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IN-VITRO EVALUATION OF PHYSICOCHEMICAL, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF POMEGRANATE (*PUNICA GRANATUM* L.) JUICE AND SEED HYDRO EXTRACTS

MANUKUMAR H.M[1]* AND THRIBHUVAN K.R[2]

1 Pristine Laboratories, Kodigehalli Gate, Bangalore-560092, Karnataka, India,
2 AGMARK Grading Laboratory, Karnataka State Agricultural Marketing, Board, Bangalore- 560022, India

ABSTRACT

This study evaluated, for the physicochemical, antioxidant, and anti-inflammatory activities using vitro assays of water extracts from juice and seed of commercially grown pomegranate fruit. The fruit weight, fruit length, fruit volume, fruit length and fruit diameter, were in the range of 258.33 g, 264.77 cm^3, 72.42 mm and 76.03 mm respectively. Total soluble solids content, pH, titratable acidity and total sugars are matching the literature reviewed. Juice having higher total phenolic content and fewer flavonoid contents and visa versa in seed. Antioxidative property was deliberated based on their free radical scavenging ability and reducing power, in which the juice exerted higher (48% and 310µg/ml) potential than seed (36% and 297 µg/ml) respectively. The investigated results against normal erythrocytes exposed to both heat and hypotonic induced lyses exhibited juice (49.23% and 29.65%) have higher potency than seed (36.25% and 19.57%) at 1mg/ml compared to the standard drug acetyl salicylic acid (64.28% and 52.29%) at 0.1mg/ml respectively.

KEYWORDS: **Pomegranate, Antioxidant, Anti-inflammatory, Erythrocyte**

MANUKUMAR H.M
Pristine Laboratories, Kodigehalli Gate, Bangalore-560092, Karnataka, India,

***Corresponding author**

INTRODUCTION

Plants are always a rich source of macro nutrients that do not appear in essential primary metabolism, including secondary metabolites. Phytochemicals are often referred to non-nutritive compounds thought to be produced by plants as means of protection against such dangers as harmful ultraviolet radiation, pathogens and herbivorous predators. The consumption of a plant-based or phytochemical-rich diet has been associated with a reduced risk of chronic human illnesses such as certain types of cancers, inflammation, cardiovascular and neuro degenerative diseases[1]. Therefore, the chemistry and biology of phytochemicals are of highest importance for evaluation of their potential health benefits to humans. Phenolic compounds, including flavonoids, anthocyanins and tannins, are the main group of antioxidant phytochemicals with interesting properties and have deep value due to their biological and free radical scavenging activities[2]. The antioxidative phytochemicals, especially phenolic compounds, found in vegetables and fruits have received increasing attention for their potential role in the prevention of human diseases[3, 4].Medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, lesser or no side effects and economic viability [5]. Furthermore, the effect of any synthetic and herbal agents on the stabilization of erythrocyte membrane exposed to hypotonic solution has been studied extensively because the erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane[6]. The pomegranate (*Punica granatum* L.), which belongs to the Punicaceae family, is a nutrient dense food source rich in phytochemical compounds[7,8]. Pomegranates are popularly consumed as fresh fruit, as beverages (for example, juices and wines), as food products (for example, jams and jellies), and as extracts wherein they are used as botanical ingredients in herbal medicines and dietary supplements. The major source of dietary pomegranate phytochemicals is the fruit (peel, seeds and juice). Pomegranate has gained popularity in recent years due to its multifunctional and nutritional benefit in the human diet. The fruit is rich in tannins and other biochemicals, particularly phenolics, which have been reported to reduce disease risk[9,10]. Edible parts of pomegranate fruit comprise 78% juice and 22% seed[11]. Pomeganate seeds are rich in sugers, vitamins, polysaccharides, polyphenols and minerals. They have low oil content but are rich in polyunsaturated fatty acids[12]. The presence of antioxidants has been reported from pomegranate juice[13,14]. Pomegranate contains some species of flavonoids and anthocyanidins in its seed oil and juice and shows antioxidant activity three times greater than green tea extract [15]. Pomegrante juice contains tannins, ellagic tannis, anthocyanins, catechins, gallic and ellagic acid as antioxidant chemicals. Pomegrante seeds are known to contain esterogenic compounds. One of the most remarkable characteristics of pomegranate fruit is that its seeds are the richest plant source of esterogen[15]. Furthermore, it inhibits breast cancer cell proliferation and invasion and promotes breast cancer cell apoptosis[16]. The consumption of pomegranate has been associated with beneficial health effects, such as prevention of oxidation of both low and high density lipoprotein, blood pressure, inflammatory, atherosclerosis, prostate cancer, heart disease, and HIV-1[17-20]. Seeram[21] reported that pomegranate juice has greater antioxidant capacity than other fruit juices and beverages.Recent studies in pomegranate fruits have shown that cultivar may also substantially influence the antioxidant activity and other physicochemical properties, such as skin and juice percentage, dry matter, pH, total soluble solids (TSS), total sugars, titratable acidity (TA), total phenolics, and anthocyanins[22-28]. These parameters may provide important information to the consumer in terms of recognizing a more nutritional fruit.If fruit of pomegranate show

