



• Antioxidant activities of pomegranate peel methanolic and water extracts by *in vitro* methods

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NATURAL SCIENCE AND DISCOVERY



RESEARCH ARTICLE

Antioxidant activities of pomegranate peel methanolic and water extracts by *in vitro* methods

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ABSTRACT

In this study, the antioxidant activities of pomegranate (*Punica granatum*) peel extracts were investigated using different antioxidant activity methods. The antioxidant activities of extracts prepared at different concentrations (100-1000 μ g/ml) were compared with α -tocopherol, ascorbic acid, BHT, and BHA standards. The methanol extract of peels had the highest yield (45.5%). Chelating activities (Fe²+) of methanol extract at 100-1000 μ g/ml concentrations ranged between 16.63 and 46.45%. However, the chelating activities of all test extracts were lower than the standards. The reducing power of all extracts increased concentration-dependent. DPPH" radical scavenging activity of methanol extract varied in the range of 18.53 to 88.75%. The highest percentage inhibition of linoleic acid oxidation was found in 250 μ g/ml concentrations. Trolox equivalent (TEs) antioxidant capacity values of methanol extract ranged from 3.09 to 15.27 μ M TEs 10 g¹. It was determined that methanol extract showed a dose-dependent inhibition of the antioxidant activities.

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1. Introduction

Pomegranate (*Punica granatum* L. Family *Punicaceae*) includes several anatomical elements together with seed, juice, peel, leaf, flower, bark, and roots may be a natural product. Food products of pomegranate such as peel, seed, and juice contain various phenolics that have been associated with antioxidant activity (*Derakhshan et al., 2018*). Pomegranate gives a blend of different bioactive compounds and is utilized as a social drug for quite a long time. The pomegranate peel contrasts with the seed and is known as a cancer prevention agent between folk (*Ravikumar et al., 2019*).

Pomegranate peels mainly contain tannins, flavonoids, polyphenols, anthocyanins as delphinidins, cyanidins, etc. It has revealed that pomegranate peel extracts include antimicrobial, antioxidant, antifungal, anti-diarrheal, anti-diabetic, anti-inflammatory, anti-infective, apoptotic, and anti-genotoxic substances. Pomegranate pe-

* Corresponding author: E-mail address: filizucan@kilis.edu.tr (F. Ucan Turkmen) ISSN: 2149-6307 doi: http://dx.doi.org/10.20863/nsd.v4i1.59 © 2021 NSD. Published by Lycia Press London. All rights reserved. els have also produced enthusiasm for analysts, given their potential use as a nutraceutical and regular nourishment fixing (Kaur and Kausal, 2018).

The consumable parts of the natural fruit product (50%) comprise 40% arils and 10% seeds. Pomegranate peel includes about 50% of the absolute organic product weight of its. It is a crucial supply of minerals, especially potassium, calcium, phosphorus, magnesium, and sodium. They also contain phenolics, flavonoids, mixtures of proanthocyanidins and ellagitannin (ETs, e.g., punicalagin and its isomers), and complex polysaccharides punicalin alone, galactic abrasive, ellagic abrasive, and ellagic abrasive glycosides, and various bioactive mixtures. (Jalal et al., 2018).

It is trusted that the pomegranate tree started in Iran and North West of India centuries prior and then spread from the Mediterranean Sea bowl nations (Mohamed et al., 2018). Pomegranate is one of the most seasoned nourishments local to the Middle East, and its utilization has become widespread in Western nations because of its potential restorative properties, including the anticipation of malignancy, treatment of cardiovascular illnesses and dental

conditions, and security from ultraviolet radiation (Hanani et al., 2019).

The essential goal of this study is to research the antioxidant activities of methanol and distilled water extracts of pomegranate peels and reveal the relationship between the various antioxidant methods.

2. Materials and methods

2.1 Preparation of pomegranate peel extracts

Pomegranates (*P. granatum* L., *Punicaceae*) were obtained from the Cavak village of Mersin, in the East Mediterranean Region of Turkey. They were dried in shadow at room temperature. After that, they were preserved in brown glass jars for further analysis. Dried pomegranate peels were powdered by using a grinder (Arçelik K 3104). Powdered peels were extracted with methanol and distilled water solvents [1:10 (w/v)] for 72 h under shaking conditions at +25 °C. After that, extracts were filtered from the filter paper (Whatman filter paper No.1). The solvents were evaporated according to the boiling temperature of each solvent. Then, samples were suspended in methanol at the 100 mg/ml final concentration and stored at +4 °C. For antioxidant activity analysis, 100-1000 µg/ml concentrations of extracts were prepared.

