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ORIGINAL ARTICLE

In vitro Evaluation of Antioxidant Potential of Isolated Compounds and Various Extracts of Peel of *Punica granatum* L.

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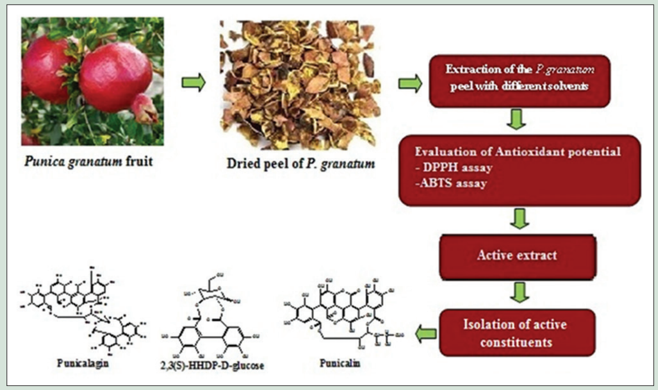
ABSTRACT

Background: *Punica granatum* L. (*Lythraceae*) peel has been proven to exhibit widespread pharmacological application against multitude of diseases due to the presence of bioactive principles. **Objective:** The objective is to isolate the bioactive compounds from the pericarp of *P. granatum* and to evaluate the antioxidant activity of various extracts. **Materials and Methods:** Dried peel of *P. granatum* was extracted with aqueous acetone and chromatographed on Diaion HP-20. Enriched fractions were rechromatographed on Sephadex LH-20 and purified on preparative high-performance liquid chromatography to identify individual compounds. The dried peel was extracted with different solvents to evaluate the antioxidant activity of the extracts. **Results:** On the chemical investigation, three compounds were isolated and characterized as punicalagin, 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, and punicalin, using various spectroscopic techniques. **Conclusion:** Results indicate that the isolated compounds have possessed antioxidant activity, and aqueous, methanol, and aqueous acetone extract showed significant scavenging of 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radicals.

Key words: 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, antioxidant, nuclear magnetic resonance, *Punica granatum*

SUMMARY

- *In vitro* antioxidant activity of *Punica granatum* extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) assay
- Dried peel of *P. granatum* was extracted with different solvents to evaluate the antioxidant activity of the extracts
- Aqueous acetone extract was found to be most active and chromatographed further to afford punicalagin, 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, and punicalin
- The presence of antioxidant properties of three compounds in the peel of *P. granatum* has been demonstrated.



Abbreviations Used: HPLC: High-performance liquid chromatography; HHDP: Hexahydroxydiphenoyl; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); UV: Ultraviolet; PDA: Photodiode array; LC: Liquid chromatography; NMR: Nuclear magnetic resonance; MHz: Megahertz; w/v: Weight by volume; MS: Mass spectra.

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INTRODUCTION

Natural products have proven to be an alternative and potential source of synthetic drugs.[1,2] Research outcomes have exhibited that crude extracts or purified chemical constituents of various medicinal plants were more effective antioxidants than some synthetic antioxidants.[3-6] *Punica granatum* found to be rich in the phenolic compound may contribute directly to antioxidant activity due of the presence of hydroxyl functional groups around the nuclear structure that are potent hydrogen donators.[7]

Plants have been used traditionally for many centuries for preventing diseases, and recent scientific studies have shown that the existence of a good correlation between traditional or folkloric application of some of these plants further strengthens the search for pharmacologically active compounds from plants.[8]

P. granatum L. is a shrub or small tree belonging to the family *Lythraceae*, and its fruit is a rich source of bioactive phytochemicals such as tannins (punicalin, pedunculagin, punicalagin, gallic acid, gallagic acid, and ellagic acid esters of glucose) and other phenolics including flavonoids. It is native from the Himalayas in Northern India to Iran but has been cultivated and naturalized over the entire Mediterranean region and has been used extensively in folk medicine of some countries in Asia and other parts of the world.[8,9] Phenolic compounds from plants exhibit various physiological properties such as anti-allergenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, and vasodilatory effects.[5,10,11] *P. granatum* fruits contain secondary metabolites such as tannins, alkaloids, flavonoids, steroids, phenolics, terpenes, volatile oils, mineral elements, amino acids, glycosides, and

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sterols which are responsible for wide variety of activities.[8,12] This has created interest among researchers, product developers, and consumers on pomegranate plant.[13]

This study was focused on the evaluation of the antioxidant activity of various extracts and isolation of bioactive compounds from the fruit peel of *P. granatum*.

