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Antioxidant Activity Directed Isolations from *Punica granatum*

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Abstract. The extracts derived from pomegranate juice following antioxidant activity directed isolation were screened for their antioxidant activity through their ability to scavenge 2,2- diphenyl-1-picrylhydrazyl (DPPH) radicals. Only fractions which exhibited >50% DPPH scavenging effect at each step of isolation were selected for further purification and their ability to reduce peroxide formation (peroxide value) in heated corn oil. Phytochemical analysis of the pure compounds finally obtained, revealed the presence of pelargonidin-3- galactose (Pg-3-galactose), cyanidin-3-glucose (Cy-3-Glucose), gallic acid, quercetin and myricetin in the fractions exhibiting >50% DPPH scavenging potential. The order of antioxidant activity of these pure compounds by DPPH method was found to be gallic acid> quercetin> myricetin> Cy-3-galactose> Pg-3-Glucose while order with respect to reduction in peroxide value (PV) was the reverse of DPPH.

Keywords: *Punica granatum,* antioxidant activity, pelargonidin-3-galactose, cyanidin-3glucose, gallic acid, quercetin, myricetin

Introduction

The excess production of active oxygen species, such as $^{\cdot}OH$, O_2, singlet oxygen and other free radicals, causes damage throughout the cell by oxidizing a variety of molecules, including unsaturated lipids. Lipids are major membrane components and their oxidation leads to significant changes in membrane properties. These changes initiate processes leading to carcinogenesis, mutagenesis, aging and arteriosclerosis (Cutler, 1992; Stadtman, 1992; Pryor, 1986). Free radicals are also involved in the deterioration of food and oil (Naz *et al.,* 2008; 2005; 2004). Cells have limited possibilities for eradicating free radicals, hence it is believed that endogenous antioxidants enhances its ability to protect vital biological functions (Osawa *et al.,* 1990; Kohen *et al.,* 1988; Cutler, 1984). There is an increasing interest in the application of naturally occurring antioxidants as therapeutic agents. Fruit and vegetable antioxidants play an important role in reducing the risk of degenerative diseases such as cardiovascular disease and various cancers and neurological diseases (Ames *et al.,* 1993). Ascorbate is the most studied antioxidant vitamin for its role in reducing the risk of degenerative diseases (Fraga *et al.,* 1991). However, recent studies have shown that fruit and vegetable total phenolics and anthocyanins contribute more to the antioxidant capacity than ascorbate (Connor *et al.,* 2002; Kang and Saltveit, 2002; Deighton *et al.,* 2000; Kalt *et al.,* 1999).

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Pomegranate fruit, which contains a high proportion of polyphenolic compounds (gallocatechins, delphinidin, cyanidin and pelargonidin) is very well known for its therapeutic uses. Pomegranate juice and peel provide protection against hepatotoxicity (Murthy *et al.,* 2002), methicillin-resistant *Staphylococcus aureus* (Machado *et al.,* 2002), Human Immunodeficiency Virus (HIV) (Lee and Watson, 1998), genital herpes virus (Zhang *et al.,* 1995) and tumors (Boukharta *et al.,* 1992). They exhibit estrogen like activity (Maru *et al.,* 2001), reduce systolic blood pressure (Aviram and Dornfeld, 2001), and decrease Low Density Lipoprotein (LDL) susceptibility to aggregation and retention (Aviram *et al.,* 2000). They also exhibit strong antioxidant potential against lipid peroxidation, LDL and High Density Lipoprotein (HDL) oxidation, which justifies their use as anti atherosclerotic agent and as biopreservative in food (Plumb *et al.,* 2002, Aviram, 2002; Singh *et al.,* 2002; Aviram and Dornfeld, 2001). Pomegranate extract exhibits antienzymic activity against glycosyl transferase (Oshima and Mitsunaga, 1999), collagenase (Kawakami, 1995) and also activity against eicosanoid (Schubert *et al.,* 1999). Due to being a collagenase inhibitor, pomegranate extract has been successfully used in skin cosmetics.

In view of its high medicinal potential and previous findings, the study was designed to isolate various fractions from the fruit following antioxidant activity

so that its further possible uses in medicines, therapeutics and food preservation could be determined.

