Research Paper

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Evaluation of Antioxidant and Free Radical Scavenging Activities of Hydroethanolic Extracts of Punica Granatum Leaves, Seeds and Rind

Biochemistry

KEYWORDS : Punica granatum(PG), Antioxidant, free radicals, oxidative stress.

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ABSTRACT

Oxidative stress caused by free radicals is associated with various degenerative diseases. Antioxidants are the substances that are responsible for scavenging free radicals. The aim of the present study was to evaluate the phytochemical and in vitro antioxidant properties of hydroethanolic extract of Punica granatum leaves, seed and rind. The results showed the presence of phenols, flavanoids and tannins in all the extracts and the free radical scavenging activity of the extracts were found to be increased in a dose-dependent manner. So the results obtained in the present study indicated that the hydroethanolic extracts of Punica granatum can be a potential source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stress mediated diseases.

Introduction:

Free radicals are electrically charged molecules with at least one unpaired electron in the outer most shell that cause them to seek and capture electrons from other substances to neutralize themselves (1). The role of free radical mechanism in disease pathology is well established, that these mechanisms are necessary for normal metabolism but can be detrimental to health as well. Those free radical reactions cause diseases such as atherosclerosis, aging, ischemic heart diseases, inflammation, diabetes, immunosuppression, neurodegenerative diseases etc., (Harman, 1988; Maxwell, 1995). Free radicals are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function (4). Antioxidants are the substances that neutralize free radicals and their action (5). The imbalance that occurs between the antioxidants and free radicals results in oxidative stress, leading to cellular damage (6). Antioxidants play important role in reducing oxidative stress that is thought to cause damage to biological molecules (7). Some of the synthetic antioxidants developed were suspected to have some adverse effects. Therefore, in search of suitable alternative natural antioxidants have received much attention to identify and develop more potent antioxidants of natural origin to replace synthetic ones (8).

Medicinal plants are the important source of antioxidants that contain some organic compounds which produce physiological action on the human body and bioactive substances include tannins, Carbohydrates, alkaloids, terpenoids, glycosides, saponins and flavanoids (9). Several studies have reported the antioxidant properties of medicinal plants rich in phenolic compounds (10, 11). The secondary metabolites like phenolics and flavanoids from plants have been reported to have potent free radical scavenging activity. They were found in all parts of the plants such as leaves, fruits, seeds, roots and bark (12). Phenolic compounds from medicinal plants possess strong antioxidant activity and help to protect cells against the oxidative damage caused by free radicals (13).

Punica granatum, popularly known as pomegranate belongs to the family Punicaceae, which has been used as a natural source of medicine. The use of plant compounds for pharmaceutical purpose has been increased gradually worldwide. This plant consists of chemical components that possess various pharmacological activities (14).The potential therapeutic property of pomegranate includes treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction and protection from UV radiations. And also it plays a role in the treatment of brain ischemia, male infertility, Alzheimer's disease, arthritis and obesity (15). Therefore, the purpose of the present investigation was to evaluate the antioxidant activity of leaf, seed and rind extracts of PG.

Materials and methods:

Chemicals

All the chemicals of analytical grade were purchased from Himedia Laboratories Pvt. Limited India.

Plant material and extraction

Punica granatum was collected from local areas of Coimbatore, Tamil nadu, India. The plant was identified and certified (BSI/SRC/5/23/2013-14/Tech/483) by the Taxonomist, Botanical Survey of India (BSI), Southern Regional Centre, Coimbatore, Tamil nadu, India. The leaf, seed and rind of PG(1 kg each) were shade dried. Powdered in a mixer grinder and stored in air tight container (16).The dried powder was mixed with various solvents (1:3 ratio) namely water, ethanol, 50% ethanol, acetone, chloroform and petroleum ether, filtered and the phytochemical analysis was done to identify the efficient plant extracts for the study.

Phytochemical analysis

Various extracts of PGleaf, seed and rind were studied for their phyto-constituents using different phytochemical tests (17).

