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Comparison of five solvents in the extraction of phenolic antioxidants from pomegranate (*Punica granatum* L.) peel

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ABSTRACT

Background: Pomegranate peels are rich in bioactive compounds and could be an alternative natural source such as antioxidants. **Aims:** The aim of the present study was to assess the abilities of five solvents to extract phenolic antioxidants from pomegranate peels. **Methods and Material:** Pomegranate peels powder was subjected to extraction and the extraction yield was compared. The total phenolic, flavonoid, condensed and hydrolysable tannins contents were analyzed. The antioxidant activity of the extracts was evaluated by two methods (DPPH* and ABTS*+ scavenging capacities) and results were then compared. **Results:** Results showed that the mixture methanol/water (50:50) allowed higher extraction yield ($37.33 \pm 5.3\%$) than the others solvents ($P < 0.05$). Further, the total phenolic, flavonoid and condensed tannins contents were the highest in mixture water/methanol (50:50) extract. Phenolic antioxidants showed a distinct reducing capacity and a high DPPH* inhibition values were recorded for all extracts with no significant differences ($P > 0.05$) between ethanol and mixture water/methanol (50:50) extracts. All extracts exhibited high inhibition against ABTS*+ but with a considerable variation. Phenolic content and antioxidant activities were well positively correlated with each other. **Conclusions:** Our findings revealed that the choice of the extracting solvent affects considerably the extraction of phenolic antioxidants from pomegranate peels.

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1. INTRODUCTION

Pomegranate (*Punica granatum*) is considered by several religions and brotherhoods as a sacred fruit. In the *Holy Quran* (*Surat Ar-Rahmân, Chapter 55, Verse 68*), pomegranate fruit is promised like one of Eden fruits. The use of pomegranate fruit for folk medicine dates from ancient times and reports of its therapeutic qualities have echoed by several scientists throughout the ages [1]. Pomegranate has shown an explosion of interest during the last decade and has gained a tremendous popularity, because of its numerous health effects [2]. Pomegranate peel, seed and juice contain considerable amounts of phenolic compounds and have antioxidant activity [3].

Pomegranate peels are by-products of pomegranate juice processing, extensive research Al-Zoreky; El-said *et al.* [4-5] have revealed that added-value products could be made from those wastes. Nowadays, pomegranate peel and its extracts are tested in several products such as fish [4], bread [6], juice [7], yoghurt powder [8], etc. This increasing interest is due to the different properties of peel particularly the antioxidant ones. Indeed, actually it is well established that pomegranate peel extract has a strong antioxidant activity [5, 9-11]. In the work conducted by Li *et al.* [9] the total phenolics content of peel extract was nearly 10-fold as high as that of pulp extract. Iqbal *et*

al. [12] recommend pomegranate peel as a potent source of antioxidants for the stabilization of food systems. One of the main compounds responsible for most of the functional properties of numerous foods are phenolic compounds in any of their forms [13]. Several studies [14, 15] have revealed that antioxidant activities of pomegranate peel are associated with their phenolic contents. Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites and constitute an integral part of both human and animal diets [16]. Furthermore, they represent a diverse class of compounds, many of which occur naturally in a range of food plants [17]. The extraction of phenolics from source materials is the first step involved in their analysis. Therefore, there is a need for systematic investigation for sample preparation and for determination of food phenolics [18]. The extraction technology is a key element for sustainable development of agri-food industries [19]. Polyphenols are conventionally extracted from plant materials by organic solvents [20]. Nevertheless, the optimization of the extraction protocol before any qualitative and quantitative study will guarantee an accuracy of results.

Among all the investigated variables (pre-treatment of the sample, solvent/sample ration, type of solvent, time, and temperature of extraction), to ensure the efficiency of extraction, type of solvent has been the most studied factor [5, 21-23]. According to Malviya *et al.* [21] selection of solvent is an important step for obtaining extracts with acceptable yields and strong antioxidant activity. However, the discordance until now between authors deserves thorough studies.

The objective of the current study was the extraction of phenols from pomegranate peel (*Punica granatum* L.) with different solvents in order to find the most beneficial, in terms of extraction yield, phenolic, flavonoid, hydrolysable and condensed tannins contents, and antioxidant activities.