2.2. In vitro antioxidant activity analysis

2.2.1. DPPH' radical scavenging activity

The Blois method (Blois, 1958) was used to measure the free radical scavenging activity of the extract with 1,1-diphenyl-2-picrylhydrazine (DPPH'). The test is based on electron transfer; in the presence of antioxidants, the free red color will be reduced. Therefore the purple solution is colorless. Prepare a methanol solution of 0.1 mM DPPH' and add 1 ml of this solution at various concentrations (100-1000 $\mu g/ml$) to the 1 ml extract. The absorption reached 100 °C after 30 minutes. Measure 517 nm, use 1 ml methanol instead of the sample as control and calculate the inhibition percentage according to the following equation (1).

Inhibition (%) =
$$[(A_{control} - A_{sample})/A_{control}] \times 100$$
 (1)

2.2.2. Metal chelating activity

The chelating interest of extracts becomes searched via way of means of the approach of Dinis et al. (1994). The approach is primarily based totally on the opposition of steel-binding compounds withinside the medium with a robust iron-chelating agent, ferrozine reagent, to bind Fe²+ ions (Isbilir, 2008). If chelating energy is high, the formation of crimson Fe²+ ferrozine complicated is prevented. 3.7 ml of deionized water and a hundred μl of 2 mM FeCl² answer have been delivered to at least 1 ml sample. Then incubated at room temperature for 30 min, 200 μl of 5 mM ferrozine answer become delivered and mixed. After 10 min, the absorbance of the aggregate becomes measured at 562 nm. Control becomes additionally done by the usage of 1 ml of deionized water in preference to the sample. As standard, EDTA answers at 50-250 $\mu g/ml$ concentrations have been used. According to the following equation, the steel chelating interest of extracts becomes calculated (2).

Metal chelating activity (%) =
$$[(A_{control} - A_{sample})/A_{control}] \times 100$$
 (2)

2.2.3. Reducing power assay

The regeneration ability was measured according to the method of Oyaizu (1986). The reducing agent in the medium reduces Fe $^{3+}$ ions to Fe $^{2+}$ ions, and the absorbance of the Prussian blue complex formed by adding FeCl $_3$ is measured. The high absorbance value is an indication of the high reduction capacity. Mix different concentrations (100-1000 $\mu g/ml$) and standard solutions (20-400 $\mu g/ml$) (1 ml) of pomegranate peel extract with 2.5 ml 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml solution. 1% potassium ferricyanide, incubate the mixture at 50 °C for 20 minutes, add 2.5 ml of 10% trichloroacetic acid (w/v), and centrifuge the mixture at 2500 rpm for 10 minutes. The upper layer (5ml) is mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride, and the absorbance is measured at 700nm. The test is performed in triplicate. The results are expressed as the average \pm standard value. The deviations of BHA, BHT, ascorbic acid, and α -tocopherol are used as standard.

2.2.4. 6-carotene/linoleic acid assay method

In this assay, 2 mg of β -carotene became dissolved in 10 ml chloroform. After 1 ml of this answer became introduced to 40 mg of linoleic acid and 400 mg of Tween-20 as an emulsifier. Chloroform became evaporated by the use of a vacuum evaporator. Then, 100 ml of oxygen-saturated distilled water was introduced. Aliquots (4.8 ml) of the organized emulsion have been transferred to a sequence of tubes containing 0.2 ml of samples. After setting the check tubes in a water bath at 50°C; the absorbance of every tube progressively became measured with the use of a spectrophotometer (Hitachi U-1900, Japan) at 470 nm with the aid of using beginning 0-time absorbance of the samples for zero and 100 and 20 min. BHT became used as a standard (Turan, 2016). The θ -carotene bleaching became calculated the use of the following equation (3):

Rate of
$$\theta$$
-carotene bleaching $R=\ln(A/B)/t$ (3)