Preparation of Hydroethanolic extracts of PG

The Hydroethanolic extract which showed the presence of most phytochemical was prepared in large scale as above, cold macerated for 72 hours withoccasional stirring, filtered and the filtrate was then concentrated to dryness under reduced pressure in a water bath to obtain the crude hydroethanolic extract.

Quantitative phytochemical analysis

Total phenol determination

The total phenolic content present in the 50 % ethanolic extract of PG leaf, seed and rind was determined using Folin-Ciocalteau reagent (18). Using standard curve, total phenolic content was calculated and expressed as catechol equivalent in mg/g of extract.

Flavanoids determination

Flavones and flavanols in the 50% ethanolic extracts of PG was determined using Aluminium Chloride colorimetric method (19).

Determination of Tannins

Tannin was determined by Folin-Danis method (20). The absorbance was measured at 700nm and the tannin content was evaluated from tannic acid standard graph.

Invitro antioxidant assay

DPPH assay (2,2-Diphenyl-1-picryl hydrazyl)

a) Dot Plot assay:

An aliquot (3µl) of each sample was carefully loaded onto the pre-coated TLC plate and allowed to dry. After 3 minutes TLC plate was placed upside down for 10 seconds in 0.04 mM DPPH

solution in methanol. Discoloration of DPPH indicates scavenging potential of the substances tested (21).

b) Photometric assay:
The stable DPPH radical was used for the determination of free radical scavenging activity of 50% ethanolic extracts of PG leaf, seed and rind (21). Briefly, 1 ml of DPPH solution in methanol was added to 0.5ml of plant extracts in different concentrations. After 15 minutes, the absorbance was recorded at 517nm, using ascorbic acid as a positive control.

Nitric oxide Radical Scavenging activity
Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which was measured by Griess reaction (22). The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

Reducing power assay
Hydroethanolic extracts of PG showed potent antioxidant power by reducing power ability and measured according to Jayaprakesh*et al.,* 2001 (23).

Statistical analysis
All the data were expressed as mean ± Standard deviation (SD) of the number of experiments (n = 3).

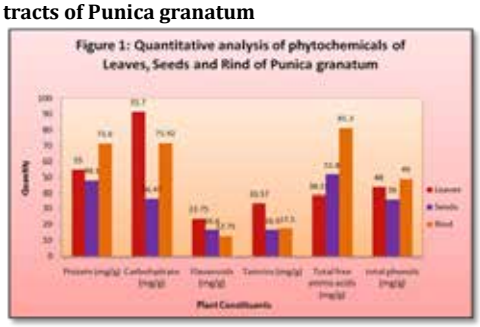
Results:
Phytochemical analysis
Phytochemical evaluation was performed for qualitative detection of various chemical constituents that aid in tracing the presence of active compounds that elicit a major pharmacological response. The results on the qualitative analysis of phytochemical constituents of different extracts of PG leaf, seed and rind are shown in Table-1.

Table 1: Qualitative Phytochemicals analysis

Plant Constituents	Extractive solvents																				
	Aqueous			Ethanol			50% ethanol			Acetone			Benzene			Petroleum ether			Chloroform		
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
Carbohydrate	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Proteins	+	-	+	+	-	+	+	+	+	+	-	-	-	-	-	+	-	-	+	-	-
Flavanoids	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+
Phenolics	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-
Saponins	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	+	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Alkaloids	-	-	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Thiols	+	-	+	+	-	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-
Triterpenoid	-	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-
Diosgenin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-

Quantitative Phytochemical analysis:
Figure 1 showed the availability of quantifiable, total phenol, flavanoids and tannin. All the three extracts showed higher amount of phenolics when compared to flavanoids and tannins.