potential to improve human health, their utilization should be encouraged during fruit processing. In the quest to promote the development of functional foods with health-benefiting properties, we investigated the physicochemical properties, antioxidant and anti-inflammatory activities of extracts from juice and seed, using in vitro assays.

MATERIALS AND METHODS

Plant materials

The pomegranate fruit was procured from a local market in Bangalore and washed with water. Juice and seeds separated. Seeds are air dried and powder (10 g) and 100ml juice were extracted and stirred with 100 ml of water at 30°C for one night. In each case, the solution was covered with parafilm to prevent the solvent evaporation and taken in continuous agitation for one night. Phenolic compounds extractions were done under homogenous conditions. The extracts were filtered through Whatman No.1 filter paper for removal of particles. Then extracts were pooled and concentrated under vacuum.

Chemicals

Spectrophotometric measurements were performed on Shimadzu ultraviolet (UV)-1800 spectrometer (Shimadzu, Kyoto, Japan). Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Gallic acid, Butylated Hydroxytoluene (BHT), Rutine and FeCl3 were purchased from Sigma Chemical. Sodium Carbonate from Merck Chemical Supplies. All the other chemicals used including the solvents, were of analytical grade.

Physical properties

Physical properties were determined for pomegranate. Fruit weight was measured by an electronic balance with an accuracy of 0.001 g. Fruit length and diameter were measured by using a digital vernier caliper with a sensitivity of 0.01 mm. Fruit volume was measured calculated by a liquid displacement method described by Westwood[29]. The measurement of fruit length was made on the polar axis, i.e., between the apex and the end of stem. The maximum width of the fruit, as measured in the direction perpendicular to the polar axis, is defined as the diameter.

Qualitative phytochemical screening

Each extract was screened for the presence of key families of phytochemicals according to the method cited by Marzouk[30] and previously reported by Trease and Evans[31] and Sakar and Tanker [32] using the related reagents and chemicals. Alkaloids were analyzed using Dragendorff‟s reagent confirmed with Bouchardat‟s (I2/MgI2) and with Meyer's reagents (KI/MgCl2).

Determination of total polyphenol content (TPP)

TPP were estimated by the Folin-Ciocalteu method reported in Elfalleh[33]. From each sample, 0.5 ml of methanolic solution to 0.5 ml of Folin- Ciocalteu (Prolabo) reagent was added. We add 4 ml of a solution of sodium carbonate (1 M). TPP of each fraction were converted into mg gallic acid equivalents per g dry weight (mg GAE/g DW).

Determination of total flavonoids content (TF)

The amount of TF in the extracts was measured spectrophotometrically following the method of Djeridane[34]. This method was based on the formation of a complex flavonoid–aluminium, having the maximum absorbance at 430 nm. Rutin was used to make a calibration curve. 1 ml of methanolic extract was mixed with 1 ml of 2% AlCl3 methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm using a Shimadzu 1600-UV spectrophotometer. TF was expressed as mg rutin equivalents per g DW (mg RE/g DW).