2. MATERIAL AND METHODS

2.1 Plant materials

Sweet pomegranate fruits (*Punica granatum* L.) were collected in Bouira (Algeria; 36°22'N3°54'E) between October and November. Fruits were washed and manually peeled. Peels were air-dried for about 02 weeks in a clean and dry place (temperature: 20-25°C; hygrometry: about 60%). Dry peels (moisture content: 8.64±2.94%) were ground and the powder was passed through a 500 µm sieve. The powder was conserved in closed glass bags and kept in the dark in cold conditions (4-6°C) until analyses.

2.2 Preparation of pomegranate peel extracts

The extract was prepared in five types of solvents i.e. 96% ethanol, methanol, acetone, water, and mixture water/methanol (50:50). Five (05) grams of peel were extracted into 100 mL of solvent. The mixtures were subjected to shaking at room temperature overnight at a speed of 500 rpm. The mixtures were filtered by Whatman No.1 paper. Then, the filtrate was subjected to rotary evaporator at 40°C under vacuum for the removal of solvent. The extracts were weighed to calculate the yield and were stored at -18°C prior to further analyses.

2.3 Determination of extraction yield

The percentage yield of extracts was expressed as the weight of extract relative to the weight of the starting plant material. The yield is expressed in percentage (%) and calculated as shown in formula (1):

$$Y = \frac{W_e}{W_p} \times 100 \dots\dots\dots (1)$$

Where "Y" is the extraction Yield (%), "W_e" is the weight of the extract (g), and "W_p" is the weight of peel (g).

2.4 Determination of total phenolic content

Total phenolic content was determined according to Singleton *et al.* [24]. Total phenolic content was expressed as gallic acid equivalents in mg per g of dry extract (GAE/g).

2.5 Determination of total flavonoid content

The total flavonoid content of the extracts was determined using the aluminium trichloride assay as described by Djeridane *et al.* [25]. The results were expressed as quercetin equivalents in µg per mg of dry extract (QE/mg).

2.6 Determination of hydrolysable tannins content

Hydrolysable tannins were determined by the method of Willis and Allen [26]. The results were expressed as tannic acid equivalents in mg per g of dry extract (TAE/g).

2.7 Determination of condensed tannins content

Condensed tannins content was measured as previously described by Broadhurst and Jones [27]. Results were expressed as mg catechin equivalents per g of dry extract (CE/g).

2.8 Antioxidant properties

DPPH radical scavenging activity

The scavenging activity on DPPH radical of different extracts was determined following the method reported by Brand-Williams *et al.* [28].

The radical scavenging activity (RSA %) was calculated as reported in formula (2):

$$\text{DPPH RSA (\%)} = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100 \dots\dots\dots (2)$$

Where A_{blank} represents the absorption of the DPPH* solution and A_{sample} represents the absorption of the DPPH* solution after the addition of the sample.

The concentration providing 50% of inhibition (IC_{50}) was investigated by plotting the RSA percentage against PPP extract concentration.

ABTS•+ scavenging ability assay

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical scavenging assay was measured according to the method of Re *et al.* [29]. The ABTS•+ radical scavenging ability of the sample was calculated by the following equation:

$$\text{ABTS RSA (\%)} = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100 \dots\dots\dots (3)$$

Where A_{blank} represents the absorption of the ABTS•+ solution and A_{sample} represents the absorption of the ABTS•+ solution after the addition of the sample.

2.9 Statistical analysis

All tests were run in triplicate. All data were expressed as mean $\pm$ standard error of mean. One-way analysis of variance (ANOVA) test for comparisons was performed to determine significant differences ($\alpha = 0.05$) between results using STATISTICA 7.1 (Statsoft Inc, France, 2005). Pearson correlation coefficients were used for the determination of the correlations among means with the same software.

3. RESULTS

3.1 Extraction yield

The extraction yield of every solvent is shown on Figure 1. The statistical analysis of results revealed that the selection of solvent influences significantly ($P < 0.05$) the extraction yield. Our results showed that the mixture water/methanol (50:50) allowed the highest extraction yield ($37.33 \pm 5.3\%$). Methanol and ethanol provided similar yield without significant differences, with 27.21 ± 0.47 and $26.20 \pm 0.45\%$, respectively. In the present study, it was observed that acetone displayed the lowest yield ($6.63 \pm 0.71\%$), which was approximately 6 times lower than the one recorded for the mixture water/methanol (50:50). This weak yield allowed us to predict that acetone is not recommended for the extraction of phenolic compounds from pomegranate peel. However, the nature of phenolic compounds contained in the extract and their antioxidant activity remain the determining elements for solvent choice.

