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Original Article

EVALUATION OF *PUNICA GRANATUM* FRUIT PEELS EXTRACTS FOR ITS FREE RADICAL SCAVENGING AND ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: To evaluate free radical scavenging and anti-inflammatory activity of petroleum ether, ethyl acetate, aqueous, methanol:water and methanol extracts of *Punica granatum* fruit peels (PGFP) (Family: Lythraceae) by *in vitro* methods.

Methods: The free radical scavenging effect was studied using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) and nitric oxide radical scavenging assay. Anti-inflammatory activity was evaluated by HRBC membrane stabilization assay.

Results: All the extracts of PGFP exhibited significant free radical scavenging effect. The methanol extract exhibited maximum significant DPPH and nitric oxide radical scavenging activity with IC_{50} value of 24.43 and 45.56µg/ml and maximum stabilization (86.96%) of HRBC membrane at 80 µg/ml among all the extracts of PGFP.

Conclusion: Methanol as an extraction solvent was found to be the best in obtaining the extract of PGFP rich in radical scavenging and anti-inflammatory phytoconsituents.

Keywords: *Punica granatum* fruit peel, DPPH assay, NO assay, HRBC membrane, Antioxidant, Anti-inflammatory.

INTRODUCTION

Under physiological conditions inflammation is an immune response of tissues to harmful stimuli such as microbes, damaged cells or irritants in order to restore the cells to normal state [1]. But in pathological state, inflammatory response damages the normal tissues through the release of free radicals such as superoxide, nitric oxide, etc and lysosomal enzymes from lysosomes [2, 3]. Thus neutralization of free radicals and inhibition of release of lysosomal enzymes by stabilization of lysosomal membrane can help to reduce inflammation. The currently used anti-inflammatory drugs such as NSAIDs, aminosalicylates and steroids to treat inflammation have been associated with severe side effects. So, there is an urgent need to find safer anti-inflammatory compounds [4].

Medicinal plants have gained increased attention in recent years. According to WHO, more than 80% of the population within developing countries uses herbal and other traditional medicines to treat their common ailments and are potential sources of antioxidants and anti-inflammatory compounds [5]. The therapeutic effects of many medicinal plants are usually due to their antioxidant and anti-inflammatory properties [6]. The phytochemicals such as phenols, tannins, flavonoids and terpenoids are responsible for antioxidant and anti-inflammatory effects [7, 8].

The fruits are widely consumed fresh or as juice. The fruits are used to prepare beverage products, jam, jelly and for flavoring and coloring beverage products [9]. Studies have demonstrated its beneficial effect as antioxidant, antibacterial, anti-inflammatory, anticancer, antidiabetic, anti-atherosclerotic, estrogen-like activity, cardiotonic, hepatoprotective, antiviral and aphrodisiac [10]. In folk medicine the fruit peels are used in treatment of diarrhea, dysentery, helminthiasis, acidosis, hemorrhage and respiratory pathologies [11]. Bioactive phytoconstituents such as tannins, phenols, flavonoids, glycosides and terpenoids have been reported to be present in fruit and its peel [10].

To best of our knowledge there are no reports on comparative studies of various extracts of *Punica granatum* fruit peels (PGFP) prepared using solvents of different polarities (petroleum ether, ethyl acetate, methanol, methanol: water and water) demonstrating free radical scavenging and anti-inflammatory activity. Thus the objective of the present study was to determine the free radical scavenging and anti-inflammatory potential of different extracts of PGFP.

MATERIALS AND METHODS

Plant material

Punica granatum fruits were purchased from local market in Navi mumbai. The PGFP were authenticated at Agharkar Research Institute, Pune, Maharashtra. The voucher specimen number is F-196.

Reagents and chemicals

All the reagents, chemicals and solvents used for the experiments were of analytical grade and obtained from Sigma Aldrich (USA).

Plant extraction

PGFP were dried at 40° C for 48 hours in hot air oven. Dried peels were powdered using an electric mixer (Remi). Soxhlet extraction technique was used to prepare petroleum ether (PE-PGFP), ethyl acetate (EA-PGFP) and methanol (ME-PGFP) extracts. Extraction was carried for 18 hours at a temperature not exceeding the boiling point of the solvent. Water/Aqueous (AQ-PGFP) and methanol-water (MEW-PGFP) extracts were prepared by refluxing the peels powder with water and methanol:water (1:1) for 8 hours. The extracts were filtered using Whatman filter paper (No.1), concentrated in vacuum under reduced pressure using rotary flask evaporator, and dried in a vacuum desiccator. The extracts were stored in amber colored bottles at 2-4 °C until further use.