MATERIALS AND METHODS

Plant material

Fresh fruits of *P. granatum* were collected from Nilgiris District, Tamil Nadu, India, and identified by Dr. Santhan, Taxonomist, Natural Remedies Pvt Ltd., Bengaluru. A voucher specimen has been deposited at the Agronomy Department of Natural Remedies Pvt. Ltd., Bengaluru.

General experimental procedures

High-performance liquid chromatography (HPLC) study was carried out using Shimadzu HPLC system LC-2010HT with ultraviolet and photodiode array detector in combination with Class LC solution software and Kromasil C18, 5 µ (250 mm × 4.6 mm) column. 1Hnuclear magnetic resonance (NMR) and 13C-NMR spectra were recorded on Bruker 400 MHz spectrometers. Mass spectra were measured with an LCQ Fleet - Thermo Fisher Scientific instrument. Absorbance was measured at 510 nm using a microplate reader - Versamax microplate reader (Molecular Devices, USA). Standards Gallic acid (Natural Remedies, Pvt., Ltd.,), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), phosphate-Buffered Saline (Sigma, USA), ammonium persulfate, Rankem, India, Microwell plate: Ninety-six well flat, clear plate, Tarsons were procured. All other chemicals and reagents utilized were of AR grade purchased from Rankem, India.

Preparation of extracts

Air-dried coarsely powdered fruit peels of *P. granatum* were extracted with each of the following solvents: chloroform, ethyl acetate, methanol, aqueous acetone, and water, in 1:4 (w/v) ratio of *P. granatum* part to solvent, for 24 h with periodic shaking at regular intervals. After the extraction, the contents were filtered and concentrated at 60°C under vacuum in rotary evaporator. The dried extracts were then used for further analysis. The aqueous acetone extract was found to be most active, which was chromatographed to afford active compounds.

Isolation

One kg of air-dried coarsely powdered fruit peel of *P. granatum* was extracted for three times (3 L) with 75% acetone/water at 60°C for 1 h by reflux method. The extracts were filtered and concentrated at 60°C under vacuum. The extract was chromatographed on Diaion HP-20 resin and rechromatographed on Sephadex LH-20 and further purified by Preparative HPLC [Figure 1]. The purity of the compounds was determined by HPLC.

Compound 1

The extract (350 g) was chromatographed over Diaion HP-20 resin and eluted using water and acetone with decreasing polarity. The fractions were collected and monitored by HPLC. The fractions enriched with compound 1 were repeatedly chromatographed over Sephadex LH-20, eluted in decreasing polarity with water/acetone mixtures. The 20% acetone in water fraction yielded the compound 1 (900 mg) which was identified as punicalagin ($\alpha + \beta$) [Figure 2].

Compound 2 and Compound 3

The 10% acetone in water fraction from the Diaion HP-20 column was enriched with Compound 2 and 3. The enriched fraction from the Diaion HP-20 column was rechromatographed on Sephadex LH-20 repeatedly. The Compound 2 was obtained in the pure form after the repeated purification over Sephadex LH-20. The compound was identified as 2,3-(*S*)-hexahydroxydiphenoyl-glucose (405 mg) [Figure 2] by comparing their spectral data with the values reported in the literature.[14]

The fractions enriched with Compound 3 were subjected to preparative HPLC, and the compounds were purified over Phenomenex C18 preparative HPLC column (250 mm × 21.2 mm, 5 µ). An isocratic method of 5% Acetonitrile in water was used as mobile phase. This purification step afforded the Compound 3 (265 mg), which was identified as punicalin ($\alpha + \beta$) [Figure 2]. The isolated compounds were identified by comparison with the previously reported spectral data.[14,15]

Punicalagin (Compound 1)

Dark yellow powder; m/z 1083.42[M-H]; α-isomer-1H NMR (400 MHz, Acetone) δ 7.03 (1H, s, Hd), 6.60 (1H, s, Hc), 6.58 (1H, s, Hb), 6.51 (1H, s, Ha), 5.21 (1H, t, J = 9.6 Hz, H-3), 5.12 (1H, d H-1), 4.83 (1H, m, H-2), 4.77 (1H, m, H-4), 4.19 (1H, m H-6b), 3.28 (1H, br t, J = 9 Hz, H-5), 2.10 (1H, m, H-6a).). 13C NMR (300 MHz, Acetone) δ 168.46 (C-7), 167.83(C-7), 167.4(C-7'), 166.94(C-7'), 157.2 and 156.66 delta-lactone, 89.45 (C-1), 76.1(C-3), 73.65 (C-2), 70.34 (C-4), 66.16 (C-5), 63.39 (C-6).