DPPH Free radical scavenging activity

The scavenging activity of DPPH free radicals by different plant extracts was determined according to the method reported by[35]. Fifty microliters of the plant extract in methanol, yielding 100 µg/ml in each reaction, was mixed

with 1 ml of 0.1 mM DPPH in methanol solution and 450 µl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µl) only was used as the experimental control. After 30 min of incubation at room temperature, the reduction in the number of DPPH free radicals was measured, reading the absorbance at 517nm. BHT were used as controls. The percent inhibition was calculated from the following equation:

% Inhibition = [Absorbance of control – Absorbance of test sample/Absorbance of control] x 100

Reducing power assay

The method of Shinde [36] was used with modifications. 2.5ml of extract was mixed with 2.5ml of sodium phosphate buffer (0.2M pH 6.6) and 2.5ml of potassium ferricyanide (1% in water) in a test tube and reacted for 20 min at 50^{o}C.The mixture was cooled using crushed ice and 0.5ml of trichloroacetic acid (10% in water) was added and the set up was centrifuged for 10 min. One ml of the supernatant was collected and an equal volume of water was added 0.2ml of 0.1% ferric chloride. The absorbance was read at 700nm against the reagent blank. Tannic acid was used as the standard. Increased absorbance reading indicates increased reducing power.

Effect on hemolysis

Human Erythrocyte suspension

The whole blood was collected from a healthy volunteer had not taken any NSAIDS for 2 weeks prior to the experiment and collected in heparinzed vacutainer. The blood was washed three times with 0.9% saline and centrifuged simultaneously for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and a 40% v/v suspension made using isotonic phosphate buffer which was composed of 154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4 used as Stock erythrocyte or RBC suspension.

Hypotonic solution-induced hemolysis

The membrane stabilizing activity of the extract was assessed according to the method described by Shinde[37] with slight modifications. The test sample consisted of stock erythrocyte (RBC) suspension 0.50ml mixed with 5ml of hypotonic solution (50mM NaCl in 10mM Sodium Phosphate Buffered saline at pH 7.4) containing juice and seed sample concentration 1mg/ml. The control sample consisted of 0.50ml RBC suspension mixed with hypotonic buffered solution alone. The standard drug acetylsalicylic was treated at 0.1mg/ml concentration. The experiment was carried out in triplicate. The mixtures were incubated at 10 minutes at room temperature, centrifuged for 10 minutes at 3000rpm and absorbance of the supernatant was measured spectrophotometrically at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated by following equation.

% Inhibition of haemolysis = 100 x [A 1 –A 2 /A 1]

Where:

A1 = Absorbance of hypotonic buffered solution alone

A2 = Absorbance of test /standard sample in hypotonic solution.

Heat-induced hemolysis

Aliquots (5 ml) of the isotonic buffer containing 1mg/ml of different extractives of samples were put into two duplicate sets of centrifuge tubes [37]. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (500 µl) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54^{o}C for 20 min in a water bath. The other pair was maintained at 0-5^{o}C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The standard drug acetylsalicylic was treated at 0.1mg/ml concentration. The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

% Inhibition of hemolysis = 100 x [1- (OD2-OD1/ OD3-OD1)]

Where,

OD1 = test sample unheated,

OD2 = test sample heated and

OD3 = control sample heated

Statistical Analyses

Assays were performed in triplicate, and the results were expressed as mean values with standard deviations (SD).

RESULTS AND DISCUSSION

The physical properties of pomegranate were measured showing fruit weight (258.33g) and fruit volume (264.77cm^3). Thus, there was a close relationship between the fruit weight and volume (Table 1) Fruit length (72.42mm) and fruit diameter (76.03mm) values were near to the values reported by Sarkhosh [38].Pomegranate juice was revealed for pH, TSS, TA and total sugar (Table 2). The pH value (3.43) (Table 2), our result near to the values observed (2.82-3.81) by Cam et al [39] on pomegranate cultivars grown in Turkey. The TSS content (13.15°Brix), which is in agreement with the results of Fadavi et al[40]. The TA was 0.91g/100 g. Fadavi et al[40] reported that TA values of some pomegranate cultivars in Iran were between 0.40 and 2.45 g/100 g. The maturity index (TSS/TA) is one of the important factors influencing the taste and flavor of pomegranate. The level of total sugars of pomegranate was 17.25g/100g. In another study, the total sugar's values of some pomegranate cultivars growing in Turkey were between 13.9 and 16.1 g/100g [41].

