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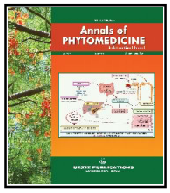

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Phytochemical analysis and antioxidant efficacy of methanol and acetone extracts of *Punica granatum* L. peel

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Abstract

The peel of the Pomegranate, *Punica granatum* L., is a rich source of bioactive substances with strong biological effects. Whereas, *P. granatum* peel, constitutes about 30-40 % of fruit weight, discarded as biological debris. The current research was intended to collect valuable information regarding phytochemical constituents and antioxidant efficacy of *P. granatum* peel. The methanol and acetone extracts of *P. granatum* peel were assessed for total phenolic, total flavonoid, total sugar, reducing sugar and non-reducing sugar contents. Phytochemical analysis results indicated that methanol and acetone extract possess total phenolics (244.11 and 196.47 mg GAE/g), total flavonoid (106.50 and 63.35 mg CE/g), total sugar (525.89 and 215.76 mg/g), reducing sugar (471.68 and 192.14 mg/g), non-reducing sugar (54.21 and 23.62 mg/g). The antioxidant efficacy was assessed through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-based assays. In DPPH and ABTS assays, it was observed that methanol extract (IC_{50} 75.63 µg/ml and IC_{50} 77.54 µg/ml) exhibited a significantly higher degree of free radical scavenging activity than acetone extract (IC_{50} 104.19 µg/ml and IC_{50} 105.63 µg/ml). Thus, *P. granatum* peel offers the opportunity to be utilised in the production of novel nutraceuticals, functional foods and other valuable products, providing opportunities for the food, cosmetic, and pharmaceutical sectors.

1. Introduction

P. granatum is one of the oldest fruits that has been cultivated and is a member of the Punicaceae family. It belongs to the category of fruits that possesses highest pharmacological activities due to the high concentration of phytochemical compounds. *P. granatum* peel, an agro-waste product generated during juice production, is used as animal feed after industrial processing (Zarfeshany *et al*., 2014). *P. granatum* peel is a potential source of various phytochemicals that are responsible for its antioxidant, wound healing, antimicrobial, anti-atherosclerotic, immune modulatory and antibiofilm activity. Phytochemicals such as polyphenolics, ellagitannins (gallotannins, gallic acid, kaempferol, ellagic acid, punicalagin, punicalin, quercetin, luteolin, glycosides and pedunculagin), flavonoids, and carbohydrate composite present in *P. granatum* peel (Zarfeshany *et al*., 2014; Seeram *et al*., 2005). Solvent extraction, most frequent method to recover plant antioxidants, significantly affected by the polarity of the solvent used and chemical nature of extracted compounds (Devi *et al*., 2023; Moond *et al*., 2023).

Namely, earlier studies proved that, *P. granatum* peel has highest concentration of phenolic compounds than other fruit parts, with hydrolysable tannins being the main component (Brighenti *et al*., 2017). In a recent study, Alexandre *et al*. (2019) performed high pressure (300 MPa) assisted extraction of *P. granatum* peel to improve its antioxidant efficacy and to reduce the possibility of microbial contamination (Dalal *et al*., 2022; Moond *et al*., 2023). In another study, ethanol extract of *P. granatum* peel was proved to exhibit significant antioxidant, antiproliferative and antimicrobial activity (Peršuriæ *et al*., 2022). Ethanol (70%) extract of *P. granatum* peel provided maximum extract yield (29.46%), total tannin (0.76 ± 0.02 mg GAE/g DM) and total phenolic content (10.61 ± 0.15 mg GAE/g DM) than methanol and acetone. However, methanol extract yielded better total phenolic and tannin content than acetone extract (Magangana *et al.,* 2021). Freeze dried powder of *P. granatum* peel exhibited highest concentration of quercetin (2.5 mg/g), punicalagin (15.2 mg/g), ellagic acid (13.6 mg/g), and gallic acid (32.2 mg/g) than ethanol, aqueous and acetone extract (Kumar *et al*., 2022). Phenolics as antioxidants promote cellular health, immunological function and slow down the ageing effects (Moond *et al*., 2023). Antioxidant action of phenolics is due to their redox mechanism which causes free radical scavenging. The current research was intended to collect valuable information regarding phytochemical constituents and antioxidant efficacy *via* DPPH and ABTS assays.