β-isomer-1H NMR (400 MHz, Acetone) δ 7.16 (1H, s, Hd), 6.66 (1H, s, Hc), 6.59 (1H, s, Hb), 6.51 (1H, s, Ha), 4.88 (1H, t, J = 9.3 Hz, H-3), 4.81 (1H, m H-4), 4.67 (1H, m, H-1), 4.63 (1H, d, H-2), 4.22 (1H, m H-6b), 2.67 (1H, t, H-5), 2.19 (1H, m, H-6a). 13C NMR (300 MHz, Acetone) δ 168.46 (C-7), 167.83(C-7), 167.4(C-7'), 166.94(C-7'), 157.2 and 156.66 delta-lactone, 93.63 (C-1), 78.14(C-3), 75.64 (C-2), 71.85 (C-5), 70.11 (C-4), 63.39 (C-6).

2,3-(S)-Hexahydroxydiphenoyl-D-glucose (Compound 2)

Dark yellow powder; m/z 481.42 [M-H]; α-isomer-1H NMR (400 MHz, DMSO) δ 6.43 (1H, S, ArHb), 6.329 (1H, S, ArHa), 5.24 (d, 2.4, H-1), 5.09(t, 9.6, H-3), 4.70 (1H, dd, J = 2.8 and 9.6 Hz, H-2), 3.73 (1H, m, H-5), 3.68 (1H, m, H-6), 3.50 (1H, m, H4), 3.37 (1H, m, H-6). 13C NMR (100 MHz, DMSO) δ 168.9 (C-7'), 168.5 (C-7), 144.5(C-4'), 144.5(C-4), 144.2 (C-6), 144.1(C-6'), 134.8 (C-5'), 134.7 (C-5), 125.3(C-2), 124.76 (C-2'), 113.7 (C-1'), 113.6 (C-1), 113.4 (C-3'), 105.1(C-3), Glu δ 89.6 (C-1), 77.05 (C-3), 76.5 (C-2), 72.1(C-4), 66.7 (C-5), 60.2(C-6).

β-isomer-1H NMR (400 MHz, DMSO) δ 6.44 (1H, s, ArHb), 6.32 (1H, s, ArHa), 4.92 (1H, m, H-1), 4.82 (1H, m, H-3), 4.47 (1H, t J = 9.25, H-2), 3.70 (1H, m, H-6), 3.652 (1H, m, H-4), 3.57 (1H, m, H-5), 3.37 (1H, m, H-6). 13C NMR (100 MHz, DMSO) δ 168.9 (C-7'), 168.5 (C-7), 144.5(C-4'), 144.5(C-4), 144.2 (C-6), 144.1(C-6'), 134.8 (C-5'), 134.7 (C-5), 125.3(C-2), 124.76 (C-2'), 113.7 (C-1'), 113.6 (C-1), 113.4 (C-3'), 105.1(C-3), Glu δ 93.1 (C-1), 79.4 (C-3), 76.8 (C-2), 74.1(C-4), 66.9 (C-5), 60.4(C-6).

Punicalin (Compound 3)

Yellowish-green powder; m/z 781.50 [M-H]; α-isomer-1H NMR (400 MHz, Acetone) δ 4.78 (1H, d H-1), 4.332 (1 H, m, H-6b), 4.046 (1H, t, J = 11.1 Hz, H-4), 3.94 (1H, t, J = 10.2 Hz, H-3), 3.32 (d, J = 8.5 Hz H-2), 3.141 (1 H, m, H-5), 2.751 (1H, d, 6a). 13C NMR (300 MHz, Acetone) δ 168.45 C-7. 4, 6-gallagyl, 66.86 C-7', 157.28 and 146.89 lactones, 87.67 (C-1), 74.14 (C-4), 71.97 (C-2), 71.71 (C-3), 68.36 (C-5), 62.22 (C-6).

