Advancement in Medicinal Plant Research
Vol. 8(1), pp. 1-13, February 2020
ISSN: 2354-2152
Full Length Research Paper

Phytochemical investigation of *Punica granatum* pomace: As source of bioactive and medicinal natural products

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Accepted 14 January, 2020

ABSTRACT

Punica granatum L. is a native plant that is greatly dispensed in south of Iran. The massive pomace of *Punica* exhibits a challenging losses exposure difficulty for the processing industries. The resent study was aimed to investigate the composition and quality of *P. granatum* pomace and understand its bioactive compounds to introduce it to different industries such as pharmaceutical, food, medicinal, agricultural etcetera for optimum use. The SHS (static headspace) GC-MS on the Combi PAL system method was used to identify and analyze the volatile components (VCs). Four different extracts (60°C water, distilled water, methanol and methanol 80%) were analyzed for polyphenol compounds identified by HPLC-DAD, total flavonoid content (TFC), total phenolic content (TPC) and antioxidant capacity (DPPH(2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay). The relationship between TFC, TPC and antioxidant activity were statistically investigated. The dominant VCs of *P. granatum* were limonene (50.6%) followed by β-pinene (25.1%), γ-terpinene (6.79%), α-pinene (5.7%) and sabinene (2.59%), respectively. Gallic acid was major polyphenol compound in all studied extracts of marc. 60°C water extract from *P. granatum* pomace represented higher antioxidant capacity, TPC and TFC than other investigated extracts. This studied marc can be introduced to different industries as a source of bioactive and medicinal natural compounds because of its valuable phytochemical characterization.

Keywords: *Punica granatum,* phenolic compounds, antioxidant capacity, flavonoids.

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INTRODUCTION

The pomegranate (*Punica granatum* L.) from the punicaceae family, is greatly grown in various subtropical and tropical territories and is the oldest edible fruits (Salaheddin and Kader, 1984). The pomegranate is a fruit-bearing deciduous small tree or shrub and is best reconciled to cool winters and hot summers. Pomegranate can be grown from plains to an elevation of 2000 m. The pomegranate is native to the territories from Iran to north India and is thought to have been first cultivated 5000 to 6,000 years ago (Ebrahimi, 2015). It is estimated that total production amounts to around 3 million tons of pomegranate are produced in the world, annually (Jaime et al., 2013), of which Iran produces approximately 28%; annual production of pomegranate has been recorded as 10,866,300 tons (Anonymous 2015). Pomegranate juice and peel comprise fundamental quantities of polyphenolic tannins, for example gallic acid and ellagic acid (Loren et al., 2005). It has been used in the procurement of food instructions and therapeutic, cosmetic and tinctures formula (Finkel

