Antioxidant Activity, Phenol and Flavonoid Contents of Seeds of *Punica Granatum* (Punicaceae) and *Solanum Torvum* (Solanaceae)

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Summary

Antioxidant activity of seeds of *P. granatum* and *S. torvum* were performed by DPPH method, reducing power method, scavenging activity against hydrogen peroxide method and estimating total antioxidant activity. The highest radical scavenging activity was observed with *S. torvum* extract and showed a significant linear co-rrelation with phenolic and flavonoid contents. The total phenolic and total flavonoid contents were expressed in terms of the reference compounds. The greater the amount of these compounds more potent is the radical scavenging effect.

Introduction

The importance of reactive oxygen species (ROS) has attracted attention globally over the past decade. The human body produces reactive oxygen species such as superoxide anion radical, hydroxyl radical and hydrogen peroxide by many enzymatic systems through oxygen consumption. Larger amounts of these ROS are dangerous because of their ability to attack numerous molecules such as proteins and lipids thereby contributing to more than one hundred disorders in humans including atherosclerosis, arthritis, cellular aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (1,2). Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Catalase and even hydroperoxidase enzymes convert hydrogen peroxide and hydroperoxides to non radical forms and function as natural antioxidants in human body. Due to depletion of immune systems natural antioxidants in different maladies, consuming antioxidants as free radical scavengers may be necessary (1,3,4,5). It has been suggested that the intake of fruits and vegetables is associated with a low risk of cancer and cardiovascular disease (6). A large number of plants world wide show a strong anti-oxidant activity (7,8). The widely used synthetic antioxidants like butylated hydroxyl anisole, butylated hydroxyl toluene, tertiary butylated hydroquinone and gallic acid esters, are associated with many ill-effects (9,10). With their limited applications, there is a need to substitute them with naturally occurring antioxidants.

Moreover synthetic antioxidants show low solubility and moderate antioxidant activity (11,12). There has been an upsurge of interest in the scientific community to produce agents from medicinal plants as effective antioxidants in reducing free radical induced tissue injury. It has been mentioned that the antioxidant activity of plants might be due to their phenolic compounds (2). Flavonoids are a group of polyphenolic compounds with known properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (13).

Punica granatum (Punicaceae) commonly known as pomegranate is rich in antioxidant of polyphenolic class which includes tannins and anthocynins (14) and flavonoids (15,16). Content of soluble polyphenols in pomegranate juice varies within the limits of 0.2–1.0%, depending on variety and include mainly tannins, ellagic tannins, anthocyanins, catechins, gallic and ellagic acids (17,18). There are many evidences that flavonoids interact with various biological system (19). *Solanum torvum* Sw. (Solanaceae), commonly known as Turkey berry is native and cultivated in Africa and West Indies (20). The fruits and leaves are widely used in Camerooninan folk medicine. It has antioxidant (21) properties. *Solanum torvum* contains a number of potentially pharmacologically active chemicals like isoflavonoid sulfate and steroidal glycosides (22,23), chlorogenone and neochlorogenone (24), triacontane derivatives (25), 22-β-O-spirostanol oligoglycosides (26), 26-O- β-glucosidase (27). Flavonoid intake appears inversely related with mortality from coronary heart disease in epidemiological studies (28).

In this study we examined the antioxidant capacity of extracts from these plants using several tests: DPPH assay, reducing power, scavenging activity against H_20_2 radical, total antioxidant activity. The total flavonoid and total phenolic contents were also estimated.

