Research Article

Assessment of Antioxidant Potential of Various Extracts of *Punica granatum* Linn: An Invitro Study

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ABSTRACT

Antioxidants protect against free radicals and they are therefore essential in obtaining and preserving good health. In order to identify the most potent antioxidants, the antioxidant profiles of numerous compounds are frequently compared. The objective of the present study was to evaluate the antioxidant potential of various extracts of *Punica granatum* Linn. using invitro antioxidant assay systems. In vitro antioxidant properties of the extract were analyzed by DPPH, OH radical scavenging and reducing power assays. The various extracts tested, showed good radical scavenging and reducing property. The present study showed that among various extracts tested, the pomegranate peel ethanolic extract possess notable antioxidant property.

Keywords: Antioxidant, *Punica granatum* Linn, invitro, reducing power.

INTRODUCTION

It is increasingly being realized that many of today's diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of prooxidants. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases[1, 2].

Phytochemicals, especially phenolics are suggested to be the major bioactive compounds having health benefits. Clinical trials and epidemiological studies have established an inverse correlation between the intake of dietary antioxidants and the occurrence of oxidative stress related diseases. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipooxygenase and scavenge free radicals or prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans[3,4]. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to development of degenerative diseases. Hence the rationale for the use of antioxidants is well established in prevention and treatment of diseases where oxidative stress plays a major aetiopathological role[5,6]. Antioxidants may protect the body against ROS toxicity either by preventing the formation of ROS, by bringing interruption in ROS attack, by scavenging the reactive metabolites or by converting them to less reactive molecules[7-9]. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in prevention of diseases.

The pomegranate, Punica granatum, an ancient, mystical, and highly distinctive fruit, is the predominant member of Punicaceae family[10].

The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, and protection from ultraviolet (UV) radiation[11-14].

Previous studies have investigated the antioxidant potential of pomegranate peel and seeds invitro using different antioxidant assay systems. However there has been no report on

the comparative antioxidant studies of pomegranate peel, whole fruit (peel + seed) and seeds.

The objective of the present study was to evaluate the antioxidant potential and free radical scavenging activity of ethanol and aqueous extracts of Punica granatum peel, whole fruit and seeds. The extracts were examined for different reactive oxygen species (ROS) scavenging activities including DPPH, Hydroxyl and Ferric reducing power by using invitro assays such as DPPH radical scavenging, Hydroxyl radical scavenging and Reducing power assays.

MATERIALS AND METHODS

Collection of Plant Material

The fruits of Punica granatum were collected from the local market, Mangalore and the specimens were identified.

Preparation of Plant Extracts

Ethanolic extract

The peel, whole fruit and seeds of P.granatum were dried in hot air oven at 40°-50°C for a period of one week. The dried plant material was powdered using mixer grinder, and subjected to soxhlet extraction with 99% ethanol for 24 hours. The mixture was evaporated to dryness in a rotary flash evaporator and stored in refrigerator. The condensed extracts were used for invitro antioxidant studies.

Aqueous extract

The peel, whole fruit and seeds powder was boiled in distilled water for 15-20 minutes, kept in room temperature overnight and filtered. The filtrate was evaporated to dryness in hot air oven and stored in refrigerator. The condensed extracts were used for invitro antioxidant studies.

Antioxidant Activity

DPPH assay

Antioxidants react with DPPH, a stable free radical which is reduced to DPPH-H and as a consequence the absorbance is decreased from the DPPH radical to the DPPH-H form. The degree of discolouration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

The free radical scavenging capacity of P.granatum peel, whole fruit and seeds ethanolic and aqueous extracts were determined using DPPH according to the method of Blois.[15] DPPH solution (0.004% w/v) was prepared freshly in 99% ethanol and added to sample solutions at the concentration of 100µg/ml in ethanol. The mixture was allowed to stand at room temperature in dark for 20 mins. Then the mixture was vortexed and the absorbance was read at 517nm using spectrophotometer. Ellagic acid was used as a reference standard. Control sample was prepared containing the same volume without any extract and 99% ethanol was used as blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. All tests were performed in duplicates. Percentage scavenging of the DPPH free radical was measured using the following equation, DPPH radical scavenging activity (%) = $(A_{control}-A_{Test})/A_{control}$ X 100. Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the extracts or standard.