β-isomer-1H NMR (400 MHz, Acetone)δ 4.37 (1H, m, H-1), 4.306 (1 H, m, H-6b), 4.16 (1H, t, J = 9 Hz, H-4), 3.47 (1H, t, J = 7.5 Hz, H-3), 3.07 (1H, m, H-2), 2.997 (1 H, m, H-5), 2.53 (1H, m, 6a). 13C NMR (300 MHz,

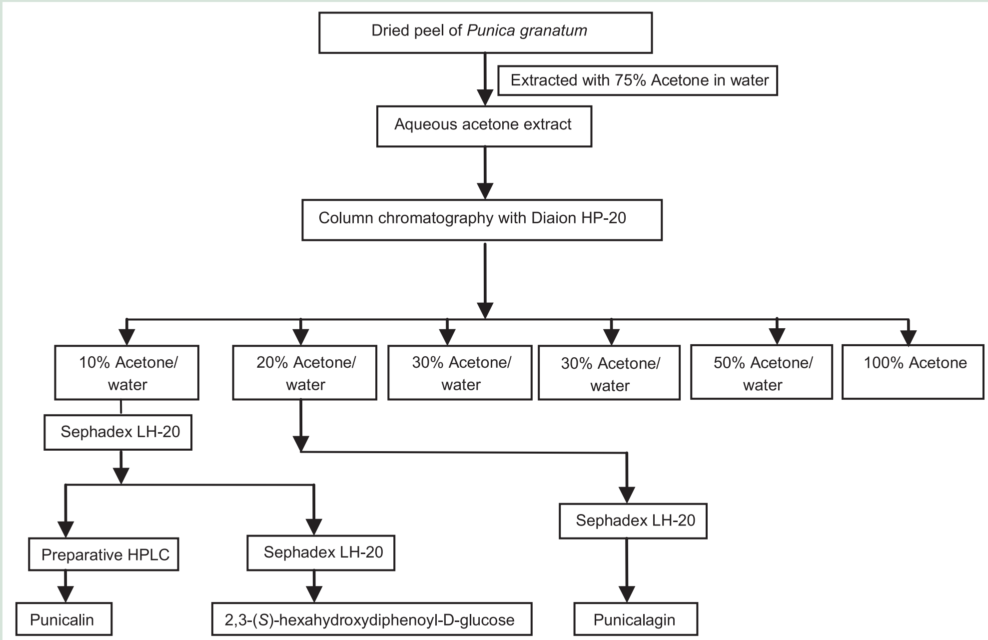

Figure 1: Scheme of isolation process

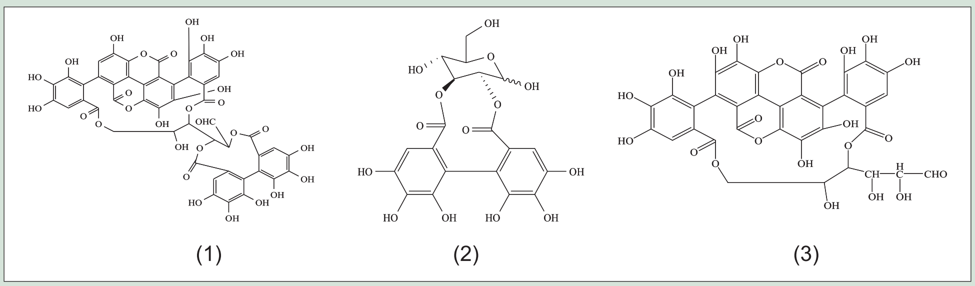

Figure 2: Structure of the isolated compounds - punicalagin (1), 2,3-(*s*)-hexahydroxydiphenoyl-D-glucose (2), punicalin (3)

Acetone) δ 167.61C-7. 4,6-gallagyl, 158.32 C-7', 157.18 and 146.79 lactones, 96.27 (C-1), 74.57 (C-4), 73.14 (C-2), 74.57 (C-3), 70.92 (C-5), 64.56 (C-6).

Evaluation of antioxidant activities

2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity

The antioxidant activities of the extracts and compounds have been measured in terms of hydrogen-donating or radical-scavenging ability using the stable radical DPPH.[16] It determines the ability of the samples for trapping this unpaired electron to the disappearance of radical color.[17]

The DPPH radical-scavenging activity was determined according to the method described by Hiraganahalli *et al.* with slight modifications.[18] Reaction mixture containing methanol, different concentration of test solutions (extracts/compounds), and DPPH (0.659 mM) were incubated in a dark place at 25°C for 25 min. Gallic acid was used as positive control. Using Versamax microplate reader, the samples, positive control, and the blank were recorded at 510 nm. Assay was performed in triplicates, and the percentage inhibition was calculated by the formula ([absorbance of control − absorbance of test sample]/[absorbance control]) × 100.