Table 1
Fruit weight (FW), fruit volume (FV), fruit length (FL) and fruit density (FD) of pomegranate.

FW (g)	FV (cm^3)	FL (mm)	FD (mm)
258.33±0.25	264.77±0.26	72.42±0.21	76.03±0.24

Table 2
pH, total suspended solids (TSS), total acidity (TA) and total sugar (TS) of pomegranate juice.

pH	TSS (°Brix)	TA (g/100g)	TS (g/100g)
3.43±0.02	13.15±0.02	0.91±0.00	17.25±0.02

Table 3
Qualitative phytochemical screening in pomegranate juice and seed.

Antioxidant contents	Alkaloids	Flavonoids	Saponins
Juice	++	+++	++
Seed	+	+	+++

(+) indicates presence, (++) indicates present in high, (+++) indicates present in very high

Table 4
Total phenolic and total flavonoids content of juice and seed aqueous extract of pomegranate

Pomegranate	Phenolic content (mgGAE/g)	Flavonoids content (mgRE/g)
Juice	89.52±0.21	32.60±0.21
Seed	6.73±0.07	2.82±0.02

Table 5
Effect of extractives of pomegranate on hypotonic solution and heat-induced hemolysis of erythrocyte membrane

Pomegranate	Concentration (mg/ml)	Hemolysis inhibition (%)	
		Heat induced	Hypotonic solution induced
Juice	1	29.65±0.07	49.23±0.14
Seed	1	19.57±0.01	36.25±0.03
Acetyl salicylic acid	0.1	52.29±0.09	64.28±0.02

Results (Table 3) show that there are differences noted between plant parts: alkaloids and flavonoids in juice higher than seed. Saponins were highly present in seed than juice. Indeed, alkaloids are commonly found to have antimicrobial properties[42]. Flavonoids have been found to possess antitumoral, anti-allergic, and anti-inflammatory activities[43]. For these reasons, activity cannot be imputed to one family of phytochemicals. The difference between parts can explain and support the uses by old people of specific part of the plant to treat specific illness and disease.The studied phenol contents (Table 4) varied according to plant parts were proved. Stintzing et al[44] suggested that a considerable diversity of opinion exists on the appropriate method to assess these antioxidants in plant tissues. Phenolic acids and polyphenols are plant derived secondary metabolites. Dark coloured samples are reported to contain more phenolic acid derivatives and less flavonoids compared to light coloured ones and visa versa[45]. In pomegranate as shown in Table 4, the TPP expressed as mg GAE/g are highest in juice (89.52), than seed (6.73). The antioxidant activity depends largely on their chemical composition, such as phenolics, flavonoids, enzymes, organic acids, amino acids, Maillard reaction products, ascorbic acid, carotenoids[46]. Flavonoids are the largest group of polyphenolic compounds found in higher plants and synthesized from the shikimic acid and malonic acid pathways[47].Flavonoids possess free radical scavenging activities which prevent oxidative cell damage, have anti-inflammatory, anticancer activities as well as protection against the different levels of carcinogenesis. The seed sample contains fewer amounts (2.82 mgRE/g) of flavonoids than juice (32.60 mgRE/g), this agreeing the results of Amiot et al[45]. There are certain problems associated with use of animals in experimental pharmacological research such as ethical issues and the lack of rationale for their use when other suitable methods are available, or could be investigated. Hence, in the present study the hemolysis was selected for in vitro assessment of anti-inflammatory property in pomegranate juice and seed.Hydro extract of pomegranate juice and seed at a concentration of 1mg/ml was studied and well established in the table 5. Here individual hydro extract were establishing it's potency through protection of RBC hemolysis in hypotonic solution. Juice sample showing hemolysis protection 49.23%, it is higher than seed 36.25%. Results were compared with standard Acetylsalicylic acid which showed 64.28% protection against the lyses at 0.1mg/ml as shown in table 5. The erythrocyte membrane is analogous to the lysosomal membrane[48] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Inhibiting the heat induced hemolysis at 1mg/ml concentration provides evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. The present finding demonstrated that the extract of juice and seed has the capacity to stabilize red blood cell membrane against stress, which indicates the ability of the extract to prevent hemolysis or rupture of RBCs. Test extract juice inhibited the heat induced hemolysis of RBCs 29.65% and seed 19.57% at concentration of 1mg/ml compare to the standard Acetylsalicylic acid 52.29% at 0.1mg/ml. The significant membrane stabilizing activity of the extract may be is due to the presence of polyphenolic content of the extract[49].From the methodological point of view the DPPH method is one of the shortest available to investigate the overall hydrogen/electron donating activity of single