Hydroxyl radical scavenging assay

This was assayed as described by Elizabeth and Rao[16] with a slight modification. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton Reaction). The reaction mixture contained, in a final volume of 1 ml, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–200 µg/ml) of the test sample or reference compound. After incubation for 1 hour at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. Reaction mixture without test substances/extracts was used as control. All tests were performed in duplicates. Ellagic acid, a classical OH scavenger, was used as a positive control. Lower absorbance of the reaction mixture indicated higher OH radical scavenging activity. Percentage inhibition was evaluated by comparing the test and blank solutions. Percentage scavenging of the OH radical was measured using the following equation, OH radical scavenging activity (%) = $(A_{control}-A_{test})/A_{control}$ X 100. Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample or the extract.

Reducing power (FRAP) assay

The Fe3+-reducing power of the extracts was determined by the method of Oyaizu[17] with a slight modification. Different concentrations (0

- 400µg/ml) of the extracts (0.5ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH6.6) and 0.5 ml potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 minutes. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 minutes at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed in duplicates. A higher absorbance of the reaction mixture indicated greater reducing power. Ellagic acid was used as a positive control.

Statistical Analysis

All values were expressed as Mean±SD of two measurements. Comparison between standard and extracts were performed by using One Way ANOVA. In all these tests, criterion for statistical significance was $P<0.05$.

RESULTS

DPPH radical scavenging assay

DPPH test provides simplified version to detect the antioxidant properties of various molecules present in the extracts. A DPPH solution is decolourized when the odd electron becomes paired off in the presence of a free radical scavenger. The colour becomes light yellow from deep violet. The results of the assay are given in table1. The percentage of radical scavenging property of peel, whole fruit and seeds ethanolic extracts; peel, whole fruit and seeds aqueous extracts and standard Ellagic acid in this assay were 89.3 ± 0.975, 82.205 ± 2.87, 61.08±3.25; 89.01 ± 0.60, 75.65 ± 1.944, 43.35 ± 0.07 and 93.36 ± 1.15 at 100µg/ml respectively.

Hydroxyl radical scavenging assay

This assay shows the abilities of the extracts and standard Ellagic acid to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Table 1. The percentage of radical scavenging property of peel, whole fruit and seeds ethanolic extracts; peel, whole fruit and seeds aqueous extracts and standard Ellagic acid in this assay were 86.88±2.12,65.68±2.03,55.53±1.03;73.73±0.56, 59.1±1.27,40.96±1.36, and 87.86±2.07 at 200µg/ml respectively.

Reducing power assay

The extracts showed potent antioxidant power by reducing power ability. Results of reducing power assay are shown in table 1. The EC50 value of peel, whole fruit and seeds ethanolic extracts; peel, whole fruit and seeds aqueous extracts and standard Ellagic acid in this assay were 105.225±0.75, 146.16±2.79, 153.88±9.92; 107.40±1.81, 176.025±7.34, 266.33±18.54 and 104.88±0.92 µg/ml respectively.

DISCUSSION

The oxidative stress, defined as ‘‘the imbalance between oxidants and antioxidants in favour of the oxidants potentially leading to damage’’ has been suggested to be the cause of aging and various disease in humans. In modern medicine, the balance between antioxidation and oxidation is believed to be a critical concept maintaining a healthy biological system[18].

In recent years much attention has been devoted to natural antioxidant and their association with health benefits[19]. Plants are potential sources of natural antioxidants. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive[20]. ROS, which include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non free-radical species such as H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process. Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Antioxidant is one of the most essential ingredient of today’s menu/therapy because the antioxidative system protects the animal against reactive oxygen species (H_2O_2, superoxide, OH, singlet oxygen & nitrogen species) induced oxidative damage. Various synthetic antioxidants (BHT) are on use, but they are suspected to be carcinogenic[21]. Natural antioxidants, therefore, have gained importance.