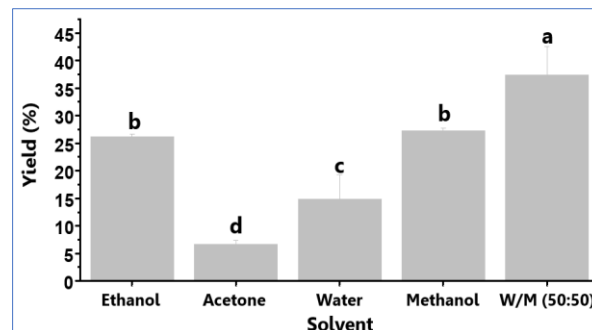


Figure 1: Extraction yield from pomegranate peel in different solvents. Different letters represent statistical different ($P < 0.05$) using the Duncan's multiple range test; W/M (50:50) - water/methanol (50:50)

Water furnished an extraction yield of $14.87 \pm 4.42\%$ which is weaker than the mixture water/methanol (50:50), methanol and ethanol solvents. Moreover, the evaporation of water took more time and required more consumption of energy, which reduces its use. For that reason, some authors advice against using water [15] for phenol extraction, despite its safety and low price.

Our results are in agreement with previous studies which consider that the choice of solvent constitutes a significant element during the extraction of phenolics. Indeed, several works have been documented, Iqbal *et al.* [12] recommend methanol for the extraction of phenols from pomegranate peel. In our situation, methanol allowed to obtain the second highest extraction yield ($27.21 \pm 0.47\%$). However, this solvent is expensive, not safe, and requires more precaution measures. Malviya *et al.* [21] obtained the highest yield with the mixture water/ethanol (50:50) and the lowest with water. More recently, Masci *et al.* [15] estimated that the mixture ethanol: acidified water with acetic acid increases the extraction yield. Whereas, Orak *et al.* [14] and Singh *et al.* [22] reported that water offers the highest yield compared to certain organic solvents (ethanol, acetone and methanol). While, Yasoubi *et al.* [23] reported that acetone provides an extraction yield higher than that obtained with methanol, ethanol or water. The inequality between extraction yields could be explained by the difference between the solubility of phenols among solvents [9]. The mixture usage of solvents, as reported in our experiment, will undoubtedly provide answers in this field and will maximize the extraction yield of phenols and antioxidant molecules.

3.2 Total phenolic content

As can be observed in Table 1, the total phenolic content varied from 242.05 ± 7.99 to 638.17 ± 10.59 mg GAE/g. The statistical analysis clearly revealed that the solvent influences the extraction of phenolics. The total phenolic content in ethanolic and mixture water/ethanol (50:50)

extracts were the highest with 638.17 ± 10.59 and 625.525 ± 6.83 mg GAE/g, respectively ($P > 0.05$) followed by acetone (580.43 ± 6.49 mg GAE/g), methanol (472.46 ± 2.39 mg GAE/g), and water (242.05 ± 7.99 mg GAE/g). To the best of our knowledge, this protocol is the first that investigates the mixture of methanol and water (50:50) in the extraction of phenolics from pomegranate peel. This mixture offered the best extraction yield and, the best phenolic content too, that could be explained by a synergetic effect of water and methanol. According to Li *et al.* [9], the mixture of different solvents (acetone, methanol, water and ethanol) is more powerful in recovering antioxidants from Chinese pomegranate than are individual solvents. Our results are in agreement with those of Singh *et al.* [22] who stated that ethanol gives highest phenolic content than water and acetone. In our study, ethanol offered the possibility to obtain a highly rich extract in phenols ($62.82 \pm 1.06\%$). Wang *et al.* [30] and Malviya *et al.* [21] suggested that water is not recommended for the extraction of polyphenols from pomegranate peel.

