS) of rutin, which is prepared using a procedure analogous to that described above. The RSS of rutin is prepared from an accurately weighed amount (~0.070 g) of the working standard substance dried for 3 h at 135°C immediately before measurements. This sample is dissolved on heating in 85 ml of 96% ethanol in a 100-ml measuring flask, after which the flask is filled with the same ethanol to the mark.

$$X = \frac{D_x m_0 \text{ X } 50 \text{ X } 100 \text{ X } 100}{D_0 m_x \text{ X } 100 \text{ X } (100 \text{ X } W)}$$

Using the results of optical density measurements, the percentage content of flavonoids *X* in the sample of plant extract is calculated using the formula where D_x and D_0 are the optical densities of the sample solution and the RSS of rutin, respectively; m_x and $m0$ are the exact weights of the extract tested and the rutin standard, respectively and *W* is the percentage weight loss upon drying of the plant material.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The amount of total phenol content was determined by Folin-Ciocalteau reagent method[10]. The different concentration of flavanoid extracts (25-100 µl) and 1 ml of 50% Folin-Ciocalteau reagent was mixed and the mixture was incubated at room temperature for 15 mins. Then 2.5 ml of sodium carbonate solution was added and further incubated for 30 mins at room temperature and the absorbance was measured at 760 nm. Total phenol values are expressed in terms of catechin equivalent (mg/g of extracted compound).

REDUCING POWER ASSAY

The reducing power capacity assessment was determined using the modified method[11]. The different concentration of flavanoid extracts (25-100 µl) was mixed with 2.5 ml of phosphate buffer (200 mM and pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM). The mixture was incubated at 50^0 C for 20 mins. Then 2.5 ml of trichloroacetic acid (600 mM) was added and centrifuged for 10 mins at 5000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 6mM). The absorbance was measured at 700 nm. Ascorbic acid was used as positive control.

METAL CHELATING ASSAY

To determine metal chelating activity of flavanoid extracts was estimated using the method[12]. The different concentration of extracts (25-100 µl) was added to a solution of 0.1 ml of 2 mM $FeCl_2$.This was followed by the addition of 0.2 ml of 5 mM ferrozine solution, which was left to react at room temperature for 10 mins.

The absorbance was measured at 562 nm. The percentage inhibition of Ferrozine–Fe^{2+} complex formation was calculated using the formula:

$$\% \text{ Inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \text{ X } 100$$

EDTA was used as a positive control

ANTIOXIDANT ACTIVITY IN HEMOGLOBIN INDUCED LINOLEIC ACID

The antioxidant activity of flavanoid extracts was determined by modified method[13]. The flavanoid extracts (25-100 µl) 1 mmol/l of linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH-6.5) and 0.0016% hemoglobin was mixed thoroughly and incubated at 37°C for 45 minutes. After incubation, 2.5 ml of 0.6% Hcl in ethanol was

added to stop the lipid per oxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm, after the addition of 0.02 mol/l $FeCl_3$ and 100 µl of ammonium thiocyanate (15g/50ml). Ascorbic acid was used as positive control.

SCAVENGING OF HYDROGEN PEROXIDE ACTIVITY

The hydrogen peroxide scavenging assay was carried out by using the method of[14]. The flavanoid extracts (25-100 µl) was added to hydrogen peroxide solution (0.6 ml, 40mM). The absorbance of hydrogen peroxide was measured at 230 nm. Ascorbic acid was used as a standard. The percentage of H_2O_2 scavenging assay was calculated as;

$$H_2O_2 \text{ Scavenging effect (\%)} = [A_0\text{-}A_1/A_0] \text{ X } 100$$

A_0 = Absorbance of the Control

A_1 = Absorbance in the presence of sample or standard.