A:Initatial absorbance B:absorbance at 120 min t:120 min

The following equation was also used to calculate the antioxidant activity (Zengin, 2010):

Inhibition value=
$$[(Rcontrol-Rsample)/Rcontrol] \times 100$$
 (4)

2.2.5. Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity assay of extracts was measured using the method of Apaydın (Apaydın, 2008). 7 mM ABTS solution containing 2.45 mM potassium persulfate was prepared. This solution was kept in an incubator set to 20 °C for 12 and 16 h to produce the ABTS radical. PBS (phosphate buffer; Phosphate Buffer Saline) solution, used for diluting the radical solution, samples, and Trolox standard, is prepared. 8.77 g of NaCl was added to 0.1 M phosphate buffer. It was adjusted to a pH of 7.4. Before starting the analysis, 1 ml of ABTS radical solution was taken and diluted with approximately 90-100 ml of PBS to an absorbance of 0.700 \pm 0.02 at 734 nm and equilibrated at 30 °C. 1 ml of diluted ABTS radical solution was placed to the microplate reader spectrophotometer (Multiskan GO UV/Vis Spectrophotometer, Thermo Scientific, Finland). And the initiation absorbance value was measured. As soon as 5 µl of the sample was added to the radical solution in the cuvette, the stopwatch is run. The absorbance was measured per minute for 6 min. The results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity) value.

2.2.6. Statistical analysis

The significant differences (p<0.05) between extracts were determined by using variance analysis (ANOVA) and Tukey multiple comparison tests.

3. Results and discussion

3.1. Total phenolics, total flavonoids, ascorbic acid, and DPPH' radical scavenging activity

The phenolic content can be an essential indicator of the antioxidant capacity of any product when intended for use as a natural source of antioxidants in functional foods (Jalal et al., 2018). Total phenolics of pomegranate peels in methanolic extracts ranged from 0.09 to 0.21 mg GAE/g (Table 1). The highest total phenolics detected in 1000

 μ g/ml concentration of methanolic and distilled water extracts (0.21 mg GAE/g). In distilled water extracts, total phenolics ranged from 0.08-0.21 mg GAE/g. Total phenolic values increased based on the concentration of extracts in both distilled water and methanol extracts. These increases statistically were significant (p<0.05).

As seen in Table 1, while total flavonoids ranged from 0.055 to 0.067 mg RE/g in methanolic extracts, total flavonoids ranged from 0.050 to 0.056 mg RE/g in aqueous extracts (p<0.05).

It was an insignificant difference between ascorbic acid contents of extracts in both distilled water and methanol extracts (p>0.05). The ascorbic acid contents ranged from 56.73 to 59.43 mg/l. The highest ascorbic acid content was determined as 59.43 mg/l in distilled water extracts (Table 1).

Table 1. Total Phenolics, total flavonoids, ascorbic acid, and DPPH radical scavenging activity values of pomegranate peel extract 1

Total phenolics (mg GAE/g)					
Extracts	100 μg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1000 μg/ml
Methanol	0.09±0.00 ^d	0.11±0.01 ^c	0.18±0.01 ^b	0.19±0.01ab	0.21±0.00°
Distilled water	0.08±0.00 ^d	0.11±0.00°	0.13±0.01°	0.17±0.01 ^b	0.21±0.00 ^a
Total flavonoids (mg RE/g)					
Extracts	100 ml/ml	250 ml/ml	500 ml/ml	750 ml/ml	1000 ml/ml
Methanol	0.061±0.00b	0.060±0.00b	0.055±0.00°	0.058±0.00bc	0.067±0.00a
Distilled water	0.053±0.00ab	0.050±0.00b	0.052±0.00ab	0.056±0.00°	0.053±0.00ab
Ascorbic acid (mg/l)					
Extracts	100 ml/ml	250 ml/ml	500 ml/ml	750 ml/ml	1000 ml/ml
Methanol	56.73±0.00°	58.56±1.44a	57.79±1.06 ^a	57.88±0.77 ^a	57.60±0.29 ^a
Distilled water	58.65±0.38ª	57.98±0.29a	58.75±0.10 ^a	59.42±1.15°	59.43±0.58 ^a
DPPH radical scavenging acti	ivity (%)				
Extracts	100 ml/ml	250 ml/ml	500 ml/ml	750 ml/ml	1000 ml/ml
Methanol	18.53±1.47°	43.97±3.72b	86.09±1.09 ^a	87.73±0.11ª	88.75±0.05a
Distilled water	12.11±1.60e	29.68±1.73d	46.54±2.56°	74.56±8.53b	87.35±0.27a

The data shown are mean values of n = 3. The difference between the different symbols (a-d, a-b, a-c, and a-e) in the same lines in the graph is significant (p<0.05).