In the present study, ethanolic and aqueous extracts of P.granatum peel, whole fruit and seeds has been studied for its antioxidant properties using different invitro antioxidant methods. Potent antioxidant activity of ethanolic and aqueous extracts of P.granatum extracts were analysed by making use of three different methods. However, the efficiency of the extracts differed against various free radicals depending on the specific assay methodology, which reflects the complexity of

the mechanisms and diversity of the chemical nature of the plant material.

Relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of single compounds as well as the different plant extracts. In this study, free radical scavenging activities of P.granatum extracts and standard ellagic acid were determined by using DPPH method. The addition of P.granatum extracts and ellagic acid to the DPPH solution induced a rapid decrease in the optical density at 517 nm. Our investigation showed that P.granatum peel ethanolic and aqueous extracts exhibited potent free radical scavenging activity compared to whole fruit and seeds ethanolic and aqueous extract. Also the radical scavenging activity of peel extract was almost similar to that of standard Ellagic acid.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. Though the DPPH radical scavenging potential of the extracts were less than Ellagic acid at 0.1 mg/ml, the study showed that the extracts have good proton-donating ability and could serve as free radical inhibitors.

Hydroxyl radical formation can occur in several ways by far the most important mechanism in vitro is the Fenton reaction where a transition metal is involved as a pro oxidant in the catalysed decomposition of superoxide and hydrogen peroxide. Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism among the ROS, which could be formed from superoxide anion and hydrogen peroxide, in the metal ions, such as copper or iron and cause the ageing of human body and some diseases. [22] The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[23]. In the present study, P.granatum extracts were evaluated for hydroxyl radical scavenging activity. The result obtained in the study indicates that the extracts showed good OH radical scavenging activity. Among all the extracts tested, the peel ethanolic extract exhibited highest antioxidant activity almost similar to standard Ellagic acid.

Antioxidants present in the sample reduce $Fe^{3+}$to$Fe^{2+}$by donating electrons. Amount of Fe^{2+} can be assessed by measuring OD at 700nm. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging.

In this study we used FRAP assay because it is quick and simple to perform, and reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present. This method was initially developed to assay plasma antioxidant capacity, but can be used to measure the antioxidant capacity from a wide range of biological samples and pure compounds to fruits, wines, and animal tissues[24]. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method Antioxidant to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700nm, it is possible to determine the concentration of Fe^{3+} ion. In this study the extract showed high absorbance than blank indicating the ability of the extract to take part in electron transfer reaction. This shows that the extract exhibited reductive activity. Among all the extracts tested, the peel ethanolic extract exhibited highest reductive potential almost similar to standard Ellagic acid.

CONCLUSION

Through our systematically comparative study of various extracts of P .granatum, the data obtained in the present study shows that the pomegranate peel extract is an excellent free-radical scavenger and a potent natural phenolic antioxidant.

Table 1: Comparison of radical scavenging property and reducing property *Punica granatum* extracts with Ellagic Acid

	DPPH radical scavenging capacity	OH radical scavenging capacity	FRAP Assay
Ethanolic extract	% of radical scavenging property/100µg of extract	% of radical scavenging property/200µg of extract	EC50 conc. µg/ml
Peel	89.3±0.975	86.88±2.12	105.225±0.75
Whole fruit	82.205±2.87	65.68±2.03	146.16±2.79
Seeds	61.08±3.25	55.53±1.03	153.88±9.92
Aqueous extract			
Peel	89.01±0.60	73.73±0.56	107.40±1.81
Whole fruit	75.65±1.944	59.1±1.27	176.025±7.34
Seeds	43.35±0.07	40.96±1.36	266.33±18.54
Ellagic Acid	93.36±1.15	87.86±2.07	104.88±0.92

P<0.05, P<0.05 is statistically significant. Data are expressed as Mean±SD.

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