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ANTIOXDANT STATUS AND RADICAL SCAVENGING ACTIVITY OF GANESH VARIETY OF THE *PUNICA GRANATUM* RIND EXTRACTS

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ABSTRACT

Oxidative stress caused by free radicals is associated to various diseases. Modern research is directed towards finding naturally-occurring antioxidants of plant origin. The aim of the present study was to evaluate the *in vitro* antioxidant activities of *punica granatum rind* extracts of ganesh variety. The present investigation was to examine the free radical scavenging activity of various extracts of *punica granatum rind* by different *in-vitro* methods. The antioxidant activity was evaluated by DPPH assay, FRAP assay, Hydrogen peroxide radical scavenging activity, Nitric oxide radical scavenging activity. The methanolic extract of *punica granatum rind* was found to more effective in the radical scavenging activity. All the above invitro studies clearly indicate that the methanolic extract of *punica granatum rind* has a significant antioxidant activity. These invitro assays indicate that this rind extracts is a better source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses and also support the ethnomedical use of this rind to promote good health for humans.

Keywords*:* Antioxidant, oxidative stress, free radicals, *punica granatum* rind, DPPH, FRAP.

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INTRODUCTION

Free radical is defined as, it is a any atom (Eg 0_2, N_2) with at least one unpaired electron in the outermost shell, and is capable of independent existence. A free radical is simply formed when a covalent bond between entities is broken down and one electron remains with each newly formed atom. They are highly reactive due to the presence of unpaired electrons. Free radicals are chemical entities that can exist separately with one or more unpaired electrons. A part of the oxygen taken into living cells is changed to severe harmful reactive oxygen species and free radicals. Once produced, free radicals can start a chain reaction leading to formation of more free radicals. Free radical reactions take place in the human body and food systems.

Free radicals, in the form of reactive oxygen and nitrogen species, are an integral part of normal physiology. An over-production of these reactive species can occur, due to oxidative stress brought about by the imbalance of the bodily antioxidant defense system and free radical formation. These reactive species can react with biomolecules, causing cellular injury and death. Free radicals have gained much importance because of their involvement in several pathological conditions such as diabetes, liver cirrhosis, nephrotoxicity, cancer, ischemia, neural disorder, metal toxicity, Alzheimer disease, Parkinson's disease etc. The consumption of fruits and vegetables containing antioxidants has been found to offer protection against these diseases. Dietary antioxidants can enhance cellular defences and help to prevent oxidative injury to cellular components.[1]

Antioxidants can cease or retard the oxidation process by scavenging free radicals. These antioxidants are considered as possible shield agents for reducing oxidative damage of human body from ROS and retard the progress of many chronic diseases. Several studies have described the antioxidant properties of medicinal plants rich in phenolic compounds.[2] Natural antioxidants such as α-tocopherol and L-ascorbic acid are widely used because they are seen as being safe and causing few adverse effects, but their antioxidants effects are however, lower than those of synthetic antioxidants such as butylated hydroxytoluene (BHT).[3] Hence, the need exists for safe and economic antioxidants with high activity from natural sources to replace these synthetic chemicals.

Modern researches with important bioactive compounds in many plant and food materials which have prominent antioxidant capacity have received much attention.[4] Polyphenols possess many biological effects. These effects are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidantion and chelating transition metals. In generally,

polyphenols all share the same chemical patterns; one or more phenolic groups for which they react as hydrogen donors and in that way neutralize free radicals.[5, 6]

In recent years, the extracts of many plants have been screened for their antioxidant activities. It has been established that the antioxidant effects are mainly due to the phenolic compounds of the plant.[7] Herbal medicinal preparations and proprietary products are being used more and more widely throughout the world, for treating various ailments. Hence evaluating and ensuring their quality becomes increasingly urgent.

In this present investigation, the free radical scavenging activities of the *punica granatum* rind extracts were analyzed via their reaction with the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical and their ferric ions reducing abilities were determined using the ferric ion reducing antioxidant potential (FRAP) assay. Results from this preliminary study will provide a better understanding of the antioxidant properties of these plants and allow the identification of plants with high antioxidant activity for further investigation and development into value-added foods and neutraceuticals.