2. Materials and Methods

2.1 Plant material and chemicals

The *P. granatum* fruits were acquired from Vegetable Science Research Farm of Chaudhary Charan Singh Haryana Agricultural University (29°.04' N latitude and 75°.69 E longitude). Using a portal (Tropicos and IPNI LSID: 554129-1), Dr. Anita, Assistant Professor,

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Department of Botany and Plant Physiology, CCS HAU, Hisar, verified the fruit samples. Fruits were peeled off, peels were collected, washed with double distilled water and shade dried for 30 days at room temperature. Then grounded into a powder and stored in sealed bottles. All analytical grade chemicals such as gallic acid, sodium carbonate, folin-ciocalteu reagent, $NaNO_2$, $AlCl_3$, NaOH, catechin, D-glucose, phenol, sulphuric acid, sodium potassium tartarate, sodium bicarbonate, $CuSO_4$, ammonium molybdate, disodium hydrogen arsenate, BHA, DPPH, ABTS and ammonium persulfate, provided by Sigma-Aldrich and Hi-media Pvt. Ltd., were utilised.

2.2 Preparation of extracts

10 g powder sample of *P. granatum* peel was taken in a thimble and inserted into a conventional Soxhlet apparatus coupled with round-bottom flask. 250 ml of solvents (methanol and acetone) were added through the column. The extraction process was lasted for 56 h, following the completion of 6-7 cycles (Moond *et al*., 2023). The volume of each solvent was recorded for further analysis of total phenolics, total flavonoids, total sugars, non-reducing sugars and reducing sugars. Extracts were concentrated using a Rota-evaporator and lyophilized up to 30 m Torr for antioxidant activity.

2.3 Quantitative phytochemical estimation

2.3.1 Total phenolics

Total phenolics of methanol and acetone extracts were quantified *via* Folin-ciocalteu approach (Singleton and Rossi, 1965). Gallic acid was applied as standard antioxidant. Methanol and acetone extracts were diluted to 50% and 20% (v/v), respectively. To 1 ml of each extract, 2 ml 20% (w/v) sodium carbonate and then 1ml Folin-ciocalteu reagent were added. The reaction mixture was finally diluted with 6 ml of distilled water and allowed to develop for 8 min. Then reaction mixture was run through a centrifuge machine at 5500 rpm for 15 min. At 730 nm, the absorbance of the supernatant solution was monitored with a Shimadzu UV-Vis spectrophotometer. A blank was prepared similarly but it contained pure solvent as a substitute of respective extract.

2.3.2 Total flavonoids

Total flavonoids were quantified *via* aluminium chloride colorimetric assay (Ribarova *et al*., 2005). Catechin was used as standard antioxidant. To 1 ml of each extract, 4 ml of distilled water and 0.3 ml of $NaNO_2$ (5%) solution were added. After 5 min., 0.3 ml of $AlCl_3$ (10%) and 2 ml of 1 M NaOH solutions were added. The reaction mixture was finally diluted with distilled water up to a volume of 10 ml. At 510 nm, the absorbance was monitored using Shimadzu UV-Vis spectrophotometer. A blank was prepared similarly but it contained pure solvent as a substitute of respective extract.

2.3.3 Total sugars

Total sugars were quantified *via* Dubois approach (Dubois *et al*., 1956). D-glucose was applied as standard. To 1 ml of each extract, 2 ml of phenol solution was poured. Immediately, 5 ml of concentrated sulphuric acid was poured and then receded to cool for 35 min. Absorbance was monitored at 490 nm using Shimadzu UV-Vis spectrophotometer. Then a blank was prepared similarly but it contained pure solvent as a substitute of respective extract.

2.3.4 Reducing sugars

Reducing sugars were quantified *via* Nelson method as modified by Somogyi (Nelson, 1944; Somogyi, 1952). D-Glucose was applied as standard. Alkaline copper reagent (2 ml) was poured to 1 ml of each extract. The reaction mixture was vortexed, wrapped in foil and sterilised for 5 min in water bath. Following cooling, 1 ml of arsenomolybdate reagent was poured and diluted with distilled water up to a volume of 10 ml. At 520 nm absorbance was monitored using Shimadzu UV-Vis spectrophotometer. A blank was prepared similarly but it contained pure solvent as a substitute of respective extract.

2.3.5 Non-reducing sugars

Non-reducing sugars were quantified using the difference between total sugar and reducing sugar content.