Materials and Methods

Antioxidant activity guided isolation. Juice of 5 kg of pomegranate was extracted with the help of a juice extractor machine. The juice was concentrated on a rotary evaporator at 37 °C.The crude juice after screening for antioxidant activity was separated by column chromatography. A column (100 × 3.5 cm) containing cellulose of mesh size 40 was used. The cellulose was soaked in a conical flask for 4 h in mobile phase-acetic acid: hydrochloric acid: water (30:3:10). The mixture was degassed and was poured into the column taking all the precautions to avoid the incorporation of the air. The column was left overnight for perfect settling of the particles. The extracted and dried sample of juice (200 g) was resolved using a flow rate of 1 mL/min. The five coloured bands (PG1, PG2, PG3, PG4 and PG5 separated within 6 h) were obtained using a fraction collector. When the fractions were screened for their antioxidant activities, PG1 and PG3 were found to be substantially active. For further separation of PG1 (39 g), flash chromatography was used. For this purpose a column (20 × 2.5 gm) filled with silica of mesh size 360 was used and the flow rate of the mobile phase butanol : acetic acid : water, 4:1:5 (BAW) was adjusted at a rate of 1mL/min. The fractions obtained were dried using rotary evaporator. The fractions PG1b and PG1c which showed significant antioxidant activity were purified further.

The dried PG1b fraction (12 g) was purified by paper chromatography, BAW was used as solvent system. The bands separated after 22-24 h on chromatographic paper were cut into small pieces and the fractions PG1b1, PG1b3 and PG1b4 were recovered by soaking the pieces in methanol:HC1 (99:1) with constant stirring for 2-3 h. Each fraction was filtered and dried on rotary evaporator at room temperature and tested for antioxidant activity. The fractions, PG1b1(1.56 g) and PG1b2 (1.2 g), which showed appreciable antioxidant activity were further purified by Thin Layer Chromatography (TLC) using silica gel as stationary and BAW as mobile phase. The fractions eluted from preparative TLC were resolved on High Performance Thin Layer Chromatography (HPTLC) using BAW. The purity of the fractions was confirmed by High Performance Liquid Chromatography (HPLC) using μ-BondaPak C-18 column (125A, 10 × 3.9 × 30 mm) using a mixture of methanol:acetic acid:water (71:7:22). Identification of the pure compounds was carried out in 99:1 methanolic HCl on a UV-visible spectrophotometer at 200-600 nm. Since the compounds were identified as anthocyanins, they were also analyzed for their carbohydrate and organic acid moieties.

The fraction PG1c was evaporated to 1/10 of its original volume in a rotary evaporator at 40 °C, acidified with 2M H_2SO_4 and then extracted with chloroform. The chloroform extract was dried (6.8 g) and then analyzed by TLC-silica using chloroform-acetic acid (9:1) as mobile phase. Among the fractions- PG1c1 and PG1c2, only PG1c1 (1.66 g) showed antioxidant potential. The fraction was scratched from the plates, dissolved in methanol:acetone (75:25), dried, washed with methanol and then dried again. The purity of the compounds was confirmed by HPTLC, HPLC and identified using R_f values and spectral properties.

PG3 (6.3 g) was further fractionated into PG3a (117 mg) and PG3b (121mg) in the same way as PG1c1 and PG1c2 from PG1c. Purity was checked by two dimensional chromatography, HPTLC, and HPLC. Purified compounds were finally run in BAW analyzed in UV and UV + ammonia, and scanned in UV-visible region in ethyl alcohol for λ_{max} for identification.

Determination of carbohydrate moiety. The purified compound (2 mg) was hydrolyzed in a mixture of 2.5 μL of methanol and 2N HCl on a boiling water bath for 30 min (Markham, 1982). The mixture was cooled down to room temperature. The aglycone was extracted in 5 μL of amyl alcohol in a separating funnel. After shaking continuously, the upper layer of amyl alcohol was collected. The lower layer containing hydrolyzed sugar was washed with a small quantity of 10% di-*n*-octylmethylamine in chloroform to remove HCl, washed with chloroform to remove traces of amines and then dried.

