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Isolation and identification of a radical scavenging antioxidant – punicalagin from pith and carpellary membrane of pomegranate fruit

Anand P. Kulkarni [a], Somaradhya Mallikarjuna Aradhya [a,*], Soundar Divakar [b]

[a] Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Mysore 570013, India
[b] Department of Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore 570013, India

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Abstract

Pith and carpellary membrane (CM) constitute 13% of pomegranate fruit composition. Pith acts as an anchoring tissue while CM acts as a protective membrane; both play significant roles in determining the health and disease status of pomegranate arils but remain unexplored for their nutraceutical properties. Activity-guided repeated fractionation of the methanol extract on a silica gel column, followed by preparative HPLC, yielded a compound which exhibited strong radical scavenging activity with EC_{50} values of 16.7 ± 2.3 and 54.2 ± 4.6 against DPPH free radical and superoxide radicals, respectively. It also showed a very strong lipid peroxidation inhibitory activity in a liposome model system with an EC_{50} value of 54.2 ± 0.9. However, it showed negligible metal chelating activity. Based on UV, IR, 2D-NMR, GC-Mass and MALDI-Mass studies, the compound was identified as punicalagin. The antioxidant activity of punicalagin and the methanol extract can be ascribed mainly to radical scavenging ability.

Keywords: *Punica granatum*; Antioxidant; Isolation; Identification; Pith and carpellary membrane; Punicalagin

1. Introduction

An antioxidant is a substance that, when present at low concentration compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1990). The possible toxicity, as well as general consumer rejection, has led to decrease in the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Namiki, 1990). Therefore a search for antioxidants of natural origin has attracted increasing attention. *Punica granatum* L., commonly known as pomegranate, belongs to the family Punicaceae, which is considered as a holy fruit in the Quran. The ancient science of Indian medicine, namely, ayurveda has identified it as a medicinal plant. Pomegranate is a rich source of polyphenols (Gil, Tomas-Barberan, Hess-Pierce, Holocraft, & Kader, 2000). Epidemiological studies show that consumption of fruits and vegetables with high phenolic contents correlates with reduced cardio-vascular and cerebro-vascular diseases and reduced cancer mortality (Hertog, sweetnam, Fehily, Elwood, & Krombout, 1997a) The edible portion of pomegranate fruit, called arils, originates from the pith and is well protected by carpellary membrane (CM). Diseased status of arils is always associated with degeneration of pith and CM. Teleologically, such tissues may be rich sources of bioactive compounds which may be able to prevent discoloration of arils and subsequent browning. Literature survey revealed that, so far, no attempt has been made to study the chemical composition and bioactive properties of pomegranate fruit pith and CM.

The present work was undertaken with the objective of isolating and identifying the potential bioactive component from pith and CM of pomegranate fruit.

* Corresponding author. Tel.: +91-821-2515653; fax: +91-821-2517233.
E-mail address: aradhyasm@yahoo.co.in (S.M. Aradhya).

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2. Materials and methods

2.1. Materials

Mature pomegranate (var. Ganesh) fruits were harvested from orchards in the Bagalkot district, India, and brought immediately to the Central Food Technological Research Institute, Mysore. Pith and CM were separated from fruit manually, dried in a hot air oven at 48 °C for 36 h and powdered to 60 mesh in an apex grinder.

2.2. Chemicals

All the organic solvents used for extraction and column chromatography were of AR grade from E. Merck (Mumbai, India). Methanol, used for HPLC, was HPLC grade from Ranbaxy fine chemicals limited (India). Nicotinamide adenine dinucleotide (reduced form, NADH), nitroblue tetrazolium (NBT), phenazin methosulphate (PMS), L-ascorbic acid, ferrozine and Tris–HCl were from Sisco Research Laboratories (India). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and BHA were from Sigma Chemical, USA. Acetic anhydride was from S.d. Fine Chemicals (India), 2-thiobarbituric acid was from ICN Biomedicals Inc. Ohio, and trichloroacetic acid and pyridine from Qualigens Fine Chemicals (India).

2.3. Isolation of bioactive compound

2.3.1. Extraction of pith and CM of pomegranate fruit

Dry powder of pith and CM was extracted with a series of solvents of increasing polarity (hexane, chloroform, ethyl acetate and methanol) in a Soxhlet apparatus. Solvent was removed by distillation and assayed for DPPH radical scavenging activity. Since methanol extract showed maximum free radical scavenging activity (Table 1), it was selected for large-scale extraction and isolation of bioactive compound.