2.4 Antioxidant efficacy

2.4.1 DPPH assay

The approach of Hatano *et al*. (1988) was modified for the measurement of DPPH free radical scavenging activity. 1 ml of each concentration of methanol (50-150 µg/ml) and acetone extract (50-150 µg/ml) was diluted with 2 ml of DPPH solution. Then, reaction mixture was subjected to incubation in dark for 30 min. Butylated hydroxyanisole (BHA) in the concentration range of 5-30 µg/ml was applied as standard antioxidant. Absorbance was measured at 517 nm using Shimadzu UV-Vis spectrophotometer. A control was prepared similarly but it contained pure solvent as a substitute of respective extract. % DPPH free radical scavenging activity was measured as:

$$\%\ \text{DPPH free radical scavenging activity} = \left[\frac{A_c - A_S}{A_c}\right] \times 100$$

where, Ac signifies the control absorbance and As signifies the sample absorbance.

2.4.2 ABTS assay

ABTS assay was assessed *via* Re *et al*. method using methanol as blank (Re *et al*., 1999). 10 ml ABTS of 7 mM concentration was mixed with 5 ml Ammonium persulfate of 2.45 mM concentration and incubated for 16 h at room temperature. Methanol was added to the ABTS solution to get an absorbance of 0.700 ± 0.002 at 734 nm. 1 ml of each concentration of methanol (50-150 µg/ml) and acetone extract (50-150 µg/ml) was diluted with 2 ml of ABTS solution and incubated at room temperature. A control was prepared similarly but had contained pure solvent as a substitute of respective extract. % ABTS free radical scavenging activity was measured as:

$$\%\ \text{ABTS free radical scavenging activity} = \left[\frac{A_c - A_S}{A_c}\right] \times 100$$

where, Ac signifies the control absorbance and As signifies the sample absorbance.

3. Results

3.1 Quantitative phytochemical estimation

Table 1 compiled the quantitative phytochemical estimation of *P. granatum* peel extracts. Total phenolics, total flavonoid, total sugar, reducing sugar and non-reducing sugar contents were calculated in methanol and acetone extracts of *P. granatum* peel.

Table 1: Quantitative phytochemical analysis of *P. granatum* peel

Plant extract	Total phenolics (mg GAE/g)	Total flavonoid (mg CE/g)	Total sugar (mg/g)	Reducing sugar (mg/g)	Non-reducing sugar (mg/g)
Methanol	244.11	106.50	525.89	471.68	54.21
Acetone	196.47	63.35	215.76	192.14	23.62

3.1.1 Total phenolics

Total phenolics were quantified using a gallic acid mediated standard curve (Figure 1). The total phenolic contents of *P. granatum* peel extracts in methanol and acetone were determined to be 244.11 mg GAE/g and 196.47 mg GAE/g, respectively.

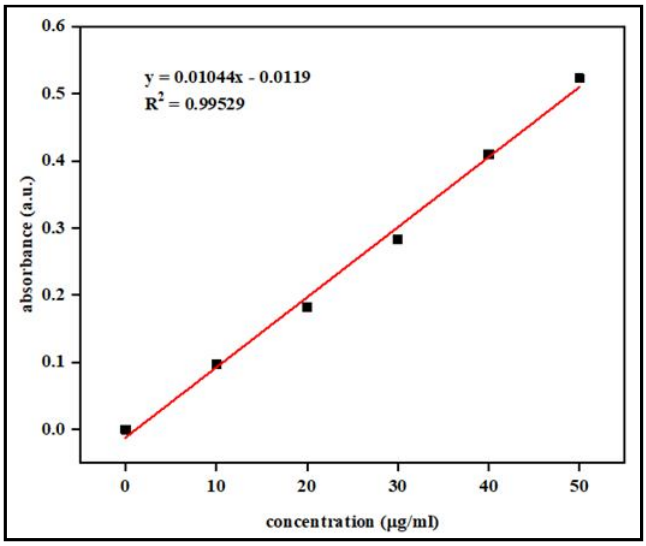

Figure 1: Total phenolics standard curve with gallic acid.

3.1.2 Total flavonoids

Total flavonoids were quantified using a catechin mediated standard curve (Figure 2) and expressed in unit of mg catechin equivalent (CE)/g. The total flavonoid contents of *P. granatum* peel extracts in methanol and acetone were determined to be 106.50 mg CE/g and 63.35 mg CE/g, respectively.