ANTIHEMOLYTIC ACTIVITY OF *PUNICA GRANATUM* FRUIT PEEL FLAVANOID EXTRACT

Antihemolytic activity of the flavonoid extract was assessed as described by[13] with a slight modification. Erythrocytes from male Wistar rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4), and diluted with phosphate buffered saline to give a 4% suspension. 1 g of extract/ml of saline buffer was added to 2 ml of the erythrocyte suspension and the volume made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in saline buffer added to induce oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of the blood cells after 240 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of hemolysis determined by measuring the absorbance, corresponding to hemoglobin liberation, at 540 nm.

RESULTS

SCREENING OF FLAVANOID AND TOTAL PHENOLIC CONTENT IN METHANOL FRUIT PEEL EXTRACT OF *PUNICA GRANATUM*

The methanol fruit peel extract of *Punica granatum* showed the presence of flavanoids and also exhibited 16% yield and 98% total phenolic catechin equivalents.

Table 1. Flavonoids screening of methanol fruit peel extract of *Punica granatum*

S/No.	CONSTITUENTS	METHANOL FRUIT PEEL EXTRACT OF POMEGRANATE
1.	**Flavonoids**	
	Alkaline Reagent	+
	Shinoda test	+
	Lead acetate	+

REDUCING POWEROF EXTRACTS

In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donation of an electron. Increasing absorbance at 700 nm indicates an increase in reductive ability. In this study, the reducing power of flavanoid extracts also increased with an increase in their concentration. The maximum reducing property was found at 100 µl/ml of flavanoid extract of *Punica granatum* (Fig-1).

METAL CHELATING ACTIVITY:

Metal chelating capacity of flavanoid extract of *Punica granatum* increased in dose dependent manner. 100 µl/ml of the fruit peel extract reduced the concentration of the catalyzing transition metal in lipid peroxidation.

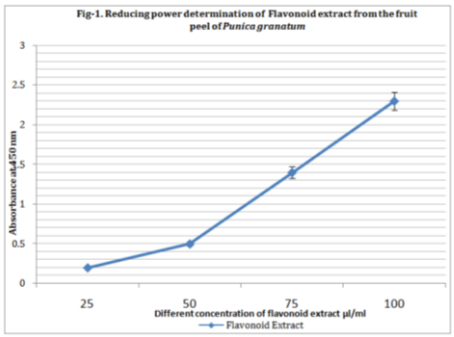


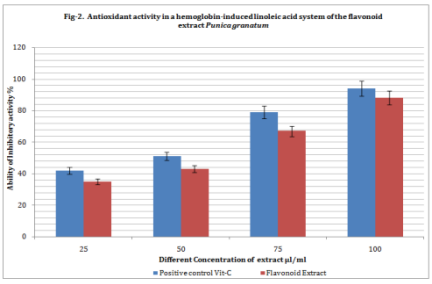
Table-2. Fe^{2+} chelating activity of flavonoid extract of *Punica granatum* fruit peel

Different concentration of *Punica granatum* flavanoid extract	[a] Fe^{2+} chelating activity
25	25±3.0
50	40±2.5
75	63±4.1
100	91±5.0
EC_{50}(µg/ml)	643

[a]Results are expressed as percentage inhibit ion of Fe^{2+} chelating with respect to control. Each value represents the mean+SD of five experiments

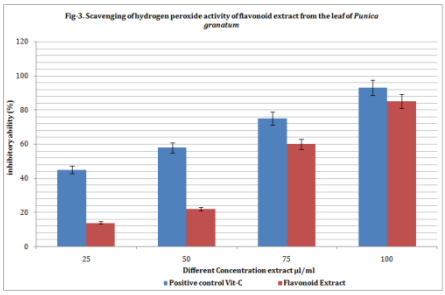
ANTIOXIDANT ACTIVITY IN HEMOGLOBIN INDUCED LINOLEIC ACID

The antioxidant activity of flavanoid extract of *Punica granatum* fruit peel was determined by using the hemoglobin induced linoleic acid system. The compounds in the extract were electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions. The maximum inhibitory activity was 88 % in 100 µl/ml concentration of flavanoid extract of *Punica granatum* (Fig-2).