2.3.2. Fractionation of methanol extract of pith and CM

Pith and CM powder of pomegranate fruit were extracted with methanol using a soxhlet apparatus and solvent was removed by distillation. About 500 g of pith and CM powder yielded about 248 g of crude methanol extract (Fig. 1). The methanol extract (≈100 g at a time) was subjected to column (450 × 40 mm) chromatography using silica gel (60–120 mesh) and eluted stepwise with hexane, chloroform and a linear gradient of chloroform: methanol: ethyl acetate (100:0:0 to 30:50:20 v/v/v). About 335 fractions, measuring 250 ml, were collected, concentrated by distillation and after analysis by TLC, pooled into 6 groups (fraction No. 1–6; Fig 1) and assayed for their DPPH free radical scavenging potential.

Table 1
Yield and 1,1diphenyl-2-picrylhydrazil (DPPH) radical scavenging effects of different solvent extracts of dry powder of pith and carpellary membrane (CM) of pomegranate fruit

Solvent	Yield% w/w	% DPPH radical scavenging activity[a]
Hexane	0.3	0.00 ± 0.00[b]
Chloroform	0.12	3.35 ± 0.04
Ethyl acetate	1.3	92.1 ± 0.32
Methanol	49.6	93.2 ± 0.02
BHA	–	94.5 ± 0.02

[a] Concentration of test sample was 50 μg ml^{-1}.
[b] Values are means ± standard deviation of 5 replicate analyses.

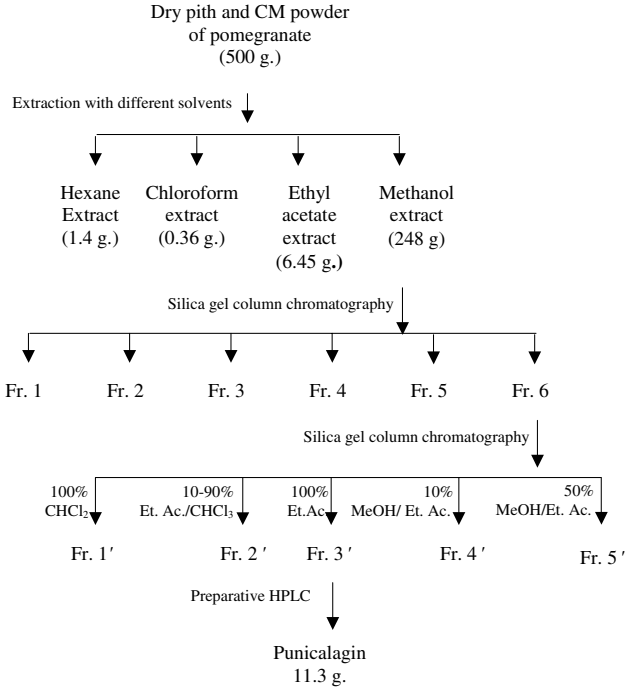

Fig. 1. Extraction scheme for the isolation of antioxidant compound from pith and CM of pomegranate fruit.

2.3.3. Thin layer chromatography (TLC)

An aliquot of column fraction was spotted on to a silica gel TLC plate (20 × 20 cm). The plates were developed in ascending direction from 18 to 19 cm of height with different proportions of chloroform and methanol as mobile phase. After air-drying, the spots on the plate were located by exposure to iodine.

2.3.4. Purification of active fraction

The active fraction (fraction No. 6), which showed maximum DPPH radical scavenging activity (Fig. 2) was subjected to further silica gel column (450 × 40 mm) chromatography and eluted first with linear gradient of chloroform and ethyl acetate (100:0 to 0:100 v/v) and then with linear gradient of ethyl acetate and methanol (90:10 to 50:50 v/v). About 218 fractions, measuring 250 ml, were collected, concentrated by distillation and,