Table 1: Total phenolic, flavonoid, hydrolysable tannins and condensed tannins contents of pomegranate peel extracts

Extract	TPC (mg GAE/g)	TFC (μ g QE/mg)	HTC (TAE/g)	CTC (CE/g)
Ethanol	638.17 ± 10.59^a	53.85 ± 1.95^a	682.39 ± 5.8^b	5.65 ± 1.2^a
Acetone	580.43 ± 6.49^b	42.86 ± 4.1^b	796.44 ± 17.4^a	2.32 ± 0.37^c
Water	242.05 ± 7.99^d	11.5 ± 0.54^d	127.59 ± 7.73^d	2.22 ± 0.14^c
Methanol	472.46 ± 2.39^c	32.6 ± 1.46^c	545.14 ± 11.6^c	3.99 ± 0.32^b
Water/methanol (50:50)	625.525 ± 6.83^a	52.68 ± 1.97^a	690.12 ± 13.53^b	6.39 ± 0.28^a

Values are expressed as Mean $\pm$ SD of three measurements; values followed by different letters in the same column differ significantly ($P < 0.05$). TPC- Total Phenolic Content; TFC- Total Flavonoid Content; HTC- Hydrolysable Tannins Content; CTC- Condensed Tannins Content.

Our results show the high phenolic content of pomegranate peel, which could be an argument for recommendation of its valorization. Indeed, if we take, for example, the phenolic content in mixture water/methanol (50:50), we can deduce, approximately, the high amount of phenolic compounds in peel powder, which is 23.35 ± 3.61 g GAE/100 g of dry peel.

In spite of the phenolic content fluctuation between extracts, our results were higher than those obtained by other authors. Fawole *et al.* [11] revealed significant cultivar differences in the levels of phenolic compounds, and reported that the phenolic content of pomegranate fruit peel, ranged between 179.3 ± 4.6 and 295.5 ± 23.91 mg GAE/g. The variability in total phenolics among studies could be partially attributed to the differences in extraction

conditions (powder bulk, agitation speed, extraction time, solid/solvent ration, etc.).

3.3 Total flavonoid content

It can be seen from the data in Table 1 that the total flavonoid content of pomegranate peel extracts (μ g QE/mg of dry extract) varied from 11.5 ± 0.54 to 53.85 ± 1.95 . The statistical analysis revealed that the selection of solvent affects the extraction of flavonoids. No significant difference ($P > 0.05$) was recorded between ethanolic and mixture water/methanol (50:50) extracts which have the highest levels of flavonoids with 53.85 ± 1.95 , 52.68 ± 1.97 μ g QE/mg, respectively, followed by methanol (32.6 ± 1.46 μ g QE/mg), acetone (42.86 ± 4.1 μ g QE/mg) and water (11.5 ± 0.54 μ g QE/mg). Previous studies focused on the influence of the solvents on the total phenolic content of extract, but to the best of our knowledge, few works have focused on the influence of the solvents' selection on flavonoid extraction. Orak *et al.* [14] reported that methanol displays the highest total flavonoid content than water and ethanol.

3.4 Hydrolysable tannins content

Hydrolysable tannins contents of various extracts are summarized in Table 1. Levels were deduced from the calibration curve generated by tannic acid ($R^2 = 0.995$). Our results showed that pomegranate peels were rich in hydrolysable tannins and the extract content was significantly influenced by the solvent choice. Acetone allowed to obtain the highest value of hydrolysable tannins with (796.44 ± 17.4 mg TAE/g). The hydrolysable tannins content of ethanol and mixture methanol/water (50:50) extracts was not significantly different ($P > 0.05$) with 682.39 ± 5.8 and 690.12 ± 13.53 mg TAE/g, respectively. As this polyphenolic class is quantitatively dominant, aqueous extract that offered the lowest phenolic content, issued the lowest hydrolysable tannins content (127.59 ± 7.73 mg TAE/g).

3.5 Condensed tannins content

Condensed tannins levels are presented in Table 1. As for hydrolysable tannins content, our results showed that condensed tannins content was affected by the extraction solvent. Among the tested solvents, the combination of water and methanol (50:50) offered the highest amount of condensed tannins with 6.39 ± 0.28 mg CE/g, whereas the lowest was unregistered for aqueous extract (2.22 ± 0.14 mg CE/g). These results are similar to those reported by a Tunisian study [31] that reported amounts around 1.5 to 7.7 mg CE/g, and explained that extraction method could lead to those variations. Çam and Hişil [20] reported that the condensed tannins content of methanol extract was nearly

3-fold as high as that of aqueous extract of pomegranate peels powder. To the best of our knowledge, no studies on the effect of acetone, ethanol, and the mixture water/methanol (50:50), in the extraction of condensed tannins from pomegranate peels, have been conducted. Our study was the first contributing to that finding.