2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity

ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to suppress the production of radical cation in a concentration-dependent manner and the color intensity decreases proportionally which can be determined spectrophotometrically at 734 nm.[19,20]

The assay was performed as per Hiraganahalli *et al.*[18] To a 250 µl, total reaction volume containing 20 µl of 10 mM phosphate-buffered saline pH 7.4/vehicle buffer/positive control (gallic acid)/test solutions of various concentrations, 230 µl of ABTS radical solution (0.238 mM) was added, mixed, and immediately read at 734 nm using microplate reader. To assess the ABTS radical-scavenging activity, the formula, absorbance of control − absorbance of test sample/absorbance control ×100 is used where absorbance of control is the absorbance of ABTS radical in methanol.

Statistical analysis

The results were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Each experiment was carried out in triplicates. Values are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The aqueous acetone extract of fruit peel of *P. granatum* afforded punicalagin, 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, and punicalin, which were identified by physical and spectral analysis. HPLC was

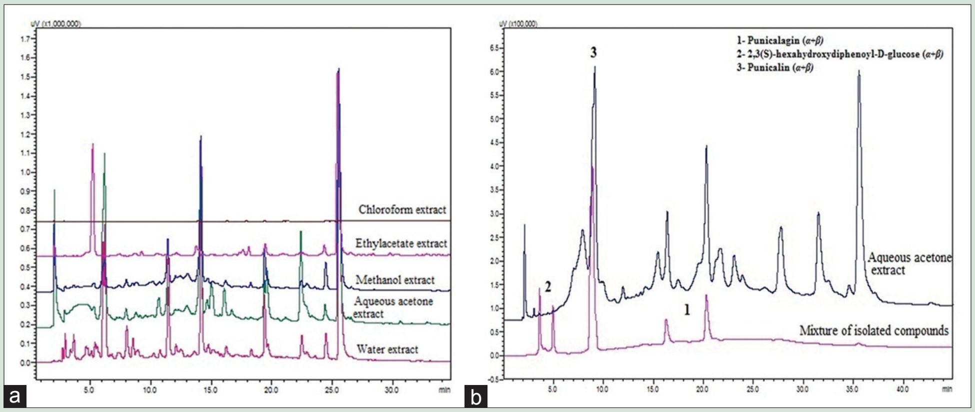

Figure 3: (a) Overlay of high-performance liquid chromatography chromatograms of various extracts of peel of *Punica granatum*, (b) Overlay of high-performance liquid chromatography chromatograms of isolated compounds from the peel of *Punica granatum*

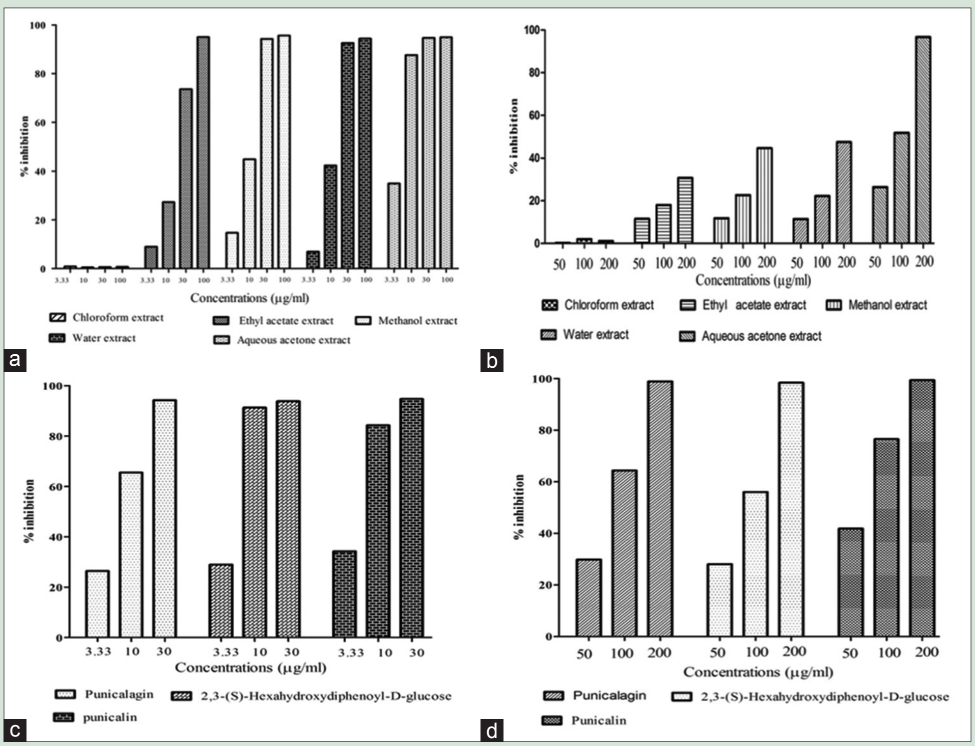

Figure 4: *In vitro* antioxidant activity of (a) various extracts of peel of *Punica granatum* by 2,2-diphenyl-1-picrylhydrazyl assay, (b) various extracts of peel of *Punica granatum* by 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) assay, (c) isolated compounds of peel of *Punica granatum* by 2,2-diphenyl-1-picrylhydrazyl assay, and (d) isolated compounds of peel of *Punica granatum* by 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) assay