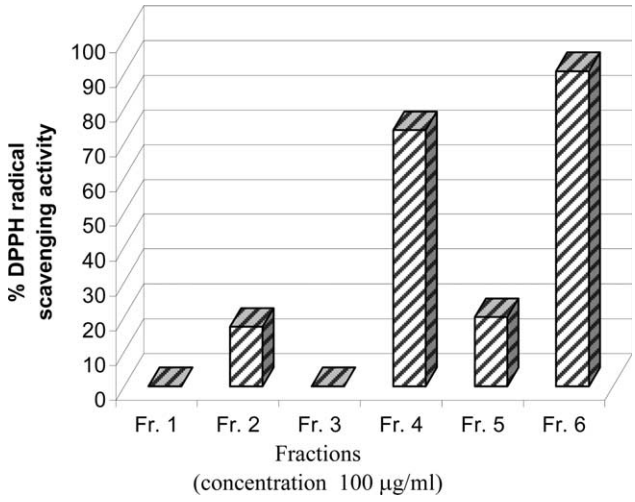

Fig. 2. DPPH radical scavenging activity of silica gel column chromatographic fractions No. 1–6 of methanol extract of pith and carpellary membrane of pomegranate fruit.

after analysis by TLC, pooled into five groups (fraction Nos. 1′–5′; Fig. 1).

2.3.5. High performance liquid chromatography (HPLC)

Partially purified active fraction (Fraction No. 3′ from the above procedure) was further purified by preparative HPLC, using a Shimadzu Preparative Liquid Chromatograph LC-8A (Shimadzu, Singapore), equipped with a Rheodyne 7725i injection valve, fitted with a 100 μl sample loop and a 250 × 20 mm, i.d. 5 μm, Shimpack C-18 column (Kyoto, Japan). The fraction was eluted with an isocratic solvent mixture comprising water: methanol: ethyl acetate (70:25:05) with a flow rate at 5 ml/min. The UV detection was carried out at 280 nm with a Shimadzu diode array detector, series SPD-M10 Avp, Shimadzu (Singapore).

2.3.6. Acetylation of purified compound

Acetylation was carried out according to the method of Markham (1982). About 20 mg of compound, dissolved in 0.5 ml pyridine, to which 0.5 ml acetic anhydride was added, was stirred in a stoppered conical flask overnight at room temperature. Reaction mixture was then poured into ice-cold water (≈50 ml) with constant stirring and left for 1 h. The insoluble acetylated compound was separated by filtration, using a Whatman filter paper No. 1.

2.4. Identification of bioactive compound

2.4.1. UV spectrophotometry

UV–vis spectra were recorded on a Shimadzu UV-160A instrument at room temperature. The region from 200–800 nm was employed for scanning. About 2 mg sample dissolved in 20 ml methanol was used for recording the spectrum.

2.4.2. Infra red spectrometry

IR spectra were recorded on a Perkin–Elmer FT-IR Spectrometer (Spectrum 2000) at room temperature. A region from 400 to 4000 cm^{-1} was scanned.

2.4.3. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analysis of the acetylated compound was carried out using an Agilent 6890 GC instrument equipped with a 5973 N mass selective detector. An HP-5 MS capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm) was used. Helium was the carrier gas employed, with 1.2 ml/min constant flow mode. The oven temperature was maintained at 50 °C for 2 min and ramped to 300 °C at 10 °C/min and held there for 5 min. Inlet temperature was 300 °C, interface temperature was 280 °C, ion source temperature was 230 °C and quadrupole temperature was 150 °C.

2.4.4. Matrix-assisted laser disorption ionization mass spectrometry (MALDI MS)

MALDI-MS analysis was carried out on a Kompact Seq, Kratos Analytical spectrometer with time of flight detector at room temperature and the matrix used was α-cyano-4-hydroxy cinnamic acid.

2.4.5. Two-dimensional heteronuclear multiple quantum coherence transfer spectroscopy (2D HMQCT-NMR)

Spectra were recorded on a Bruker DRX500 NMR instrument operating at 500 MHz for ^1H at room temperature. The region from 0 to 12 ppm for ^1H and 0–200 ppm for carbon was employed for scanning. Signals were referred to tetramethylsilane to within ± 0.01 ppm. About 30 mg sample dissolved in 0.5 ml DMSO-d_6 were used for recording the spectra.

2.5. Determination of antioxidant activity

The antioxidant activities of methanol extract and punicalagin of pith and CM of pomegranate fruit were determined by four methods.

2.5.1. DPPH Radical scavenging activity

The antioxidant activity of pomegranate pith and CM extracts on DPPH (Blois, 1958; Bondet, Brand-Williums, & Berset, 1989) radical was measured according to the method of Moon and Terao (1998). About 0.2 ml of ethanolic solution of test sample, at different concentrations, was mixed with 0.8 ml of Tris–HCl buffer (pH 7.4) to which 1 ml DPPH (500 μM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV–visible spectrophotometer (UV-160A, Shimadzu co. Japan). All measurements were made in triplicates with BHA as a

positive control. IC_{50} represents 50% of the radicals scavenged by the test sample.