Materials and Methods

Plant material and extraction

1 Kg of Pomegranate fruits were purchased from local market. The seeds were isolated and were ground to obtain juice. The juice was air dried and concentrated under reduced pressure to obtain 32 g, corresponding to a yield of 3.2 % w/w. To obtain its n-butanolic fraction, the seeds were separated from the fruits and were refluxed with 2 M HCl for 1 h at 100^0C. This gave the red colour extract from pomegranate which was cooled and filtered. The acidic extract was extracted with ethyl acetate to remove the flavones. This acidic extract was heated at 80^0C for 3 min to remove the last traces of ethyl acetate and cooled. It was then extracted exhaustively with n- butanol. The n-butanol extract (yield 1.6%w/w) was concentrated by distillation. It gave the concentrated red colored pigment which was subjected to thin layer chromatography. The Rf values obtained with BAW, forestal and formic acid was 0.86, 0.71 and 0.40 respectively which indicates the presence of anthocynidins (29). Dried fruits of *Solanum torvum* Sw. (Solanaceae) were purchased locally and authenticated by Dr. S. C. Pal, NDMVP Samaj's College of Pharmacy, Nashik, India. The voucher specimen (1731) has been deposited at Agharkar Research Institute, Pune, India. Mature fruits were dried in shade, and grounded. The powder obtained (1 Kg) was defatted using pet ether (60-80^0C). The marc was macerated in ethanol for 3-4 days at room temperature. The filtrate was concentrated under reduced pressure to obtain 120 g of extract (12.0 %w/w). The ethanolic extract was subjected to column separation and successively extracted with ethyl acetate (yield 1.88 %w/w) and methanol (yield 5.76 %w/w). The ethanolic extract of *S. torvum* (ST-extract),

methanolic fraction of ST-extract (M-ST- extract), *P. granatum* juice extract (PJ-extract), n-butanolic fraction of *P. granatum* juice extract (n-B-PJ-extract) were used for the study. Appropriate concentrations of the extracts were made in distilled water. The phytoconstituents present in the extracts were flavonoids, alkaloids, tannins and saponins (30).

Chemicals

1,1-diphenyl-2-picryl hydrazyl (DPPH), Butylated hydroxyl anisole (BHA), Butylated hydroxyl toluene (BHT) and Adrenaline were purchased from Sigma-Aldrich, Mumbai. Standard reference Rutin was obtained as gift sample from Apex, Mumbai, α-tocopherol was purchased from Merk, Mumbai, and Gallic acid was purchased from Research-lab, Mumbai. All the other chemicals were of analytical grade

Methods

DPPH (1, 1-diphenyl-2-picryl hydrazyl) Method

Scavenging free radical potential was evaluated against ethanolic solution of DPPH a stable free radical. Antioxidants react with DPPH and convert it to 1, 1-diphenyl-2-picryl hydrazine (non-radical). Degree of discoloration indicates the scavenging activity of drug. The change in absorbance produced at 517 nm, has been used as a measure of antioxidant activity (31). DPPH (2.365 mg) was dissolved in 10 ml of 95% ethanol and the extracts were dissolved in 95% ethanol to make the stock solution, which was diluted to give concentrations ranging from 50-800 ppm. The extracts of varying concentrations (1.5 ml) were added to both blank as well as in the test. DPPH (1.5 ml) was added only in test and finally 95% ethanol (1.5 ml) was added in both blank and test. The tubes were kept aside for 20 min allowing the reaction to take place. After 20 min the absorbance (A) of the solutions was recorded against the respective blanks at 517 nm (32). BHA was used as reference standard.

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing power assay

The yellow color of the test solution changes to various shades of green and blue, depending upon the reducing power of each extract. The presence of reductants (antioxidants) in the extracts causes the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form (Fe^{2+}). Therefore the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (33). The extracts (60-400 ppm) in 1 ml of phosphate buffer with 5 ml of 0.2 M phosphate buffer (pH 6.6) and 5 ml of 1% potassium ferric cyanide solution were incubated at 50^0C for 20 min. After incubation, 5 ml of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 ml) was mixed with 5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then the absorbance of the reaction mixture was read spectroscopically at 700 nm. BHT was used as reference standard.

Scavenging activity against hydrogen peroxide

The scavenging capacity of extracts on hydrogen peroxide was determined according to the method of (34). Test tubes were prepared with 2.0 ml of various extracts (50-800 ppm) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way but without H_2O_2. After incubation of the mixture during 10 min, the absorbance was recorded at 230 nm. The scavenging

activity was calculated using the following formula: % scavenging activity = [(Ac – At)/ Ac] 100, where Ac is the absorbance of the control and At is the absorbance of the extract. BHA was used as reference standard.