The free radical scavenging effects of the extracts were determined as % over the DPPH' radical (Table 1). Methanol and distilled water extracts of pomegranate peels showed intense activity in the highest concentrations (see Table 1). The % inhibition rate increased based on the concentration of extracts (p<0.05). DPPH' radical scavenging activity of methanol extracts ranged from 18.53 to 88.75%. % Inhibition values ranged from 12.11 to 87.35% in aqueous extracts. As seen in Table 1, the distilled water extract exhibited low DPPH' scavenging activity while the methanolic extract showed high activities. Values obtained from low concentrations of methanol and distilled water extracts were relatively low than high concentrations, and therefore the antiradical activity of the extracts was weak.

Hayouni et al. (2007) and Ozcan et al. (2007) reported that polar solvents are more effective than apolar solvents on % yield and antioxidant activity. Furthermore, according to the polarity of the solvents, the extraction yields vary. The most effective solvent in the present study was methanol (45.5%) according to the extraction efficiency.

Al-Zoreky (2009) reported that the activity of methanolic extracts from pomegranate peels was related to the higher content (262.5 mg/g) of total phenolics.

Pomegranate has been engendered as polyphenol-rich sustenance with well-being beneficial impacts because of its high anti-oxidative limit. Accordingly, it is usually alluded to as "superfruit". Attributable to elevated amounts of phenolic acids, flavonoids, and other polyphenolic compounds, pomegranate could be utilized as a compelling forager of a few receptive reactive oxygen species (Derakhshan et al., 2018).

Derakhshan et al. (2018) determined that the total flavonoids, the total phenolics, and the antioxidant activity of pomegranate peel ethanolic extracts ranged from 36-54 mg rutin/g, 276-413 mg GAE/g and 45-58%, respectively. They said that our results showed a positive correlation between phenolic contents and antioxidant activity.

The high antioxidant capacity of pomegranate especially peel, had shed light to use them not also as natural food preservatives but as health supplements rich in natural antioxidants (Derakhshan et al., 2018).

Mohamed et al. (2018) reported that our results showed that the ethanolic peel extract of *P. granatum* contains important amounts of polyphenols, including flavonoids, and can be considered good sources of these compounds for medicinal and food applications.

3.2. Metal chelating activity

Metal chelating activity was evaluated according to the competition Fe^{2+} ions binding of ferrozine and sour cherry stalk extracts. The decreasing absorbance in the metal chelating activity indicates that the metal ions are chelated before the ferrozine is bound. EDTA, as a suitable metal chelator, was selected as standard. A decreasing level of absorbance in the reaction mixture represents a higher metal chelating capability.

As seen in Table 2, the chelating activity of extracts increased as the concentration increased. Chelating activities of Fe^{2+} ions of all extracts were seen to be lower than standard. Chelating activities of Fe^{2+} ions of methanol extracts ranged from 16.63% to 46.45% at 100-

Table 2. Metal chelating activity of pomegranate peel extracts (%) 1

Extracts	100 μg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1000 μg/ml	
Methanol	16.63±2.77°	22.89±2.05°	35.90±1.20b	40.66±5.00ab	46.45±1.02a	
Distilled water	12.11±3.31 ^d	19.88±1.81 ^{cd}	21.93±5.90bc	29.22±0.90b	34.52±1.39a	
Standard	50 ml/ml	100 ml/ml	150 ml/ml	200 ml/ml	250 ml/ml	
FDTA	80.16+1.34 ^b	93.59+0.83	94.42+0.00a	94.83+0.00°	94.73+0.10 ^a	

¹ The data shown are mean values of n = 3. The difference between the different symbols (a-e, a-d, and a-b) in the same lines in the graph is significant (p < 0.05).