MATERIALS AND METHODS

Collection of plant material

The plant specimen for the proposed study was collected from local fruit market. Care was taken to select healthy fruits. It was identified as *P.granatum Linn* – Ganesh (yellowish red rind) belonging to Punicaceae family. The required fruit rind was cut and removed from the fruit. It was authenticated by Dr.P. Jayaraman, Director of National Institute of Herbal Science, Plant Anatomy Research Centre, Chennai. A voucher specimen is maintained in plant anatomy research centre, Chennai.

Preparation of the extracts

P.granatum rind was removed from the fruit and they were dried in shade and powdered mechanically. 5gm of Coarse powder was weighed and extracted with 50ml of each solvent (Ethyl acetate, Ethanol, Methanol, Acetone and Water) separately and kept overnight. The extract was collected after filtration using Whatman No.1 filter paper and used for phytochemical analysis. A part of extract evaporated below 40ºC was used for analysis.

Free radical scavenging activity

DPPH assay

Antioxidant activity or free radical scavenging activity of the *Punica granatum* extracts against DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) was measured according to Mensor *et al*., 2001.[8] The percentage inhibition of DPPH radical by the sample was calculated using the following formula

$$\% \text{ DPPH} = (\text{A control} - \text{A sample}) / \text{A control} \times 100$$

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) is a stable free radial with purple color (absorbed at 517nm). If free radicals have been scavenged, DPPH will degenerate to yellow color.

Preparation of sample

The evaporated extracts were dissolved in respective solvent in the concentration of 1mg/ml which was then used to determine its antioxidant activity.

Quantitative assay of antioxidant activity

The principle for reduction of the DPPH free radical is that the antioxidant reacts with the stable free radical DPPH and converts it to 2, 2- diphenyl-1-picryl hydrazine. The ability to scavenge the stable free radical DPPH is measured as a decrease in absorbance at 517 nm. To an alcoholic solution of DPPH (0.05 mM) was added an equal volume of *Punica granatum* rind extracts dissolved in water, to a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517 nm in a UV double beam spectrophotometer (UV-260). The antioxidant activity of the sample was compared with known standard (0.16%) of Butylated Hydroxy Toluene (BHT). The antioxidant activity was calculated as inhibition (%) of DPPH radical formation.

FRAP assay

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain, 1996.[9] FRAP assays uses antioxidants as reductant in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has intense blue color) can be monitored by measuring the change in absorption at 593nm. The change in absorbance is therefore, directly related to the combined or total reducing power of the electron donating antioxidants present in the reaction mixture. 50µl of extract is mixed with 1.5ml of working FRAP reagent and incubated at 37ºC for 4 minutes. After incubation the absorbance at 593nm was measured. Ferrous sulphate standard was processed in the same way and the FRAP value was calculated from the standard graph. The FRAP value was

expressed as mmol/g. The antioxidant activity of the sample was compared with known standard of Ascorbic acid.

% FRAP = (Acontrol - Asample) /Acontrol X

Hydrogen peroxide (H_2O_2) scavenging activity

Scavenging of H_2O_2 was determined by the method of Ruch *et al.*, 1989. [10] 0.6 ml of 4 mM H_2O_2 solution (prepared in PBS) was added to 4 ml solution of extract and incubated for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without H_2O_2.

% Inhibition = (Acontrol - Asample) /Acontrol X 100

Nitric oxide scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, [11] which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. [12] Sodium nitroprusside (5 mM) in PBS was mixed with methanolic extract of *punica granatum* rind and incubated at 25°C for 150 min. The samples from the above were reacted with Greiss reagent (1% sulfanilamide, 2% H3PO4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and compared with that of standard solutions treated in the same way.

% Inhibiton = (Acontrol - Asample) /Acontrol X 100

RESULTS AND DISCUSSION

Antioxidant status of the *Punica granatum* extracts

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS. [13, 14] These free radicals are the major points in lipid peroxidation. [15, 16] The antioxidants may mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions [17].Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available but are quite unsafe and

their toxicity is a problem of concern. Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive which are capable of absorb and neutralize free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Recently focus has been concentrated on identification of plants with antioxidant ability that may be used for human consumption. [18, 19, 20, 21] Thus in the present investigation the successive extraction of *Punica granatum* rind extracts was screened for *in vitro* antioxidant properties using standard operating procedures.