Antioxidant activity

DPPH radical scavenging activity

Radical scavenging activity against the stable DPPH radical was determined spectrophotometrically. The various concentrations (10-100ug/ml) of each extract prepared in methanol were added to 1 ml of 0.1 mM DPPH solution. An equal amount of methanol and DPPH was added to the control. In case of blank DPPH was replaced by methanol. The mixture was shaken vigorously and incubated for 30 minutes in dark at room temperature and then absorbance was recorded at 517 nm. Ascorbic acid was used as positive control [12, 13].

Nitric oxide scavenging activity assay

Sodium nitroprusside (10 mM, 1.5 ml) in phosphate–buffered saline (PBS) was mixed with 0.5 ml of different concentrations (10-150ug/ml) of extracts dissolved in the methanol and incubated at 25 °C for 150 minutes. The samples from the above were reacted with 1 ml Griess reagent for 15 minutes (1% Sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). Control without test compound was prepared in an identical manner. The absorbance of the chromophore formed during the diazotization of nitrite with Sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm and referred to the absorbance at standard solutions of ascorbic acid, treated in the same way with Griess reagent [14].

Percentage radical scavenging effect of DPPH and NO was calculated using the formula: [(absorbance of control-absorbance of sample)/absorbance of control] × 100.

All the test analyses were run in triplicate and IC_{50} values were calculated.

Anti-inflammatory activity

Human red blood cell (HRBC) membrane stabilization method

HRBC membrane stabilization method was used to evaluate *in vitro* anti-inflammatory potential. The blood was collected from healthy human volunteer and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and0.42% NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline solution and a 5% HRBC suspension was made in isosaline solution. The assay mixture contains 0.5 ml HRBC suspension (5% v/v), 0.5 ml of dilutions of PGFP extracts at different concentrations (10-80µg/ml) prepared in isosaline solution, 1 ml phosphate buffer (pH7.4) and 2 ml hyposaline (0.36%) solution.

The mixture was incubated at 37 °C for 30 minutes and centrifuged at 3,000 rpm for 20 minutes. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac sodium served as positive control and a negative control was prepared by omitting the extract and distilled water instead of hyposaline solution. The percentage of hemolysis was estimated using following formula by assuming the hemolysis produced in the control as 100% [15].

Percentage Stabilization/protection = 100-[(optical density of sample)/(optical density of control)] x 100

Statistical analysis

The experiments were replicated thrice for each parameter. Values are expressed as mean±SEM.

RESULTS

DPPH Radical Scavenging Activity

All the extracts of PGFP showed DPPH scavenging activity up to 100 µg/ml concentration in the following order-ME-PGFP>MEW-PGFP>AQ-PGFP>EA-PGFP>PE-PGFP (table 1) with IC_{50} value of 24.43 µg/ml, 27.40 µg/ml, 32.78 µg/ml, 81.13 µg/ml and 93.24 µg/ml respectively. ME-PGFP showed maximum scavenging effect, but was less as compared to standard antioxidant ascorbic acid, which showed scavenging effect with the IC_{50} of 11.16 µg/ml. DPPH free radical scavenging activity of extracts increased with increasing concentration showing a dose dependent effect (fig. 1).

Nitric oxide scavenging activity assay

NO generated from sodium nitroprusside is very unstable species. It reacts with oxygen to form nitrite through the intermediates NO_2, N_2O_4 and N_3O_4 [13]. It was estimated by using the Griess reagent. The nitrite produced by the reaction mixture was reduced by all the extracts of PGFP in the order of ME-PGFP>MEW-PGFP>AQ-PGFP>EA-PGFP>PE-PGFP with IC_{50} value of 45.56 µg/ml, 71.65 µg/ml, 81.10 µg/ml, 114.80 µg/ml and 121.26 µg/ml respectively. (table 1). The NO scavenging activity of PGFP extracts increased with an increase in concentration (fig. 2). Maximum scavenging effect exhibited by ME-PGFP was less as comparable to that exhibited by ascorbic acid.