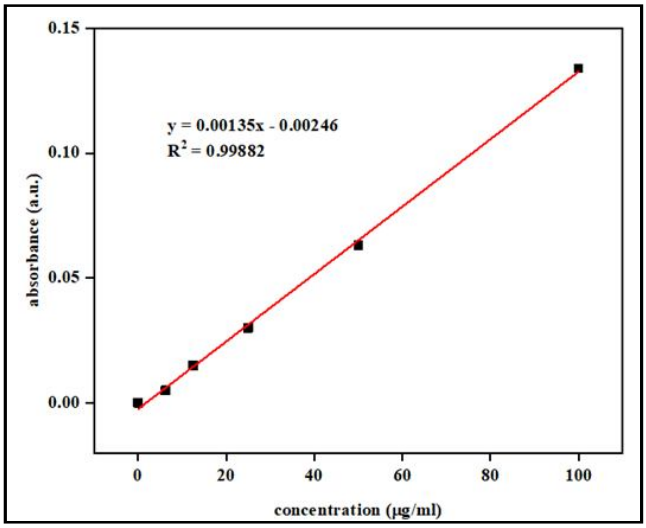

Figure 2: Total flavonoids standard curve with catechin.

3.1.3 Total sugars

Total sugars were quantified using a D-glucose mediated standard curve (Figure 3).The total sugar contents of *P. granatum* peel extracts in methanol and acetone were determined to be 525.89 mg/g and 215.76 mg/g, respectively.

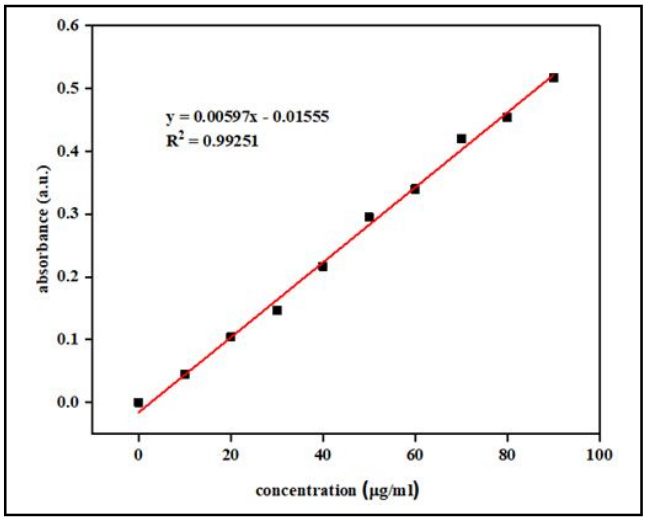

Figure 3: Total sugars standard curve with D-glucose.

3.1.4 Reducing sugars

Reducing sugars were quantified using a D-Glucose mediated standard curve (Figure 4). The reducing sugar contents of *P. granatum* peel extracts in methanol and acetone were determined to be 471.68 mg/g and 192.14 mg/g, respectively. Reducing sugar was found 89.69% of total sugar in methanol extract and 89.05% in acetone extract.

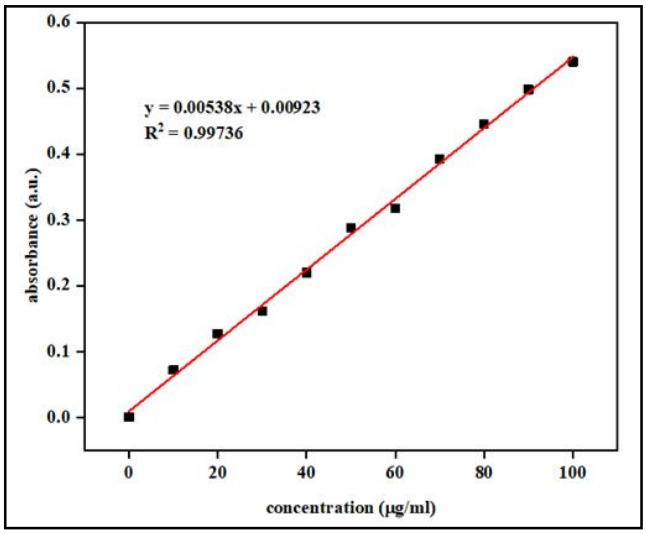

Figure 4: Reducing sugars standard curve with D-glucose.