SCAVENGING OF HYDROGEN PEROXIDEACTIVITY

The flavanoid extract of *Punica granatum* potentially removes hydrogen peroxide by converting H_2O_2 in to H_2O by donating the electron. This scavenging activity increased with increase in concentration. The maximum activity was observed at 100 µl/ml (Fig-3).



ANTIHEMOLYTIC ACTIVITY OF FLAVANOID EXTRACT OF PUNICA GRANATUM FRUIT PEEL

The flavanoid extract of *Punica granatum* fruit peel showed higher antihemolytic activity with the increase in concentration. The membrane damage induced by the H_2O_2 in the erythrocytes was inhibited by the flavanoid extract of *Punica granatum* fruit peel (Table-3).

Table-3. Antihemolytic activity of flavanoid extract of *Punica granatum* fruit peel

Different concentration of *Punica granatum* flavanoid extract	[a] Antihemolytic activity of flavanoid extract
25	17.23±3.0
50	32.1±3.5
75	56.3±6.2
100	90±2.0
EC_{50}(µg/ml)	254

[a]Results are expressed as Antihemolytic activity with respect to control. Each value represents the mean±SD of five experiments.

DISCUSSION

Medicinal plants produce biologically active substances, some of which are related to special flavour or taste. Some of the biologically active substances are found useful as antioxidants and or antimicrobial agents. Antioxidant prevents oxidation and controls the oxidative damage to cells and biomolecules. Dietary antioxidants containing phenolic compounds are the most abundant natural antioxidant[15]. These phenolic compounds function as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers and cell saviours[16].The effective activity of antioxidants in protecting oxidative induced injuries receives more attention in the fields of life sciences.

Punica granatum flavanoid extract showed high total phenol and flavanoid contents. Food products derived from plant sources exhibited significant antioxidant activity due to the presence of high content phenols and polyphenolic compounds[17]. Plant products like flavanoids improves the quality of life by directly influence the iron (III) ions level within tissues[16]. Flavanoids reduces the iron related complications by mobilizing tissue iron and excrete in faeces and urine[18]. The absorbance of Fe^{2+} ferrozine complex in the present study decreased in dose dependent manner with increasing concentration from 25 to 100 µl/ml. Metal chelating capacity was significant since the extract reduced the concentration of catalyzing transition metal in lipid peroxidation.

Punica granatum fruit peel flavanoid extract was also capable of scavenging hydrogen peroxide in a concentration dependent manner. Unsaturated fatty acids like linoleic acid and arachidonic acid undergo lipid peroxidation. Hence the membrane lipids which contain unsaturated fatty acids are involved in oxidation process[19]. *Punica granatum* fruit peel flavanoid extract potentially controls the oxidation of unsaturated fatty acids. Higher concentration of polyunsaturated fatty acids (PUFA) in membrane and oxygen transport associated with redox active hemoglobin molecules leads the free radical attack on erythrocytes. Singlet oxygen and hydroxyl radical formed from superoxide anion initiates lipid peroxidation. Thus superoxide indirectly involves in lipid peroxidation[20]. Reactive oxygen species promotes the free radical attack resulting in lipid per oxidation. The *Punica granatum* fruit peel flavanoid extract showed good activity in hemoglobin-induced linoleic acid system due to the presence of high total phenolic content in the extract and thus acting as potential antihemolytic extract.

CONCLUSION

The natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy. In this study, the flavanoid extract of pomegranate fruit peel were investigated with various antioxidant systems. The results indicated that *Punica*

granatum fruit peel possessed abundant phenolic and flavanoid contents and exhibited excellent antioxidant activities comparing to standard (Ascorbic acid). In addition, the flavanoid extract also exhibited good antihemolytic activity. Therefore, the flavanoid extract from *Punica granatum* is worthy for further studies on definitive mechanisms of its chemotherapeutic activities and potential effects in vivo are needed.

ACKNOWLEDGEMENT

My sincere thanks to the management of Gloris Biomed Research Centre (P) Ltd for providing me good lab facilities, valuable suggestion and their continuous support throughout my research study.

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