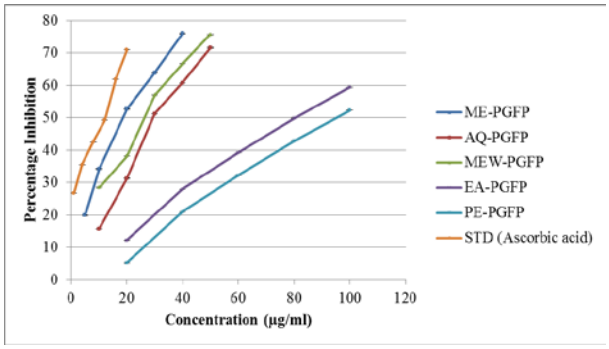

Fig. 1: DPPH radical scavenging activity of PGFP extracts

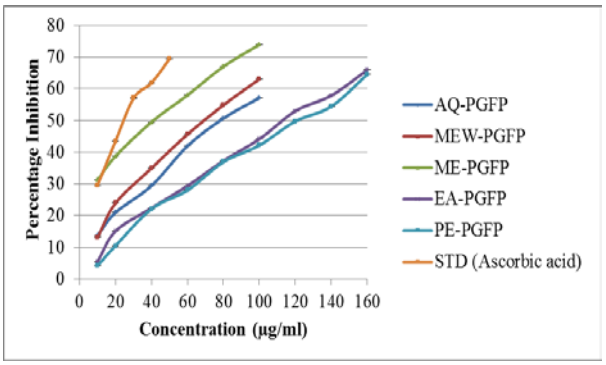

Fig. 2: NO radical scavenging activity of PGFP extracts.

Human red blood cell (HRBC) membrane stabilization method

In vitro anti-inflammatory activity of PGFP extracts was tested at concentrations ranging from 10-80 µg/ml using HRBC membrane stabilization method. The results obtained revealed that the ME-PGFP exhibited maximum stabilization against the HRBC membrane. Percentage membrane stabilization by ME-PGFP at 80 µg/ml concentrations was highest (86.96 %) and was comparable to the standard-diclofenac at 40 µg/ml, which showed 89.96 % stabilization. ME-PGFP, MEW-PGFP and AQ-PGFP extracts showed better stabilization of HRBC membrane as compared to the EA-PGFP and PE-PGFP extracts (table 2). With the increasing concentration the membrane hemolysis is decreased and membrane stabilization/protection is increased (fig. 3).

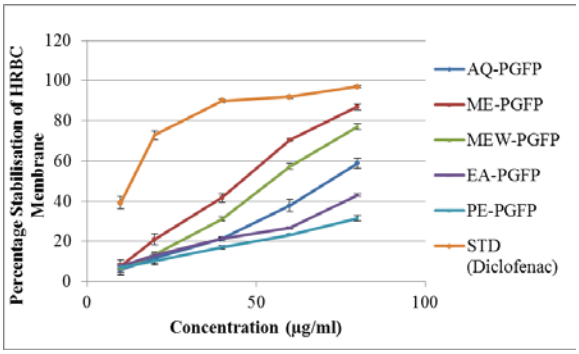

Fig. 3: Percentage stabilisation of HRBC membrane by PGFP extracts

Table 1: IC_{50} values of PGFP by DPPH and NO radical scavenging activity

S. No.	PGFP Extracts	IC_{50} values (µg/ml)	
		DPPH radical scavenging activity	NO radical scavenging activity
1	AQ-PGFP	32.78±0.357	81.10±0.271
2	ME-PGFP	24.43±0.315	45.56±0.314
3	MEW-PGFP	27.40±0.218	71.65±0.284
4	EA-PGFP	81.13±0.51	114.79±0.471
5	PE-PGFP	93.24±0.401	121.26±0.282
6	Ascorbic acid (Standard)	11.16±0.183	27.70±0.211

N=3, Values expressed as mean±SEM

Table 2: Percentage stabilisation/protection of HRBC membrane by PGFP extracts

Conc. (µg/ml)	AQ-PGFP	ME-PGFP	MEW-PGFP	EA-PGFP	PE-PGFP	Standard (Diclofenac)
10	5.81±2.715	7.80±2.532	6.34±3.031	7.85±0.418	7.03±0.503	39.26±3.009
20	11.5±0.456	20.85±3.26	13.59±2.711	13.16±0.743	10.08±1.005	72.96±2.066
40	21.16±1.088	41.74±1.039	31.16±2.089	21.36±0.382	17.13±0.944	89.96±0.823
60	37.84±1.566	70.52±2.931	57.42±0.569	26.66±0.5279	23.28±0.317	92.00±0.786
80	58.81±1.448	86.95±2.477	77.05±1.466	42.94±0.679	31.48±1.490	97.05±0.438

N=3, Values expressed as Mean % Stabilization±SEM

DISCUSSION

Free radicals are generated endogenously in many biochemical processes of the body. During inflammation excessive generation of free radicals results in tissue injury by damaging the macro molecules and lipid peroxidation of membrane phospholipids. The free radicals generated also in turn propagate inflammation by stimulating release of inflammatory and pro-inflammatory cytokines. Thus, the free radicals exert their adverse effects by reacting with different cell components, inducing loss of function and cell death at the site of inflammation [2].

DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. In this assay the DPPH radical on accepting a hydrogen (H) atom from the scavenger molecule i.e. Antioxidant, gets reduce to $DPPH_2$. The purple color of DPPH solution changes to yellow with concomitant decrease in absorbance. The color change was monitored spectro photo metrically and the, degree of discoloration indicated the scavenging potential of the different PGFP extracts in terms of their hydrogen donating ability [12, 15].

Nitric oxide radical is a signaling molecule that plays a key role in the pathogenesis of inflammation. Under physiological conditions it gives an anti-inflammatory effect. But under pathophysiological conditions over production of NO is considered as a pro-inflammatory mediator that induces inflammation [16]. In this assay the NO radical generated from sodium nitroprusside is very unstable species. It reacts with oxygen to form nitrite through the intermediates NO_2, N_2O_4 and N_3O_4 which is estimated by using the Griess reagent [13, 14]. The nitrite produced by the reaction mixture was reduced by all the extracts of PGFP.

Under physiological conditions lysosomal enzymes released from lysosomes removes cell detritus from an inflamed area. But in diseases state lysosomal enzymes cause destruction of the normal cell by digesting components of the cell itself in turn alleviating the inflammation[3]. The stabilization of lysosomal membrane will inhibit the release of lysosomal enzymes, which is important in limiting the inflammatory response. Most of anti-inflammatory drugs exert their beneficial effect by inhibiting either release of lysosomal enzymes or by stabilizing lysosomal membrane which is one of the major events responsible for the inflammatory process. HRBC membrane is analogous to the lysosomal membrane and its stabilization implies that the extracts may as well stabilize lysosomal membranes. Exposure of HRBCs to injurious substances such as hypotonic medium results in its lysis. Stabilization of HRBC membrane from hypotonicity induced membrane lysis is taken as an *in vitro* measure of anti-inflammatory activity of the PGFP extracts [15, 17]. Lysosomal membrane stabilization by PGFP extracts would thus prevent the release of lytic enzymes and leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators [3].

In earlier studies phytochemical analysis reported that PGFP extracts contains phenolic compounds, tannins, flavonoids, terpenoids, glycosides and alkaloids [18]. These compounds are known to possess antioxidant and anti-inflammatory activity [19, 20]. Hence the free radical scavenging/antioxidant and anti-inflammatory activity of PGFP extracts may be due the presence of these compounds in it. Presence of antioxidant and anti-inflammatory compounds in PGFP extracts prepared in different solvents (which differ widely in their polarity) indicate that phytochemicals contributing to the antioxidant and anti-inflammatory activity belong to different groups of plant metabolites and varies widely with respect to their chemical properties.

Methanol extract possessed maximum free radical scavenging and anti-inflammatory activity among all the extracts of PGFP in the dose dependent manner. Maximum radical scavenging activity signifies the presence of an effective antioxidant (i.e. a substance that scavenges free radical) constituents and the stabilization of HRBC membrane signifies the presence of effective anti-inflammatory constituents in it. Hence methanol as an extraction solvent was found to be the best in obtaining antioxidant and anti-inflammatory constituents from the PGFP. This study also showed that PGFP, which are waste from agro-food industry, can be used as the potential source of antioxidants and anti-inflammatory substances. Usage of PGFP can be helpful in waste management and can reduce the cost required for waste treatment.

Further *in vivo* studies are required to confirm the effect of different extraction solvents on free radical scavenging and anti-inflammatory activity.

CONCLUSION

The present study revealed that different solvents for extraction had a great influence on the antioxidant and anti-inflammatory properties of PGFP. The methanol extract of PGFP could be a potential source of natural antioxidant and anti-inflammatory agents and can be used in preventing or slowing free radical and inflammation related disorders.

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CONFLICT OF INTERESTS

Declared None

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