3.1.5 Non-reducing sugars

There were 54.21 mg/g of non-reducing sugars in methanol extract and 23.62 mg/g in acetone extract of *P. granatum* peel. Non-reducing sugar was found to be 10.31% of total sugar in methanol extract and 10.95% in acetone extract.

3.2 Antioxidant efficacy

Table 2 summarises the Antioxidant efficacy of methanol and acetone extracts of *P. granatum* peel. Antioxidant efficacy was assessed *via* DPPH and ABTS assays and results were reported in terms of IC_{50} (µg/ml).

Table 2: Antioxidant efficacy of *P. granatum* peel

Plant extract/standard	IC_{50} (µg/ml)	
	DPPH	ABTS
Methanol	75.63	77.54
Acetone	104.19	105.63
BHA	16.39	4.47

3.2.1 DPPH assay

As the concentration of methanol and acetone extracts of *P. granatum* peel increased, % DPPH free radical scavenging activity increased as well. Methanol extract exhibited highest DPPH free radical scavenging activity of 78.78% at 140 µg/ml, followed by 77.89, 75.94, 73.05, 69.52, 62.98, 55.39, 43.62, 30.78 and 20.67% at concentrations of 130, 120, 110, 100, 90, 80, 70, 60 and 50 µg/ml. Acetone extract exhibited greatest DPPH free radical scavenging activity of 59.20% at 140 µg/ml, followed by 58.70, 56.44, 53.25, 47.22, 41.96, 35.89, 25.22, 14.46 and 6.7% at 130-50 µg/ml concentration range (Figure 5). The IC_{50} concentrations for BHA, methanol extract and acetone extract were found to be 16.39, 75.63 and 104.19 µg/ml which demonstrated the greater antioxidant efficacy of BHA, followed by methanol extract and acetone extract of *P. granatum* peel (Table 2).

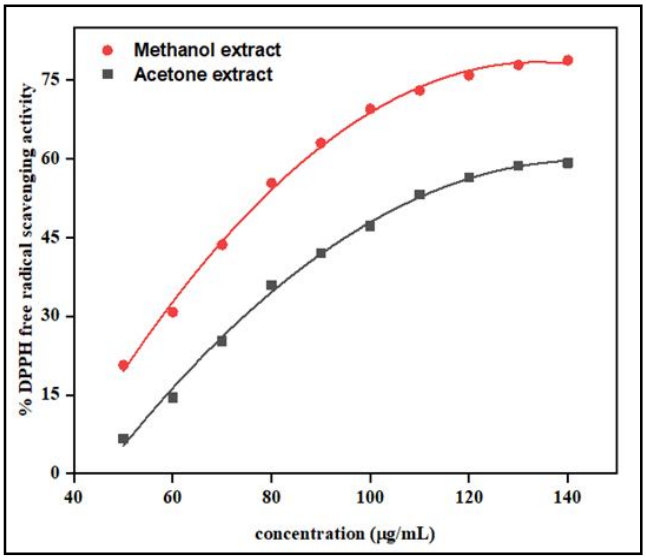

Figure 5: DPPH assay of methanol and acetone extracts of *P. granatum* peel.

3.2.2 ABTS assay

In ABTS assay, methanol extract had the maximum ABTS free radical scavenging activity of 97.98% at 140 µg/ml, followed by 95.95, 90.18, 83.89, 74.96, 63.11, 51.38, 40.14, 24.36 and 14.45% at 130, 120, 110, 100, 30, 89, 70, 60 and 50 µg/ml concentration. Acetone extract exhibited highest scavenging activity of 70.73% at 140 µg/ml, followed by 66.94, 61.08, 54.89, 45.78, 37.21, 28.08, 18.34, 7.46 and 1.06% at 130-50 µg/ml concentration (Figure 6). The IC_{50} concentrations for BHA, methanol extract and acetone extract were found to be 4.47, 77.54 and 105.63 µg/ml which demonstrated the greater antioxidant efficacy of BHA, followed by methanol extract and acetone extract of *P. granatum* peel (Table 2).