3.6 DPPH radical scavenging activity

The radical-scavenging activity on DPPH* was calculated as the concentration of the extract ($\mu\text{g/mL}$) required to inhibit 50% of the initial DPPH free radical (IC_{50}). In this test, when a solution of DPPH* is mixed with that of a substance that can release a hydrogen atom, then this leads to the reduced form with a loss of violet color to become a residual pale-yellow color. The IC_{50} of all peel extracts is shown on Figure 2. The pomegranate peel extracts showed strong antioxidant activity. The highest DPPH* scavenging activity was recorded for the ethanolic extract ($76.75 \pm 2.59 \mu\text{g/mL}$) followed by mixture 50% water: 50% methanol ($85.37 \pm 7.05 \mu\text{g/mL}$), methanol ($147.83 \pm 5.03 \mu\text{g/mL}$), acetone ($160.01 \pm 7.28 \mu\text{g/mL}$), and water ($183.71 \pm 8.71 \mu\text{g/mL}$) extracts while, the DPPH* scavenging activity of ascorbic acid, used in this study as a positive control, was lower than peel extracts ($394.87 \pm 17.35 \mu\text{g/mL}$).

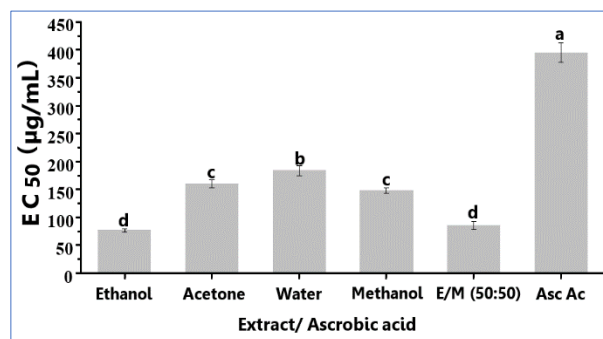


Figure 2: DPPH Inhibition activity of pomegranate peels extracts and ascorbic acid.

Different letters represent statistical different ($P < 0.05$) using the Duncan's multiple range test; E/M (50:50)- water/methanol (50:50); Asc A- Ascorbic Acid.

The statistical analysis revealed significant differences ($P < 0.05$) among some solvents. Ethanol and mixture water/methanol (50:50) solvents provided extracts with high inhibitory activity against DPPH radical than methanol, water and acetone extracts. Singh *et al.* [22] suggested that the selection of solvents affect the antioxidant activity of extract.

3.7 ABTS•+ scavenging ability

The ABTS•+ radical cation decolorization test constitutes another technique usually used to investigate antioxidant activity [22]. It can be applicable to both lipophilic and

hydrophilic antioxidants [29]. The decrease in color at 734nm was observed for all extracts, which indicates that all extracts exhibited ABTS•+ radical scavenging activity but in different degrees (Figure 3). These differences are certainly due to the content and quality of the phenols in the different extracts [32]. The ethanol extract possessed the highest ABTS•+ scavenging activity ($78.92 \pm 1.13\%$) than others extracts ($P < 0.05$), whereas the lowest was observed in the aqueous one.

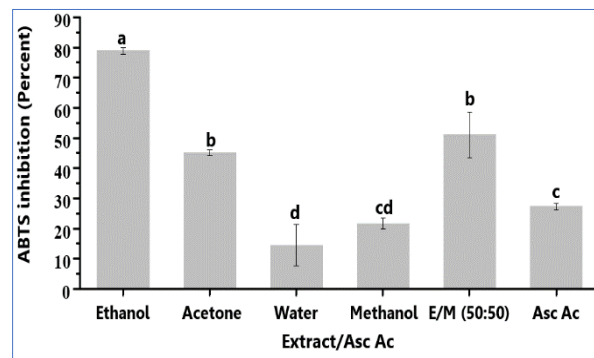


Figure 3: ABTS•+ Inhibition activity of pomegranate peels extracts and ascorbic acid.

Different letters represent statistical different ($p < 0.05$) using the Duncan's multiple range test; W/M (50:50)- water/methanol (50:50); Asc A- Ascorbic Acid.