2.5.2. Superoxide radical scavenging activity

The superoxide radical scavenging assay was carried out according to the method of Liu, Ooi, and Chang (1997). Superoxide radicals were generated in 1 ml of Tris–HCl buffer (0.02 M, pH 8.3) containing 0.1 mM NADH, 0.1 mM NBT, 10 μM PMS and crude extract of pith and CM of pomegranate and punicalagin. The colour reaction of superoxide radicals and NBT was detected at 560 nm, using a Cintra 10 UV–Vis spectrophotometer.

2.5.3. Lipid peroxidation assay

Lipid peroxidation inhibitory activity of methanol extract and punicalagin was measured according to the method of Duh and Yen (1997). Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in a dr. Hielscher Gmb_H, UP 50H ultraschallprozessor. The test samples (100 μl), of different concentrations, were added to 1ml of liposome mixture; control was without test sample. Lipid peroxidation was induced by adding 10 μl $FeCl_3$ (400 mM) plus 10 μl L-ascorbic acid (200 mM). After incubation for 1 h at 37 °C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was boiled for 15 min, then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm.

2.5.4. Metal chelating activity

The ferrous ion chelating activity was determined by the method of Dinis, Almeida, and Madeira (1994). Test samples at different concentrations were mixed with 2 mM $FeCl_2\ 4H_2O$ and 5 mM ferrozine at a ratio of 10:1:2 and the mixture was shaken. After 10 min. the Fe^{++} was monitored by measuring the formation of ferrous ion-ferrozine complex at 562 nm.

3. Results and discussion

3.1. Isolation and identification of antioxidant compound

Solvent extraction, followed by silica gel column chromatography, of dry powder of pith and CM of pomegranate, yielded a potential antioxidant compound. The dry powder was first extracted with hexane in a Soxhlet apparatus in order to separate non-polar compounds, the yield of which is negligible (0.2% w/w). Then it was extracted with chloroform and ethyl acetate which are known to extract low molecular weight phenolics; yields of extract for these solvents were also negligible (0.6% and 1.2% w/w, respectively). Yield of methanol extract was highest (49% w/w) probably due to extraction of both low and high molecular weight phenolic compounds (Hagerman et al., 1998). Table 1 summarizes the extraction yield of different solvents and their antioxidant activity. Antioxidant activities of both ethyl acetate extract and methanol extract were high but the yield of methanol extract was higher than the ethyl acetate extract. Hence methanol was selected for large-scale extraction and isolation of antioxidant compound. Methanol extract was subjected to activity guided repeated fractionation on a silica gel column and eluted with a series of solvents (chloroform, ethyl acetate and methanol) in different proportions, of increasing polarity. The active fraction obtained from repeated silica gel column chromatography was finally purified by preparative HPLC. Fig. 1 schematically represents the extraction procedure for bioactive compound. Yield of the purified bioactive compound was about 2.3% w/w.

3.2. Identification of bioactive compound

The structure of the bioactive compound was determined by various spectroscopic techniques. The purified compound exhibited absorption maxima at 217 nm and at 260 nm, indicating that it possessed phenolic groups. IR data exhibited OH stretching (3430 cm^{-1}) and carbonyl stretching (1660 cm^{-1}) besides other stretching at 2988 and 1027 cm^{-1}, among other signals.

The ^1H and ^{13}C NMR data were obtained from the detailed 2D HMQCT (two dimensional heteronuclear multiple quantum coherence transfer) spectrum. The NMR spectrum (Table 2) showed signals corresponding to glucose and gallagyl units. The C_1 signal observed at 97.6 ppm corresponded to the β-anomer of glucose. The other glucose signals are C_2 – 75.7, C_3 – 72.7, C_4 – 74.0, C_5 – 70.7 and C_6 – 62.2 ppm. The corresponding proton signals were observed in the range 2.89–4.28 ppm. Most of the glucose signals were shifted up-field due to esterification at the C_6 and C_4 OH groups and C_2 and C_3 OH groups. The aromatic carbon signals from gallagyl