Identification of sugar. Two drops of water were added to the isolated hydrolyzed sugar for preparing a concentrated solution which was spotted on Whatman chromatographic paper 1 with the standard reference sugars and was developed in BAW for 14 h. The developed chromatogram was dried and dipped in aniline-hydrogen phthalate and heated at 110 °C for 3-5 min to visualize sugar spots (Harborne, 1973). To distinguish between the sugars giving identical colour reactions and R_f values, a small quantity of sugar was treated with resorcinol-1M H_2SO_4 and aniline-hydrogen

phthalate separately and then both of the coloured complexes so formed were scanned in the visible region.

Determination of organic acid moiety. The isolated compound 2 mg was dissolved in 100 µL of ethanol and heated boiling water bath for 5 min. The two dimensional paper chromatography was carried out by using the systems-*n*-propanol: IM ammonium hydroxide (7:3) and *n*-butanol:formic acid:water (10:3:10). The chromatogram was dried in order to remove the traces of formic acid and was sprayed with bromothymol blue solution prepared by dissolving 0.04 g of bromothymol blue in 100 mL of 0.01 M sodium hydroxide (Harborne, 1973).

Spectroscopy and chromatography. Ultraviolet absorbance, λ_{max} in nm, were measured in methanol, on a Shimadzu 160A UV-Visible spectrophotometer. Merck Silicagel 160 G_{254} (20 × 20 cm) glass plates (5715) were used for analytical TLC and Macherey-Nagel HPTLC Nano-SIL 20 UV 254 (0.2 mm, 10 × 10 cm) plates (811 022) were used for two dimensional chromatography to confirm the purity of the isolated compounds. HPLC was performed on a Micromeritics instrument equipped with a 787 variable UV-visible detector, a µ-Bondapak ODS (C-18, 300 × 4.5 × 5 µm) column (Waters) and Chromatography Station for Windows (CSW32). The mobile phase used for HPLC was 50% aqueous methanol at a flow rate of 1 mL/min., pressure 8.4 bar and attenuation 0.32 mV.

DPPH scavenging activity. Reaction mixtures containing test sample [5 µL dissolved in dimethyl sulphoxide (DMSO)] and 95 µL of 316 µM ethanolic solution of DPPH in 96-well microtiter plates were incubated at 37 °C for 30 min and absorbance was measured at 515 nm (Yu *et al.,* 2002; Wettasinghe and Shahidi, 2000). Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group.

Peroxide radical scavenging activity in oil. Ten grams of fresh oil were taken into two clean and dry flasks separately (control and test). In one of them 0.1 g of the test sample was added (Test). Both were heated at 98 °C under carefully controlled aerated conditions for 2-3 h. At the end of heating, the peroxide value of each was determined after cooling the flasks to ambient room temperature (Kochhar and Rossell, 1990; Kahl and Hildebrandt, 1986).

Determination of Peroxide Value (PV). The test was carried out in diffused daylight. One gram of oil sample from control and test were dissolved separately in 10 mL of chloroform in an iodine flask by stirring. 15 mL of acetic acid and 1 mL of saturated potassium iodide solution were then added. The flasks were stoppered quickly, shaken for one min and then, kept in the dark for 5 min. After adding 75 mL distilled water, the liberated iodine was titrated with sodium thiosulphate solution using starch solution as an indicator. A blank test was carried out simultaneously without oil sample under the same conditions (IUPAC Standard Methods, 2.501, 1987).

Results and Discussion

The fractions isolated and their antioxidant activities have been depicted in the antioxidant activity directed isolation scheme (Fig.1). Flash chromatography of PG1 lead to the isolation of PG1b1, PG1b2, PG1b3 and PG1b4. The fractions could not be studied further due to their unstable character and immediate decolourization. PG1bl and PG1b2 which were further purified by TLC and identified as anthocyanins in view of their chromatographic and other properties. The purified compound from PG1b1 was identified as pg-3-galactose and from PG1b2 was identified as Cy3-glucose after comparing their R_f and spectral values with the standard compounds supplied by Fluka. Carbohydrate moieties -galactose and glucose were identified on the basis of a brownish coloured spot with aniline-hydrogen phthalate and comparison of their R_f values (R_f value was 0.12 for both galactose and glucose) with the standard sugars. On the basis of R_f values in BAW, galactose and glucose were distinguished. Glucose showed two peaks at 489 and 555 nm with an inflection at 430 nm while galactose showed peaks at 422 and 495 nm with an inflection at 550 nm on treatment with resorcinol-1M H_2SO_4. No organic acid moiety attached to the purified anthocyanin was detected as confirmed by the absence of a shoulder peak at 334 nm (Horry and Jay, 1988) (Table 1). The pure compounds from fractions PG3a and PG3b and PG1c2 were identified as quercetin, myricetin and gallic acid, respectively in view of their chromatographic and spectral properties compared to standards (Table 2).