carried out for different extracts and isolated compounds to identify the presence of phytoconstituents in the extracts [Figure 3]. All isolated compounds and extracts were tested *in vitro* for their antioxidant activity, and the results for DPPH and ABTS assay were displayed in Figure 4.

In DPPH assay, the extracts and compounds were demonstrated a dose-dependent increase at concentrations ranging from 3.33–30 to 3.33–100 µg/ml, respectively. The percentage inhibition values showed the following order of radical-scavenging activity: aqueous acetone extract > methanol extract > water extract > ethyl acetate extract > chloroform extract. Among the compounds, 2,3-(*S*)-hexahydroxydiphenoyl-glucose showed significant antioxidant and radical quenching potential [Table 1].

The ABTS assay results have shown that the extracts and compounds displayed a dose-dependent activity with different concentrations (50,100, and 200 µg/ml). Punicalin showed the significant scavenging activity when comparing with other isolated compounds [Table 2]. The order of ABTS radical-scavenging activity of all extracts was similar to that observed for DPPH. The extracts of lower polarity solvents, chloroform, and ethyl acetate showed lower antioxidant activity compared to polar solvents. The antioxidant activity shown by the polar solvent extracts may be due to the presence of highest total phenolic content.

Table 1: 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity of isolated compounds and different extracts of *Punica granatum* peel

Sample name	Percentage of inhibition			
	3 µg/ml	10 µg/ml	30 µg/ml	100 µg/ml
Punicalagin	26.45±0.55*	65.49±0.53*	94.21±0.29*	Not tested
2,3-(*S*)-HHDP-glucose	28.97±0.43*	91.31±0.25*	93.83±0.82*	Not tested
Punicalin	34.26±0.82*	84.26±0.24*	94.71±0.27*	Not tested
Chloroform extract	0.84±0.06	0.52±0.07	2.31±0.26	0.73±0.12
Ethyl acetate extract	9.01±0.22	27.25±0.50*	73.58±0.65*	94.97±0.64*
Methanol extract	14.68±0.33	44.86±0.38*	94.23±0.81*	95.60±0.37*
Aqueous acetone extract	34.91±0.10*	87.63±0.48*	94.65±0.23*	94.86±0.41*
Water extract	6.96±0.21	42.32±0.15*	92.54±0.28*	94.36±0.89*

The values are represented as mean±SD (n=3). The criterion for statistical significance was *P<0.05. HHDP: 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose; SD: Standard deviation

Table 2: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity of isolated compounds and different extracts of *Punica granatum* peel

Sample name	Percentage inhibition		
	50 µg/ml	100 µg/ml	200 µg/ml
Punicalagin	29.85±0.26*	64.36±0.44*	98.92±0.10*
2,3-(*S*)-HHDP-glucose	28.10±0.21*	56.05±0.42*	98.41±0.06*
Punicalin	41.85±0.50*	76.51±0.48*	99.38±0.50*
Chloroform extract	0.21±0.06	2.05±0.80	1.18±0.39
Ethyl acetate extract	11.59±0.50	18.00±0.92*	30.67±0.63*
Methanol extract	11.90±0.23	22.72±0.35*	44.62±0.38*
Aqueous acetone extract	26.41±0.52*	51.85±0.88*	96.67±0.36*
Water extract	11.54±0.54	22.26±0.45*	47.49±0.40*

The values are represented as mean±SD (n=3). The criterion for statistical significance was *P<0.05. HHDP: 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose; SD: Standard deviation

CONCLUSION

Pomegranate peel is a good source of phenolic compounds and has potent antioxidant activity. Here, the presence of antioxidant properties of three compounds punicalagin, 2,3-(*S*)-hexahydroxydiphenoyl-glucose, and punicalin in the peel of pomegranate has been demonstrated. Further work is in progress to find out the nutritional and therapeutic properties.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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