Table 2
^1H and ^{13}C NMR data (δ in ppm) for punicalagin from 2D-HMQCT

^{13}C NMR	Assignment	^{H}fs NMR	Assignment
62.2	[a]C_6	3.65	[a]H_{6a}
		3.43	[a]H_{6b}
70.7	[a]C_5	3.54	[a]H_5
72.7	[a]C_3	3.53	[a]H_3
74.0	C_4	3.42	H_4
75.7	C_2	2.89	H_2
97.6	C_1	4.28	H_1
105.7	C–Ar	6.30	Ar–H
106.3	C–Ar	6.44	Ar–H
108.1	C–Ar	6.43	Ar–H
110.7	C–Ar	6.74	Ar–H
		6.15	Phenolic OH (Broad)
		6.56	Phenolic OH (Broad)
		7.9–9.6	Phenolic OH (Broad)

[a]Assignments are interchangeable.

units were observed between 105.7 and 110.7 ppm. Broad phenolic OH signals were observed in the proton region between 6.15 and 9.60 ppm. The aromatic region showed only four protons in the ^{1}H NMR spectrum, at 6.30, 6.44, 6.43 and 6.74 ppm consistent with gallagyl and ellagyl units, which have only two protons each, respectively, attached to the phenyl rings. The other protons were from phenolic OH groups and there are a large number of these in the molecule, as gallagyl and ellagyl units possess ten and six phenolic OH groups, respectively. This, coupled with above-mentioned spectral data, clearly indicated the structure is one as proposed in Fig. 3.

The GC–MS data of the peracylated compound showed 100% abundance of acyl group (CH_3–CO–). The m/z peak at 98 indicated a glucopyranoside ring in the molecule. Other m/z peaks at 115, 127, 157, 169 indicated galloyl groups in the molecule. The MALDI-MS spectrum of the compound showed the mass fragment 1109 (hydrated parent ion, punicalagin).

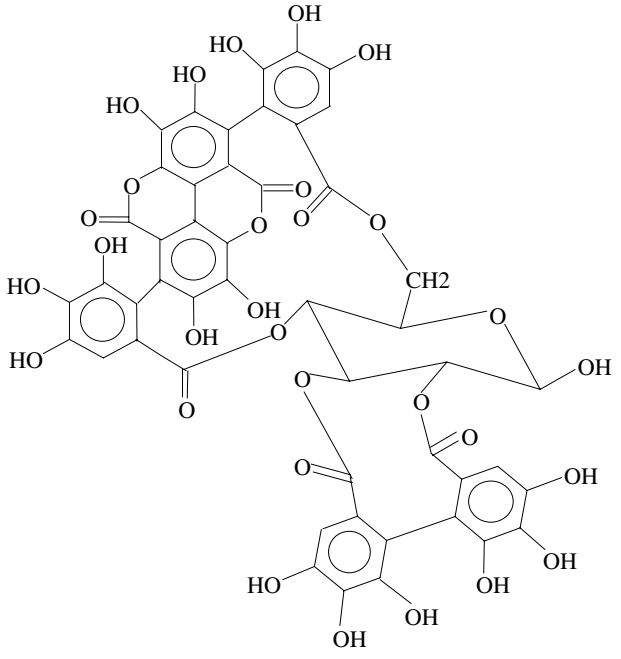

Fig. 3. Structure of an antioxidant compound, punicalagin, isolated from pith and CM of pomegranate fruit.

3.3. Antioxidant activity of pith and CM extracts and punicalagin

Antioxidants may act in various ways, by scavenging the radicals, by decomposing peroxides and by chelating metal ions. Therefore, the antioxidant activity can and must be evaluated with different tests for different mechanisms. In our study we followed most frequently used methods for measuring the antioxidant activity. Table 3 summarizes the antioxidant potential of punicalagin and methanol extract in different antioxidant assay systems.

3.3.1. DPPH radical scavenging activity

Among the different solvent extracts of pith and CM, methanol extract showed (Fig. 2) excellent DPPH free radical scavenging activity (93.2%), followed by ethyl acetate extract (92.1%). Both the extracts are very close to the radical scavenging ability of synthetic antioxidant BHA (94.5%). Chloroform extract showed very negligible DPPH scavenging activity (3.35%) and hexane extract did not show any activity. Yield of the methanol extract (dry) was the highest (49.6% w/w) whereas ethyl acetate extract was very much less (1.29% w/w). The EC_{50} value of methanol extract was found to be 8.33 μg/ml whereas that of synthetic antioxidant BHA was 5.33 μg/ml (Table 3). Punicalagin, the major chemical constituent of pith and CM, showed potent DPPH free radical scavenging activity, with an EC_{50} value of 16.7 μg/ml.