The order of antioxidant activity of these pure compounds by DPPH method was found to be gallic acid> quercetin> myricetin> Cy-3-galactose> Pg-3 Glucose (Fig. 2), while the order with respect to reduction in PV was the reverse of DPPH (Fig. 3). In general, the antioxidant capacity of

compounds possessing an *o*-diphenolic arrangement (catechol structure) is higher than in monophenols due to their ability to form *o*-quinones when reacting with free radicals. Replacing the 3-hydroxyl group of protocatechuic acid by a methoxy group as in vanillic acid had a suppressive influence on the antioxidant capacity (Rosch *et al.,* 2003). This explains the comparatively higher anti-oxidant capacity of cyanidin compared to pelargonidin. This is also in

Table 1. Spectral and chromatographic properties of the isolated anthocyanins

Criterion	Property recorded	
	Pelargonidin-3-galactose	Cyanidin-3-glucose
Colour	Orange red	Deep red
(Rf x100) in BAW	0.46	0.67
Two dimension chromatography BAW and 5% aqueous acetic acid	Best separation	Best separation
Acid/Base response	Blue/colourless in base and red in acidic medium	Blue/colourless in base and red in acidic medium
Carbohydrate moiety	Galactose: R_f 0.12, brown spot with aniline-hydrogen phthalate, Peaks at 422 and 495 nm when complexed with resorcinol-1M H_2SO_4	Galactose: R_f 0.14, brown spot with aniline-hydrogen phthalate, Peaks at 489 and 555 nm when complexed with resorcinol-1M H_2SO_4
Organic acid moiety	No organic acid moiety	No organic acid moiety
λ_{max} in HCl-methanol and then in 5% alcoholic $AlCl_3$	523 nm, no bathochromic shift as there is no catechol group in the molecule group in the molecule	523 nm, a bathochromic shift was observed due to catechol

Table 2. Spectral and chromatographic properties of the isolated flavonols

Criterion	Property recorded		
	Quercetin	Myricetin	Gallic acid
Colour	Yellow	Light brown	Light yellow
(Rf x100) in BAW	0.64	0.64	0.05
Two dimension chromatography: BAW and 5% aqueous acetic acid	No separation	No separation	No separation
λ_{max} in HCl-methanol and then in 5% alcoholic $AlCl_3$	255 and 374 nm, a bathochromic shift was observed due to catechol group in the molecule	256 and 378 nm a bathochromic shift was observed due to catechol group in the molecule	272 nm a bathochromic shift was observed due to catechol group in the molecule