Total antioxidant activity

The antioxidant activity of the extracts was determined using the thiocyanate method (35). Various concentrations (50-500 ppm) of extracts was prepared in methanol and added to a linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0) and phosphate buffer (2 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid with 0.2804 g tween-20 as emulsifier in 50 ml phosphate buffer (40 mM). The mixture was then homogenized. The final volume was adjusted to 5 ml; with 40 mM phosphate buffer, and pH 7.0. The mixed sample was then incubated at 37^0C in a glass flask for 60 h, to accelerate the oxidation process (36). One milliliter of the incubated sample was removed at 12 h interval and 0.1 ml $FeCl_2$ (20 mM) and 0.1 ml 30% ammonium thiocyanate was added. The absorbance of this was measured at 500 nm, using a spectrophotometer (Shimadzu, 2450). Alpha Tocopherol was used as a reference compound. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to linoleic acid emulsion in the test sample and the reference compound, was used. Percent inhibition of lipid peroxide generation was calculated using formula.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Estimation of Total Flavonoid Content

The aluminum chloride colorimetric method was used for determining total flavonoid content (37). Standard Rutin was used to make the calibration curve. Ten milligrams of rutin was dissolved in 80% ethanol and then diluted to 25, 50, and 100 ppm. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The same concentrations of extracts were also prepared. After incubation at room temperature for 30 min, the absorbance of reaction mixture was measured at 415 nm with a Shimadzu UV-2450 spectrophotometer (38).

Estimation of Total Phenolic Content

Total soluble phenolic compounds present in extracts were determined with the Folin-Ciocalteu reagent (39,40).The calibration curve was prepared by preparing gallic acid solutions at concentrations 0, 50, 100, 150, 250, 500 ppm in ethanol. To 0.1 ml of fraction (1 mg/ml in distilled water) one ml of Folin-Ciocalteu reagent was added. After 3 minutes, 3 ml of 2% Na_2CO_3 was added. The same concentrations of extracts were also prepared. Subsequently, the mixture was shaken for 2 h at room temperature and the absorbance was measured using Shimadzu UV-2450 spectrophotometer at 760 nm. Total phenol values were expressed in terms of gallic acid equivalent (mg/g of dry mass).

Statistical Analysis: All assays were carried out in triplicates and results are expressed as mean ± SEM. The IC_{50} values were calculated using the Graph Pad Prism 4 software.

Results and Discussion

An easy rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1, 1-diphenyl-2 picryl hydrazyl (DPPH) stable radical spectrophotometrically. The antioxidant activity of phenolic compounds is mainly due to their redox properties which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (41). A dose dependent increase in % scavenging activity was observed with various concentrations of extracts (50-800 ppm). The IC_{50} values of ST extract, M-ST extract, PJ extract and n-B-PJ extract were 180 ppm, 250 ppm, 450 ppm, and 480 ppm respectively. The IC_{50} value of standard antioxidant- BHA was found to be 80.12 ppm. The results are summarized in table 1.

Table 1- % Scavenging activity by DPPH method of various extracts

Sr.no	Concentrations (ppm)	% Scavenging activity by DPPH Method				
		ST - extract	M-ST- extract	PJ- extract	N-B- PJ- extract	BHA
1	50	39.77±0.01	11.27±0.02	17.05±0.02	32.57±0.01	38.59±0.02
2	100	43.56±0.02	22.68±0.03	33.22±0.02	33.33±0.02	62.48±0.05
3	200	51.51±0.01	38.14±0.02	39.23±0.02	35.22±0.01	78.94±0.06
4	400	70.45±0.02	62.37±0.02	49.55±0.01	46.36±0.02	92.90±0.07
5	800	90.90±0.02	92.78±0.03	62.5±0.01	62.27±0.01	99.56±0.04
6	IC_{50}	180 ppm	250 ppm	450 ppm	480 ppm	80.12 ppm

Each value in the table is obtained by calculating the average of three experiments and expressed as mean ± SEM.
ST- extract: Ethanolic extract of *S. torvum* dried fruits
M-ST-extract: Methanolic fraction of Ethanolic extract of *S. torvum* dried fruits
PJ-extract: Dried extract of *P. granatum* seed juice
N-B-PJ-extract: n-butanolic fraction of *P. granatum* seed juice
BHA- Butylated hydroxyl anisole

Table 2 shows scavenging capacity against hydrogen peroxide with various concentrations of all extracts. It is observed that IC_{50} value was minimal with ST – extract (130 ppm) amongst the four extracts as observed with DPPH assay. Hydrogen peroxide has the ability to form hydroxyl radical. It has been proven that dietary phenols protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide (42,43), indicating that the observed hydrogen peroxide scavenging activity of our plants could be due to the presence of phenols.