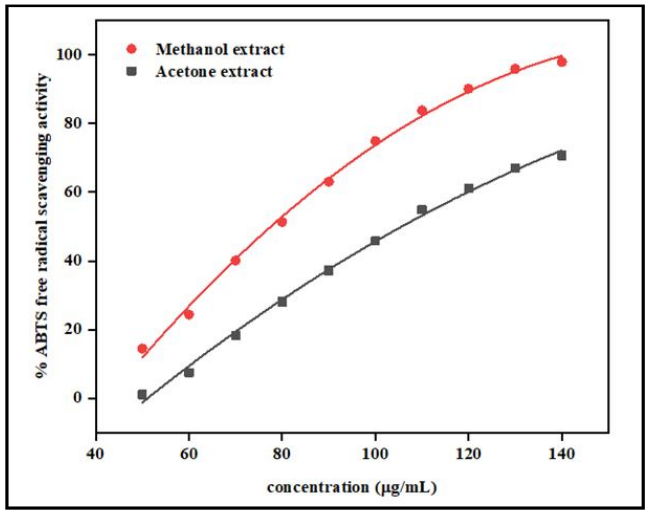

Figure 6: ABTS assay of methanol and acetone extracts of *P. granatum* peel.

4. Discussion

The quantitative phytochemical analysis of *P. granatum* peel is a vital initial step for evaluation of its biological, nutritional and technical characteristics. Table 1 displays the phytochemical screening of methanol and acetone extract of *P. granatum* peel. Both extracts were examined for important groups of phytochemicals, such as total phenolics, total flavonoid, total sugar, reducing sugar and for antioxidant activity *via* DPPH and ABTS assay.

Results of the current investigation concurred with Wang *et al.* (2011), who estimated total phenolics in *P. granatum via* different solvents and reported higher total phenolics in methanol extract than acetone extract. Fawole *et al.* (2012) also estimated total phenolics in ganesh variety of *P. granatum* using methanol extract and reported 295.5 mg GAE/ g DM total phenolics content.

Wang *et al.* (2011) estimated total flavonoids in different solvents and reported 2% in methanol extract and 1% in acetone extract of *P. granatum*. Fawole *et al.* (2012) also reported 121.1 mg CAE/g DM of total flavonoids in methanol extract of *P. granatum* (Ganesh variety). Total flavonoid results of current study were found in consistent with above findings.

Both total sugar and reducing sugar were found maximum in methanolic extract than acetone extract. Total sugar evaluation is centred on the production of hydroxymethyl furfural, which was produced when D-glucose is dehydrated in an acidic media, mixed

with phenol to produce a yellowish-brown solution with strongest absorption at 490 nm. For the assessment of reducing sugar content, alkaline copper tartrate is heated with reducing sugars, which transformed cupric ions to cuprous ions and produced cuprous oxide. Cuprous oxide reduced arsenomolybdic acid to molybdenum blue which has maximum absorbance at 520 nm (Aggarwal *et al*., 2022; Devi *et al*., 2023).

DPPH and ABTS free radical scavenging potential of *P. granatum* peel is due the higher amount of phytochemicals (Moond *et al*., 2023). Lower IC_{50} value of DPPH and ABTS assays revealed the higher total phenolics and total flavonoid in methanol extract as compared to acetone extract. Antioxidant activities results are in consistent with the results of total phenolics and total flavonoid and were found to be concentration dependent. The results indicate that methanol and acetone extract of *P. granatum* peel acts as electron donor or hydrogen donor in reducing DPPH and ABTS radicals. Many health benefits, such as anti-inflammatory, antioxidant, anticancer, and cardiovascular protective properties, have been associated with the diverse range of bioactive compounds found in *P. granatum* peel, such as phenolics and flavonoids. *P. granatum* peel bioactive compound extraction has garnered a lot of interest because of its potential use in the production of nutraceutical products.

5. Conclusion

In the current research, it is observed that *P. granatum* peel entail phytochemicals that are possibly crucial for scavenging radicals that induce oxidative stress. To figure out the medicinal applications of *P. granatum* peel, quantitative phytochemical analysis and antioxidant efficacy would be effective.The current study revealed that *P. granatum* peel methanol extract had higher total phenolics (244.11 mg GAE/g) and flavonoid content (106.50 mg CE/g) and superior antioxidant efficacy for both DPPH (IC_{50} 75.63 µg/ml) and ABTS (IC_{50} 77.54 µg/ml) than acetone extract. Pharmaceutical and nutritional supplement industries will get significant benefit from current research. However, *P. granatum* peel is a natural source of nutraceuticals, more research is needed to completely comprehend their modes of action, bioavailability, and possible drawbacks in various populations.

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Conflict of Interest

The authors declare no conflicts of interest relevant to this article.

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