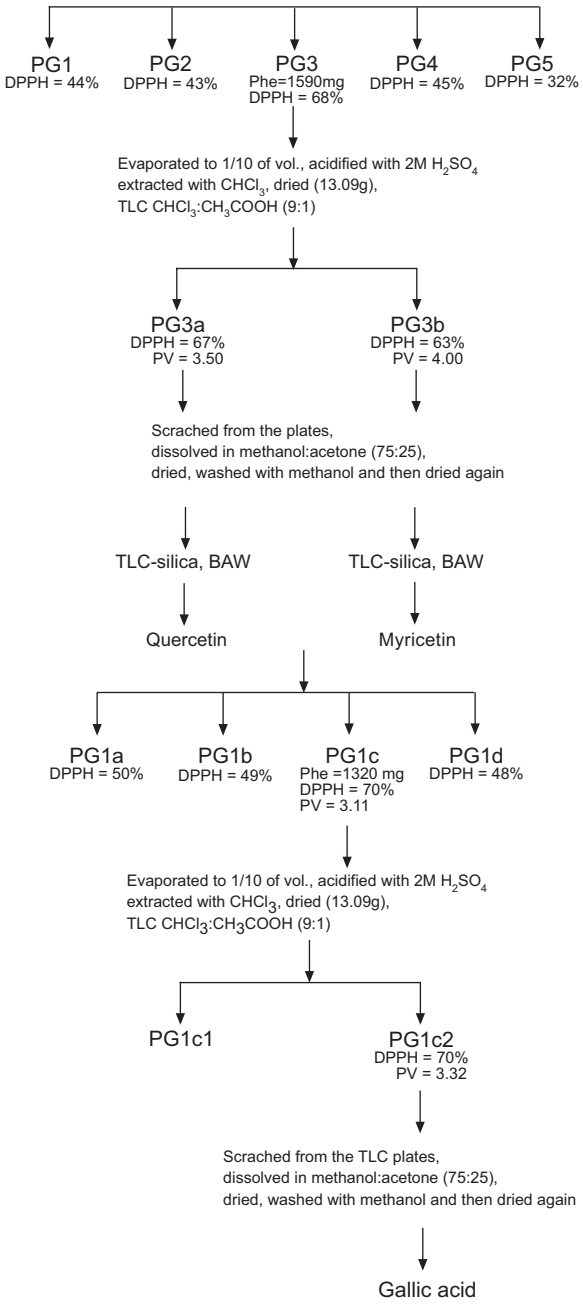

Fig. 1. Scheme for antioxidant activity directed isolations from pomegranate juice.

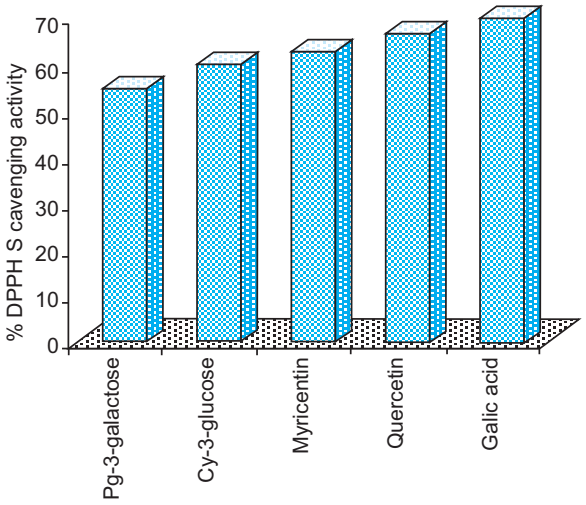

Fig. 2. Relative % DPPH scavenging activity of the isolated compounds.

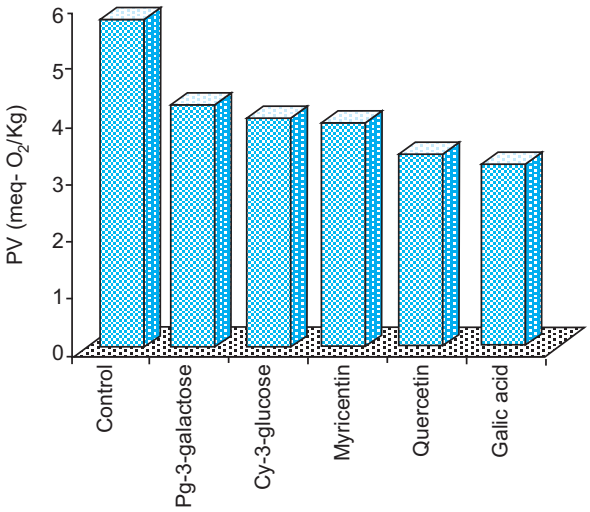

Fig. 3. Effect of the isolated compounds on the PV of the heated oil.

agreement with the findings of Noda *et al.* (2002) in which free radical scavenging activity of 70% active extract of pomegranate was determined and ID_{50} of cyanidin (22 μM) against O_2- was found to be less than pelargonidin (45 μM). The highest antioxidant activity of gallic acid is due to the presence of an additional hydroxyl group. Because of its pyrogallol structure, it shows a greater oxidizability and the quinine formed can be stabilized by resonance structures. The importance of a pyrogallol structure for maximum antioxidant activity of hydroxyl benzoic acid derivatives was also described by (Rice-Evans *et al.,* 1996) for the TEAC assay and by Cao *et al.* (1997) for the ORAC assay. A possible reason for the lower antioxidant activity of myricetin compared to quercetin could be the high oxidation sensitivity of myricetin which caused its rapid decomposition during measurement (Burda and Oleszek, 2001).

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