DPPH radical scavenging activity

Table 1 shows the *in vitro* assessment of free radical scavenging activity of *Punica granatum* rind extracts by using DPPH model. DPPH is a relatively stable free radical and used as a substrate to evaluate the anti-oxidant activity of the antioxidant. The assay is based on the reduction of DPPH solution in the presence of hydrogen donating antioxidant due to the formation of non radical form DPPH-H which is formed by the reaction and this method determines the ability of *Punica granatum* rind extracts to reduce DPPH radical to corresponding yellow coloured diphenyl picrylhydrazine. it has been found that cysteine, glutathione, ascorbic acid, tocopherol, poly hydroxyl aromatic compounds like pyrogallol, gallic acid etc reduce the DPPH by donating hydrogen.[22] DPPH was used to determine the proton radical scavenging action of extracts of *Punica granatum* rind because it shows a characteristic absorbance at 517 nm. The methanolic extract of *Punica granatum* rind showed significant antioxidant activity invitro in scavenging DPPH radical by 97%. The study showed that the extract has the proton donating ability and could serve as free radical scavengers, acting possibly as primary antioxidant.

Table 1 shows DPPH scavenging activity of *Punica granatum* rind extracts

	% of Inhibition at various timings (mins)					
Rind Extracts	**5´**	**10´**	**15´**	**20´**	**25´**	**30´**
Ethyl acetate	22.35	30.88	38.53	44.41	48.24	50.88
Ethanol	51.24	58.82	61.47	69.41	75.29	80.59
Methanol	95.59	95.88	96.47	96.76	97.06	97.35
Water	87.14	88.24	88.82	89.41	92.94	94.71
Acetone	38.24	49.71	50	62.94	62.94	62.94
BHT	12.35	34.71	50.59	62.35	70.29	75

FRAP assay

The ability of the plants extracts to reduce ferric ions was determined using the FRAP assay developed by Benzie and Strain (1996). An antioxidant capable of donating a single electron to

the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous- TPTZ (Fe (II)-TPTZ) complex which absorbs strongly at 593 nm.

The FRAP assay treats the antioxidants contained in the samples as reductants in a redox- linked colorimetric reaction and the value reflects the reducing power of the antioxidants. The procedure is relatively simple and easy to standardize. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The results were expressed as m mol ferrous iron equivalents per g of sample which was shown in Figure 1. Thus it has been used frequently in the assessment of antioxidant activity of various fruits and vegetables and some biological samples.[23] In the present study based on FRAP value the methanolic extract of *Punica granatum* rind has stronger antioxidant activity.