antioxidants and health-promoting dietary antioxidant supplements of fruit and vegetable juices or extracts. The free radical scavenging activities of samples were measured showing reduction of DPPH radicals can be observed by the decrease in absorbance at 516 nm. Results are in Fig.1 show that both extracts displayed good antioxidant activities but juice (48%) higher than seed (36%).

Figure 1
Percentage of inhibition of DPPH radical scavenging activity of pomegranate juice and seed.

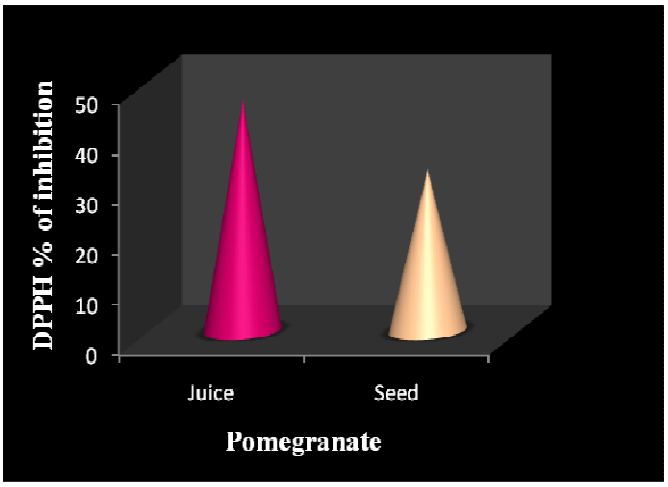
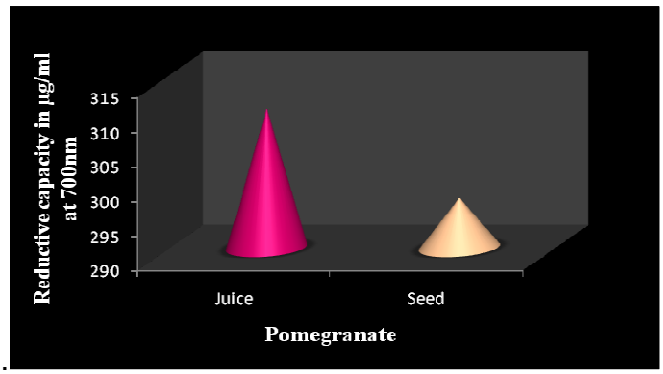


Figure 2
Reductive Capacity of pomegranate juice and seed.



Reducing power assay is a novel method that is used in the assay of the antioxidant activities of various medicinal plants and it employs the reduction of Fe3+ to Fe2+. The reducing properties are generally associated with the presence of reductones[50], which having antioxidant action by breaking the free radical chain by donating a hydrogen atom[51]. Fe2+ has been shown to produce oxyradicals and lipid peroxidation. This is because antioxidants are strong reducing agents[52]. In aqueous extract of juice exhibiting 310µg/ml higher than seed 297µg/ml shown in Fig 2.

CONCLUSION

In this study, pomegranate was analyzed for various physicochemical properties, antioxidant activity and anti-inflammatory property. The results indicate that there is a genetic heterogeneity compare to literature study. In addition, the results provide important

information of the physicochemical properties of pomegranate which can be useful for developing fruit processing industry. This investigated study has shown that the juice and seed of the pomegranate fruit possess strong antioxidant and anti-inflammatory activities. Therefore the juice and seed of the pomegranate fruit could be exploited as a potential source of natural antioxidant agents, as well as a potential anti-inflammatory agent. The findings provide scientific basis to promote value-adding of pomegranate fruit parts for pharmaceutical and cosmetic purposes. Further studies on the isolation of active ingredients, determination of its mode of action in pomegranate juice and seed extracts are warranted.

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CONFLICT OF INTEREST

There is no conflict of interest associated with the authors of this paper.

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