3.3.2. Superoxide radical scavenging activity

The superoxide radicals were generated in a PMS-NADH system and assayed by the reduction of NBT. Table 3 shows the superoxide radical scavenging ability of methanol extract and punicalagin of pith and CM of pomegranate. Both exhibited strong superoxide radical scavenging activity with EC_{50} values of and 71.0 and 58.2 μg/ml, respectively. Artificial antioxidant BHA showed very low superoxide scavenging activity with an EC_{50} value of 264 μg/ml.

3.3.3. Lipid peroxidation inhibitory activity

Lipid peroxidation is a free radical-mediated propagation of oxidative insult to polyunsaturated fatty acids,

Table 3
Antioxidant potential of methanol extract and punicalagin of pith and CM of pomegranate fruit and synthetic antioxidant BHA

Bioactive compounds	EC_{50} value (μg/ml)			
	DPPH radical scavenging activity	Superoxide radical scavenging activity	Lipid peroxidation inhibitory activity.	Metal chelating activity
Methanol extract	8.33 ± 1.6[a]	71.0 ± 3.2	32.4 ± 1.7	–
Punicalagin	16.7 ± 2.3	58.2 ± 4.6	54.2 ± 0.9	–
BHA	5.33 ± 0.6	264 ± 1.6	92.3 ± 1.9	–

[a] Values are mean ± standard deviation of three replicate analyses.

involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants (Heim, Tagliaferro, & Bobilya, 2002) To evaluate the antioxidant activity of punicalagin and methanol extract (to inhibit the lipid peroxidation in biological systems), the liposome model system was used. The lipid peroxidation inhibitory activities of punicalagin and methanol extract of pith and CM of pomegranate are shown in Table 2. The EC_{50} value for punicalagin was found to be 54.2 μg/ml for punicalagin and methanol extract showed an EC_{50} value of 32.4 μg/ml, which is more active than the pure bioactive compound. BHA showed very strong antioxidant activity against lipid peroxidation with an EC_{50} of 12.3 μg/ml.

3.3.4. Metal chelating activity

The above lipid peroxidation studies use iron as a metal ion catalyst. Thus, in principle, it could happen that the ability of punicalagin to inhibit lipid peroxidation was exerted by complexing iron in a catalytically silent form. Therefore, punicalagin and methanol extract were also tested with respect to metal binding capacity, by assessing their ability to compete with ferrozine for ferrous ion and avoiding the formation of the coloured complex with absorbance peaking at 562 nm. Both methanol extract and punicalagin showed very negligible metal chelating activity. Punicalagin showed 1.93% and methanol extract showed 6.7% metal chelating activity at the concentration of 100 μg/ml test sample.

Although punicalagin contained many phenolic OH groups capable of chelating ferrous ion, it showed very low metal chelating activity. With so many OH groups, from gallagyl and ellagyl units, the ion-chelating activity should be very high. Such low activity observed can not be explained.

The above result suggests that the lipid peroxidation inhibitory activities of punicalagin and methanol extract are not due to the iron chelation and must be due to chain termination by scavenging the peroxyl radicals. Thus both methanol extract and punicalagin exert their antioxidant activity mainly by radical scavenging activity, by donating electrons.

The protective effects of antioxidants in biological systems are ascribed mainly to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes and inhibit oxidases. The radical scavenging ability of punicalagin is due to its radical scavenging activity because of multiple phenolic hydroxyl groups which increase the antioxidative activity by additional resonance stability and *o*-quinone or *p*-quinone formation (Chen & Ho, 1997; Bouchet, Barrier, & Fauconneau, 1998). However, the region for very low metal chelating activity is not known.

Compared to punicalagin, the methanol extract showed greater ability to scavenge radicals. This may be due to the synergistic effect of other phenolics present in the crude extract, which supplement the radical scavenging ability. Both punicalagin and methanol extract showed potential radical scavenging activities and negligible metal chelating activity. The above experimental results suggest that the mechanism of antioxidant action of punicalagin and methanol extract is by donating electrons to free radicals.

This is the first report on isolation and identification of punicalagin from pith and CM of pomegranate fruit waste and its bioactive property. Our results suggest that this waste is an economically viable source of punicalagin, a natural and potent antioxidant.

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