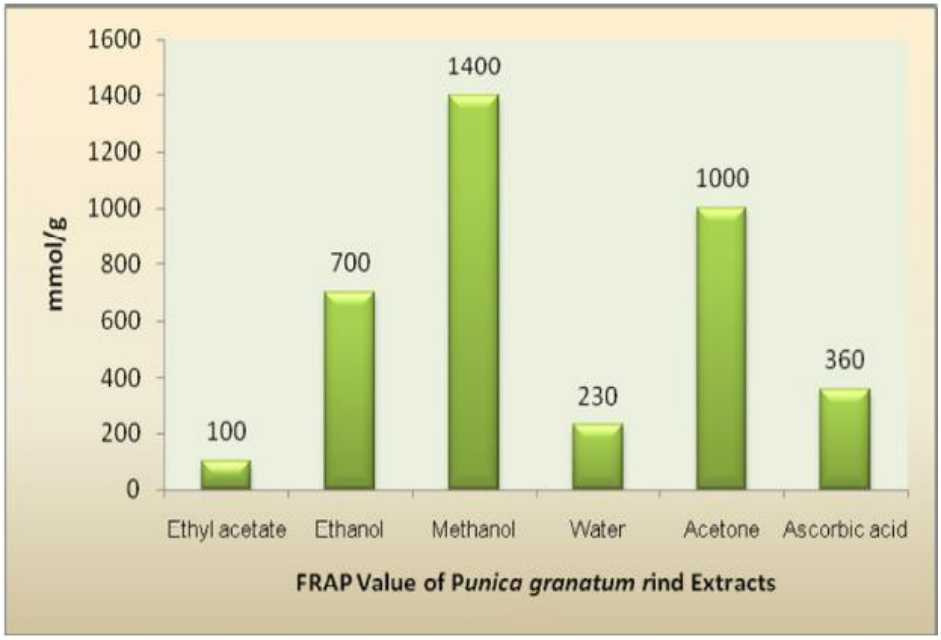

Figure 1 shows total antioxidant activity of *Punica granatum* rind extracts by FRAP assay

Hydrogen Peroxide Scavenging

H_2O_2 ultimately plays critical role in malignant transformation, but can also sensitize cancer cells to H_2O_2-induced cell death. Cellular production of superoxide anion and H_2O_2 favours the formation of other reactive oxygen and nitrogen species such as hydroxyl radical (OH^-) and peroxynitrite ($ONOO^-$) and over production of these species causes oxidative stress and plays an important role in carcinogenesis. [24] Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. [25, 26] The ability of the extracts to effectively scavenge hydrogen peroxide is determined according to the method of Ruch, [27] where they are compared with that of Quercetin as standard. The extracts were capable

of scavenging hydrogen peroxide. In the present study the methanolic extract of *Punica granatum* rind showed significant scavenging activity which is about 87.64% than other solvent extracts of *Punica granatum* rind which can be seen in Figure 2. Thus methanolic extract of *Punica granatum* have high antioxidant property due to variation in quality and quantity of phytochemicals and the active compounds present in different extracts. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important. [27] In conclusion, all extracts exhibited different levels of antioxidant activities in all the models studied.

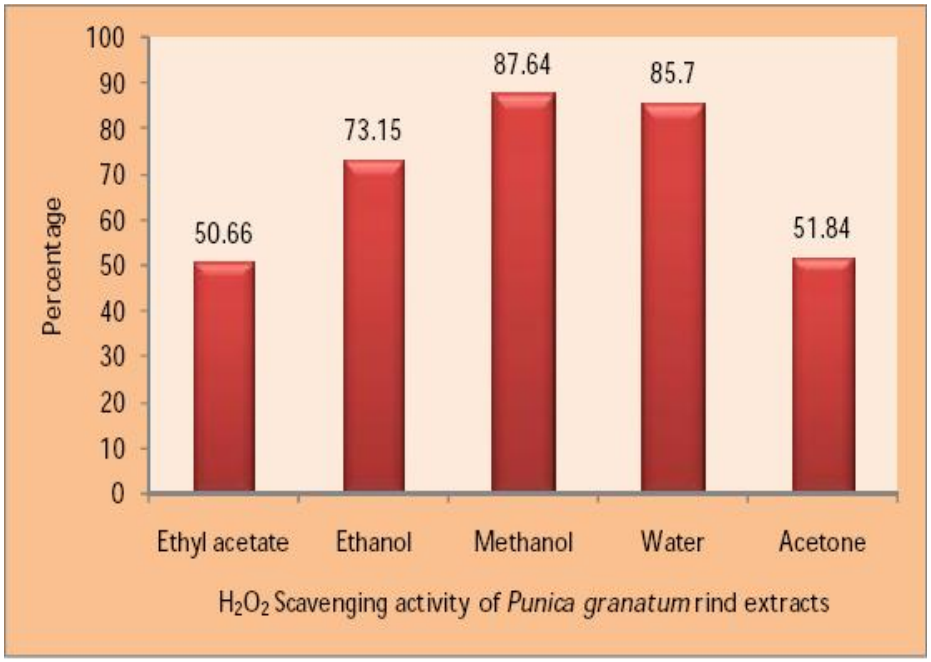

Figure 2 shows the H_2O_2 Scavenging activity of the *Punica granatum* rind extracts

Nitric oxide radical scavenging activity

NO is an important chemical mediator generated by endothelial cells, macrophages, neurons and is concerned in the regulation of various physiological processes. [28] Overload of NO in our system which may be associated with several diseases like inflammation, cancer and other pathological condition. [29]Oxygen reacts with the excess nitric oxide to produce nitrite and peroxynitrite anions which acts as free radicals. [30] Nitric oxide can react rapidly in the intracellular environment to form nitrate, nitrite and s-nitrosothiols. These metabolites play a key role in mediating many xenotoxic effects such as DNA damage. NO causes DNA injury via peroxynitrite.