Table 2- % Scavenging activity by H_2O_2 method of various extracts

Sr. no	Concentrations (ppm)	% Scavenging activity by H_2O_2 Method				
		ST -extract	M-ST-extract	PJ- extract	n-B-PJ-extract	BHA
1	50	21.54± 0.04	9.64±0.03	28.88±0.02	18.34±0.02	38.59±0.02
2	100	31.74±0.03	24.43±0.02	32.54±0.05	28.44±0.02	62.48±0.05
3	200	58.55±0.02	45.45±0.02	48.94±0.01	36.23±0.04	78.94±0.06
4	400	80.28±0.04	52.8±0.04	78.61±0.05	47.24±0.06	92.90±0.07
5	800	86.05±0.02	76.81±0.04	83.64±0.02	57.47±0.07	99.56±0.04
6	IC_{50}	130 ppm	410 ppm	210 ppm	560 ppm	80.12 ppm

Each value in the table is obtained by calculating the average of three experients and expressed as mean ± SEM.
ST- extract: Ethanolic extract of *S. torvum* dried fruits
M-ST-extract: Methanolic fraction of Ethanolic extract of *S. torvum* dried fruits
PJ-extract: Dried extract of *P. granatum* seed juice
N-B-PJ-extract: n-butanolic fraction of *P. granatum* seed juice
BHA-Butylated hydroxyl anisole

The total antioxidant activity of various extracts were determined by thiocyanate method (35). The results are shown in table 3. The total antioxidant activities were in the range of 200-300 ppm and comparable to the reference standard α-tocopherol.

Table 3- % Scavenging activity by Total antioxidant activity Method of various extracts

Sr.no	Concentrations (ppm)	% Scavenging activity by Total antioxidant activity Method				
		ST -extract	M-ST-extract	PJ -extract	n-B-PJ-extract	α-tocoferol
1	50	21.7±0.01	16.0±0.01	21.85±0.02	8.78±0.02	39.56±0.008
2	100	33.61±0.01	29.32±0.01	39.35±0.1	26.73±0.01	53.04±0.007
3	250	51.68±0.02	46.67±0.02	50.26±0.02	45.26±0.01	64.66±0.11
4	500	76.22±0.01	71.36±0.01	74.8±0.02	70.4±0.02	85.26±0.02
5	IC_{50}	245 ppm	295 ppm	250 ppm	310 ppm	85 ppm

Each value in the table is obtained by calculating the average of three experiments and expressed as mean ± SEM.
ST- extract: Ethanolic extract of *S. torvum* dried fruits
M-ST-extract: Methanolic fraction of Ethanolic extract of *S. torvum* dried fruits
PJ-extract: Dried extract of *P. granatum* seed juice
N-B-PJ-extract: n-butanolic fraction of *P. granatum* seed juice

The reducing power of various extracts are presented in Fig.1, where it is seen that the best reducing power was obtained from ST- extract. A dose dependent increase in % scavenging activity was observed with various extracts. The extracts showed a higher potency than BHT.