1000 μg/ml concentrations. The highest chelating activity was recorded for methanolic extracts by comparison with other extracts.

Metal ions can cause lipid peroxidation, which can induce the production of free radicals and lipid peroxides. Therefore, metal chelating activity indicates antioxidant and antiradical properties (Bursal et al., 2013).

Kim and Ishii (2006) reported that the purified glucosinolates exhibited moderate antioxidant activity. Heimler et al. (2007) suggested that the high chelating capacity of arugula in ethanol extract can be derived from glucosinolates.

The metal chelating activity of herbal extracts depends on the functional groups in the structure of the phenolic compounds and the functional groups' position and availability. It has been found at least two of the functional groups -OH, -SH, -COOH, -PO₃H₂, C=O, -NR₂, -S, and -O- in its structure. The structure and functional configuration of the phenolic compounds affect the chelating properties of

substances (Gülen 2013). Therefore, the diversity in the chelating activity of the pomegranate peel extracts can be explained by having different phenolic content and phenolic groups in different structures and positions of samples.

3.3. Reducing power

The reduction of Fe⁺³ ions is an indicator of the ability of a compound to provide electrons, which is an essential mechanism for antioxidant activity and is closely related to other antioxidant properties. It is accepted that the high absorbance value represents the high reducing capacity. To determine the Fe⁺³ reduction ability of the pomegranate peel extracts was studied at varying extract and standard concentrations. It is compared with BHA, BHT, α -tocopherol and ascorbic acid standards (p<0.05). All extracts and all standards observed a concentration-dependent activity (Table 3). Reducing power values ranged from 0.060 to 0.127 (abs.) in methanolic extracts. The highest values were obtained from methanolic extracts.

Table 3. Reducing power of pomegranate peel extracts (abs.) ¹

Extracts	100 µg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1000 μg/ml
Methanol	0.060±0.00 ^d	0.077±0.01°	0.099±0.00 ^b	0.120±0.00 ^a	0.127±0.01 ^a
Distilled water	0.063±0.00 ^d	0.074±0.00°	0.094±0.00 ^b	0.109±0.01 ^a	0.112±0.00 ^a
Standards	20 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml	400 μg/ml
BHT	0.07±0.00 ^d	0.09±0.00 ^d	0.14±0.00°	0.33±0.02 ^b	0.45±0.01ª
ВНА	0.22±0.01°	0.24±0.01 ^c	0.25±0.02°	0.32±0.03 ^b	0.51±0.01 ^a
lpha-tokopherol	0.13±0.00 ^d	0.15±0.00 ^d	0.16±0.01°	0.20±0.01 ^b	0.29±0.01 ^a
Ascorbic acid	0.12±0.00e	0.13±0.00 ^d	0.17±0.00°	0.25±0.00b	0.37±0.00a

¹The data shown are mean values of n=3. The difference between the different symbols (a-e, a-d, and a-c) in the same lines in the graph is significant (p<0.05).

In the determination of reduction capacity, the capability of plant extracts to convert Fe⁺³ into Fe⁺² was investigated. The Fe⁺³ reducing capacity of a compound is associated with its power of electron

transformation, giving electrons. This is an important indicator of potential antioxidant activity (Isbilir, 2008).

Table 4. Antioxidant activities of pomegranate peel extracts with β -carotene/linoleic acid assay method (%) ¹

Extracts	100 μg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1000 μg/ml
Methanol	75.49±5.11°	78.63±4.29 ^a	78.20±0.91°	71.47±2.12 ^a	72.03±1.41 ^a
Distilled water	79.41±3.61a	79.97±2.01°	76.44±4.98°	76.32±0.34a	72.21±4.77°
Standard	200 μg/ml	400 μg/ml	600 μg/ml	800 μg/ml	1000 µg/ml
BHT	51.36±0.00°	53.74±0.00°	62.14±0.00 ^b	61.03±0.00b	67.25±0.02°

 $[\]overline{1}$ The data shown are mean values of n=3. The difference between the different symbols (a-e, a-b, and a-c) in the same lines in the graph is significant (p<0.05).