Phenolic and flavonoid compounds which occur universally in plants are known to possess a diversity of biological actions. These compounds showed linear correlations with free radical

scavenging, NO scavenging and total antioxidant activities. [31] In addition, the contribution of saponins in antioxidant activity has been reported in several studies. [32] In the present study, the methanolic rind extract of *punica granatum* showed better activity with nitric oxide radical and thus inhibiting the generation of anions which can be seen in figure 3. Thus the methanolic rind extract of *punica granatum* scavenges the NO radical more effectively which may be due to the phenolics, flavanoids and saponins present in the extract than that of other extracts of *punica granatum.*

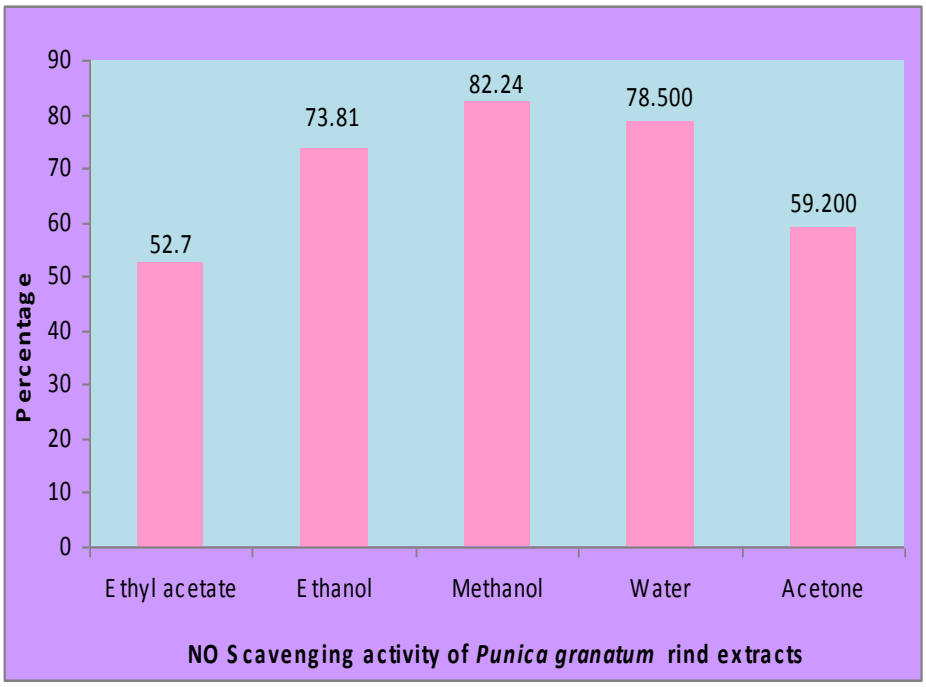

Figure 3 shows the NO Scavenging activity of the *Punica granatum* rind extracts

CONCLUSION

In this present investigation, DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH and the color changes from purple to yellow after reduction. DPPH can be used to determine the proton radical scavenging action of extracts of the leaves of the selected plants, because it possesses phenolic compounds, a proton free radical. The more the polar capacity of the extract, the greater the antioxidant activity is. The antioxidant activity of the extracts of *punic granatum* differed.

This study suggests that methanolic extract of *punica granatum* rind has highest antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress induced diseases. The results also suggest that the phytochemicals in the methanolic extract play a vital role in its free radical scavenging activity. On the other hand, in most food industries,

synthetic antioxidants e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), propyl gallate (PG) etc. are used in order to prevent the rancidity of processed foods. This experiment supports that these fruit rind can be used in such industries as natural antioxidants subjected to proper investigations and also purification of the bioactive component(s) from the extracts is underway and further investigations may improve our understanding of anti-cancerous potential.

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