Figure 1: Quantitative analysis of leaf, seed and rind extracts of Punica granatum



DPPH scavenging assay

a) Dot plot assay
Dot plot test of DPPH provides simplified version to detect the antioxidant properties of various molecules present in the extracts (Figure 2a). A DPPH solution is decolorized when the old electron becomes paired off in the presence of a free radical scavenger. So the color becomes light yellow from deep violet. Dose dependent dot assay was observed for all the extracts.

b) Photometric assay
DPPH radical scavenging assay is one of the most widely used method for screening the antioxidant activity of the plant. In the present study all the extracts were found to be effective scavengers against DPPH radical. The scavenging activity of 50% ethanolic extract of leaf, seed and rind extracts of PGwas found to be in a dose-dependent manner (figure 2b). It showed the reduction of the free radical to the corresponding hydrazine, when it reacts with the hydrogen donor present in the antioxidants. The degree of discoloration indicated the scavenging potentials of the antioxidant.

Nitric oxide Scavenging assay
The extracts showed ability to scavenge NO and inhibit nitrite formation that was generated *in-vitro* by sodium nitroprusside. The nitric oxide scavenging activity of the extracts increased with the increasing concentration of the plant extracts (Figure 2c). All the extracts showed maximum nitric oxide scavenging activity at concentration 500µg/ml.

Reducing power assay
The reducing power of 50% ethanolic leaf, seed and rind extract of PGwas determined by Potassium ferricyanide reduction method, in which the extracts showed dose-dependent reducing power activity (Figure 2d).

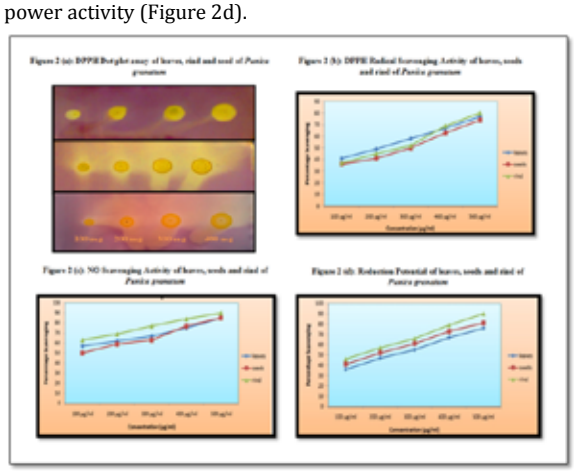


Figure 2: Free radical scavenging activity of Leaf, Seed and Rind of Punica granatum

Discussions
The result of the present study showed that the hydroethanolic extracts of PG contains highest amount of phenolic compounds exhibited the greatest antioxidant activity. Phenolics compounds are well known antioxidant and scavenging compounds against the free radicals associated with oxidative damage (24). The high scavenging property of PG may be due to hydroxyl groups existing in the phenolic compounds. Flavanoids and tannin are also present in PG appropriate quantities, which also involved in the management of oxidative stress treatment. There are numerous antioxidant methods for evaluation of antioxidant activity. For *invitro* antioxidant screening DPPH, NO and Reduction power assay are commonly used. In DPPH assay, dot plot test showed the discoloration of deep violet to yellow on dose-dependent manner. And the photometric assay also showed the reduction of free radical in a dose-dependent manner, which showed the antioxidant potential of the plant. Nitric oxide is a free radical generated from nitroprusside and reacts with oxygen to form oxides of nitrogen. This was significantly inhibited by plant extract that indicates the strong antioxidant potential of the plant. The reducing power of the extracts exhibited dif-

ferent degrees of electron donating ability in a concentration-dependent manner. The present data suggest that the leaf, seed and rind extracts of PGcould be potential source of natural antioxidant for the treatment of free radical related disease and age-associated diseases.

In conclusion, the high antioxidant activity exhibited by the extracts of PG may be due to the presence of higher quantities of phenolic compounds. The high free radical scavenging property may be due to hydroxyl groups existing in the phenolic compounds. Polyphenols are one of the major plant compounds responsible for antioxidant activity. The finding of this study suggests that all these extracts of PGcould be a potential source of natural antioxidant for treating age-associated oxidative stress related degenerative diseases. Further investigations are needed for isolation and characterization of the antioxidant constituents of this plant.

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