Because of these properties, it can be said that the extracts may play a role in the terminating of free radical chains by converting the reactive free radical species into more stable non-radical species. In some studies, it was reported that there is a strong relationship between Fe⁺³ reducing capacity and inhibition of lipid peroxidation (Juntachote and Berghofer, 2005; Hinneburg et al., 2006).

3.4. 6-carotene/linoleic acid assay method

The θ -carotene/linoleic acid method is based on the fact that peroxide radicals have occurred during linoleic acid oxidation at high temperature caused color expression in the θ -carotene molecule and measured spectrophotometrically. The linoleic acid oxidation's high inhibition rate indicates that this sample has a strong antioxidant

capacity. According to this, methanolic and distilled water extracts had the highest inhibition percentage with 78.63 and 79.97% at 250 $\mu g/mL$ concentrations, respectively (see Table 4). For all extracts, the inhibition rate of linoleic acid oxidation was found to increases and decreased based on the increase of concentration. However, the percentage inhibition of BHT, synthetic antioxidant, was determined as 51.36-67.25%, and it is clearly stated that the synthetic antioxidant had lower antioxidant capacity than the tested extracts.

Isbilir (2008) reported that the aqueous extracts of the plant samples showed lower activities than the ethanol and acetone extracts to prevent linoleic acid peroxidation. This can be expressed that the polar antioxidant compounds in the water extract can not be

sufficiently solved by the reactions that take place in the apolar phase because of the apolar linoleic acid test emulsion system.

Furthermore, in another study, the high linoleic acid and α -linolenic acid contents of the plant species were indicated to be a source of essential fatty acids (Zengin, 2010).

Bastos et al. (2015) expressed the antioxidant activity as θ -carotene bleaching inhibition at the hydromethanolic extracts of sweet cherry stems, and this value was detected as 0.30 in stems.

Table 5. Antioxidant activities of pomegranate peel extract with Trolox equivalent antioxidant capacity assay (µm TEs 10 g⁻¹) 1

Extracts	100 μg/ml	250 μg/ml	500 μg/ml	750 μg/ml	1000 μg/ml
Methanol	3.09±1.40°	6.06±1.06bc	7.97±1.12 ^b	9.29±0.53b	15.27±2.18a
Distilled water	3.68±0.20a	4.15±1.81a	5.12±1.54 ^a	8.95±4.73°	10.10±2.83a

¹ The data shown are mean values of n=3. The difference between the different symbols (a-c) in the same lines in the graph is significant (p<0.05).

3.5. Trolox equivalent antioxidant capacity assay

Trolox equivalent antioxidant capacities of extracts generally increased with the increase of concentration (Table 5). The highest antioxidant values were obtained from methanol and distilled water extracts. The antioxidant capacities of methanolic extract ranged from 3.09 to 15.27 μ M TEs 10 g-1. Values of distilled water extract ranged between 3.68 and 10.10 μ M TEs 10 g-1 (p<0.05).

In addition, the antioxidant activity observed in extracts may have resulted from the synergistic interaction of two or more compounds present in the plants. Many natural anti-oxidative compounds have been reported to generally act synergistically, thus providing an effective defense against free radicals (Zin et al., 2002).

It is well-known that each antioxidant substance in the extract may have a different reaction mechanism to free radicals. So, there may be differences in the antioxidant capacities of the extracts according to the methods performed. In brief, it is not the right approach that the antioxidant activity of extracts is determined by a single method. In addition, the determination of antioxidant activity by using different methods simulated the biochemical reactions in living systems can be a more objective approach. Supporting our results by *in vivo* and clinical studies is also essential to determine the bioavailability of plant extracts in food.

4. Conclusions

In our study, antioxidant activities of pomegranate peel extracts were investigated by using different methods. According to our results, the highest antioxidant activity was recorded for methanolic extract, a polar solvent. This high activity is explained by solving phenolic and polyphenolic compounds having antioxidant activity in pomegranate peels. More specific studies are needed to determine the isolation and characterization of bioactive constituents in pomegranate peels for use in the food system. Especially when considered the toxic effects of synthetic antioxidants, the extracts of pomegranate peels can be used effectively as a natural antioxidant source in pharmacology and the food industry.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest.

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Supplementary file

None.

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