The ABTS•+ scavenging activities of extracts are in the order: ethanol > mixture water/methanol (50:50) > acetone > methanol > water. As for DPPH assay, pomegranate peel extracts showed higher ABTS•+ scavenging activity than ascorbic acid. These results were consistent with others previously published studies. Malviya *et al.* [21] reported that ethanol extract showed higher ABTS•+ scavenging capacity than methanol and water extracts. Elfalleh *et al.* [32] observed that methanol extracts showed stronger antioxidant activities than the water ones.

3.8 Correlation study

The results of the correlational analysis are summarized in Table 2. The results show highly significant ($r = 0.99$, $P < 0.05$) correlation between phenolic and flavonoid contents that agree with those obtained by Masci *et al.* [15] who reported a high positive correlation ($R = 0.89$) between total phenolic and total flavonoid contents in pomegranate peels. Moreover, there was a high positive relationship ($R = 0.95$, $P < 0.05$) between total phenolic and hydrolysable tannins contents. This is comparable to several studies [20, 31, 32] that reported proportion of hydrolysable tannins in the pomegranate peels extracts that constituted a high amount of the total phenolics.

Table 2: Correlation matrix between polyphenol compositions and antioxidant activity

	TPC	TFC	HTC	CTC	DPPH RSA	TPC inhibition
TPC	1.00	0.99 **	0.95 **	0.68	-0.80	0.84
TFC	-	1.00	0.90 **	0.75	-0.87	0.88**
HTC	-	-	1.00	0.44	-0.58	0.69
CTC	-	-	-	1.00	-0.95**	0.65
DPPH RSA	-	-	-	-	1.00	-0.85
ABTS•+ inhibition	-	-	-	-	-	1.00

Results are expressed as correlation coefficients (r); ** Significant at $P < 0.05$. TPC- Total Phenolic Content; TFC- Total Flavonoid Content; HTC- Hydrolysable Tannins Content; CTC- Condensed Tannins Content.

In pomegranate peels powder extract, high linear correlations were observed between the DPPH RSA and phenolic content ($R = -0.80$, $P > 0.05$), and total flavonoid content ($R = -0.87$, $P > 0.05$). On the other hand, no significant correlations were observed between the DPPH inhibition and hydrolysable tannins content ($R = -0.58$). Unexpectedly, strong positive correlation ($R = -0.94$, $P < 0.05$) was observed between DPPH RSA and condensed tannins contents. The tendency of either hydrolysable or condensed tannins or of flavonoids to act as antioxidants, or to exhibit antimicrobial features, are governed by their chemical structures [33]. It should be noticed that most antioxidant compounds display distinct reducing capacity [33]. In our situation, this result could be explained by the fact that the condensed tannins compounds contained in pomegranate peel extracts display high antioxidant capacity.

A highly positive correlation ($R = 0.84$, $P > 0.05$) was found between ABTS•+ scavenging capacity and total phenolic content. Another significant correlation ($R = 0.88$, $P < 0.05$) are found between total flavonoid content and ABTS•+ scavenging capacity, which indicates that flavonoid, are antioxidant compounds that respond positively to ABTS•+ assay. Moreover, DPPH RSA was highly correlated with ABTS•+ RSA ($R = -0.85$, $P > 0.05$). This could confirm that the compounds which scavenge DPPH radical in the pomegranate peel extracts were also able to scavenge ABTS radical.

4 CONCLUSIONS

In the current study, the effectiveness of five solvents, to extract phenolic compounds from pomegranate peels powder, was assessed. The mixture water/methanol (50:50)

allowed obtaining the highest extraction yield. A higher phenolic, flavonoid, condensed tannins contents in extract, were obtained with the mixture water/methanol (50:50), and ethanol than other solvent. This research supports that pomegranate peels extracts have a considerable antioxidant capacity (DPPH and ABTS RSA). In addition, the antioxidant activities showed a marked correlation with the total phenolic, flavonoid, and tannins contents. Our findings may be used as starting point for improving protocols when utilizing solvent extraction of phenolics. However, further studies should emphasize on the effect of the mixture of solvents such as ethanol/water (50:50) and acetone/water (50:50) on the extraction of phenolic antioxidants from pomegranate peel. As well as, studies on the phenolic compounds contained in each extract are recommended.

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