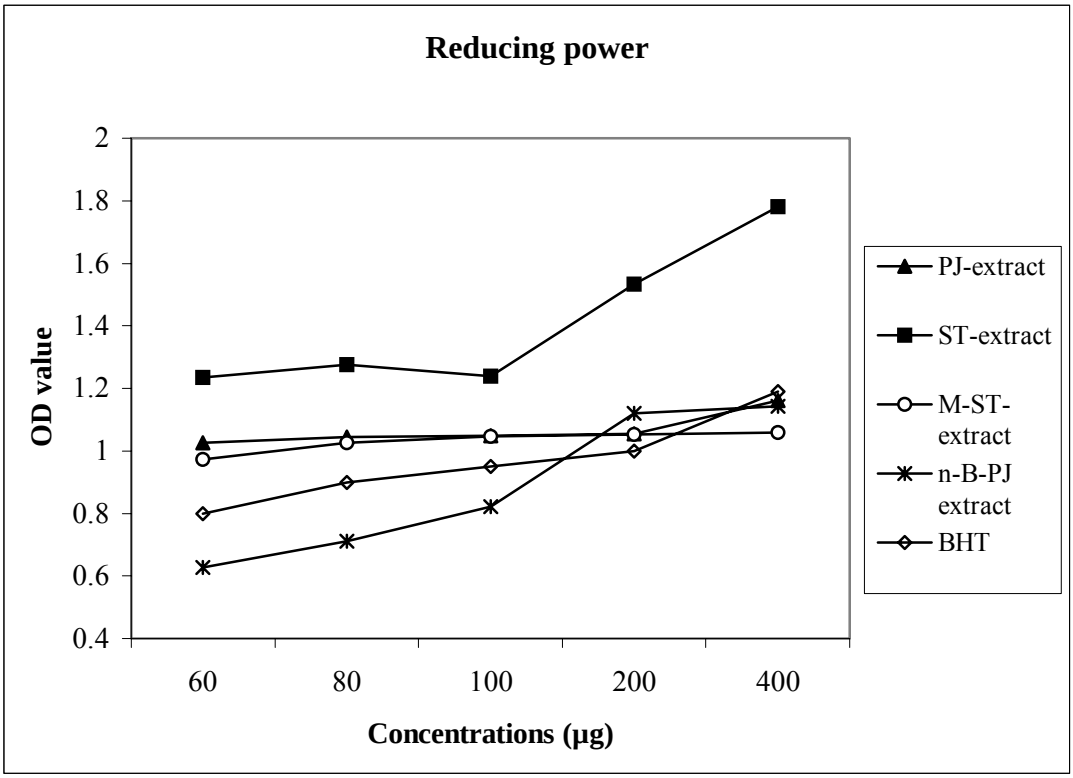

Fig. 1-Reducing power activity of various extracts
Each value in the table is obtained by calculating the average of three experients and expressed as mean ± SEM.
ST- extract: Ethanolic extract of *S.torvum* dried fruits
M-ST-extract: Methanolic fraction of Ethanolic extract of *S.torvum* dried fruits
PJ-extract: Dried extract of *P. granatum* seed juice
N-B-PJ-extract: n-butanolic fraction of *P. granatum* seed juice
BHT-Butylated hydroxyl toluene

Plant phenolics constitute one of the major constituent as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in selected plant extracts. Flavonoids are the most diverse and widespread group of natural compounds and probably the most important phenolics (44). These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. Such properties are especially distinct for flavanols. Therefore the content of flavonoid and phenolics were determined in the extracts
The total flavanoid content of PJ- extract and its n-B-PJ extract was found to be 94.15± 9.92 µg and 56.56± 3.58 µg rutin equivalent/mg of extract respectively. The

total phenolic content of PJ-extract and its n-B-PJ extract was found to be 455.7± 6.33 µg and 222.7± 2.88 µg gallic acid equivalent/ mg of extract respectively.
The total flavonoid content of ST-extract and M–ST extract was found to be 85.26 ± 0.02 µg and 65.69 ± 0.02 µg rutin equivalent/ mg of extract respectively. The total phenolic content of ST-extract and M–ST extract was found to be 99.52 ± 0.42 µg and 78.9 ±0.43 µg gallic acid equivalent/mg of extract repectively.
Thus the results suggest that ST extract was the most potent as it exhibited outstanding reducing power, scavenging activity against DPPH and hydrogen peroxide. Good co-relation was observed with radical scavenging activity of extracts and total phenolics. A potent scavenger of free radicals may serve as a possible preventive intervention for many diseases as the involvement of free radicals in the pathogenesis of a large number of diseases is well known (45).All the extracts exhibited different extent of antioxidant activity. This may be related to the high amounts of flavonoid and phenolic compounds in the extracts. The identification of specific phenolic or flavonoid compound(s) responsible for the high antioxidant activities and therapeutic use represents our future aim.

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