and Holbrook, 2000) and therefore pomegranate peel can be introduced as a good source of antioxidants (Singh et al., 2001). From epochal to recent times, different parts of pomegranate have been used for different objects such as in diets (e.g., juices, jams, jellies, dressings, marinating, and wine), or as religious symbolism (e.g., righteousness, fullness, fertility, abundance), or for its medicinal values. High nutrient composition such as, oxalic acid, potassium, folate and vitamins E, C, B_6 and A is well demonstrated in pomegranate peels (Al-Rawahi et al., 2014). Generally, low aromatic intensity is the main characteristic of pomegranate fruit (Wang et al., 2013). Moreover, antioxidant, antimicrobial and antiparasitic activities of pomegranate leaf extract have been illustrated (Rahmani et al., 2017). Extraction of solvent is more mostly applied for the extraction of secondary metabolites with distinct polarity, such as phenolic compounds, tannins, flavonoids, terpenoids etc. Various extraction methods have been used for the extraction of phenolic compounds using solvents with different polarities, like for example petroleum ether, ethyl acetate, water and methanol (Yasoubi et al., 2007). After pressing of fruits for juice or oil, the solid remains are pomace or marc. It includes the stems, seeds, pulp and skins of the fruit. Different pomegranate cultivars had different polyphenol compositions and antioxidant potential. It is considerably associated with many factors such as cultivar type, growing region, maturity, cultivation, climate, edaphic condition, and storage situation. But our aim was not to investigate the pomegranate characteristics from different cultivars. To our knowledge, this is the first report on the phytochemistry of pomaces from *P. granatum* L. All of previous researches were considered the essential oil, volatile compounds and other phytochemistry studies on different parts of fresh or dried of *P. granatum.* Pomegranate pomaces represent enormous waste from their processing. Pomace, rind, skin and seed of the fruits are the waste materials. They contain well founts of possibly worth bioactive compounds, like oils, enzymes, vitamins, dietary fibers, polyphenols and carotenoids. These phytochemicals may be used in various industries such as the textile and chemical industries, the health industry for pharmaceuticals and medicines and the food industry, for the advancement of enriched or functional foods (Sagar et al., 2018). During the *Punica* juice processing, about 40 to 50 percent of the products were retained (Animal Science Research Institute of Iran, 2015). It is possible to keep the waste of these crops one hundred thousands of tons were estimated (Animal Science Research Institute of Iran, 2015). What remains of fruit juice industry contains all parts of the fruits which are dumped as waste. Unfortunately, these valuable substances, such as fruit pomace, are unknown in different countries like Iran. Therefore, we decided to investigate the volatile compounds (VCs), total flavonoid content (TFC), total phenolic content (TPC), antioxidant capacity and phenolic compounds from *P. granatum* marcs for better applications.

MATERIALS AND METHODS

Plant materials

Pomaces of *P. granatum* (Farough variety) were gathered from a juice factory in Jahrom, Fars province (south of Iran). Pomaces were sliced into small chunks and shaded at room temperature (20 to 25°C) for 10 days. The experiments (TPC, TFC, polyphenol and antioxidant capacity) were designed with four treatments (60°C water, distilled water, methanol and methanol 80%) with three replications.

Standards and chemicals

Homologous series of C_8-C_{25} n-alkanes were purchased from Sigma (Sigma–Aldrich, Steineheim, Germany). Folin-Ciocalteu's phenol reagent was provided from Merck (Darmstadt, Germany). Methanol and reference standard of 17 polyphenols (gallic acid (≥99%; Sigma-Aldrich), catechin hydrate (90%; Fluka), caffeic acid (≥98%; Sigma-Aldrich), chlorogenic acid (≥95%; Sigma-Aldrich), quercetin (≥ 95%; Sigma-Aldrich), hesperidin (≥ 80%; Sigma-Aldrich), coumarin (≥99%; Merck), sinapic acid (≥98%; Sigma-Aldrich), trans-ferulic acid (99%; Sigma-Aldrich), vanillin (99%; Sigma-Aldrich), p-coumaric acid (≥98%; Sigma-Aldrich), eugenol (99%; Sigma-Aldrich), hesperetin (≥80%; Sigma-Aldrich), ellagic acid (≥95%; Sigma-Aldrich), rosmarinic acid (≥98%; Sigma-Aldrich), thymol (≥98.5%; Sigma-Aldrich) and carvacrol (98%; Sigma-Aldrich) were collected for HPLC grade analysis. Deionized water was provided using Millipore Direct-Q UV. All other reagents were of analytical grade.

Static headspace (SHS) volatiles extraction

Based on our previous work (Bahmanzadegan and Rowshan, 2018), the SHS analysis was performed on the Combi-PAL model type (CH-4222 Zwingen, Switzerland). 1 gram of pomace with 1 µl of n-heptadecane including n-hexadecane (as internal standard, 200 ppm) were put in headspace vial. The incubation temperature was at 85°C for 15 min, the temperature of the sampling needle was 90°C, fill speed was 150 µl/s and the injection volume was 1000 µl. Peak area in the GC-MS chromatogram relative to that of the internal standard was used to calculate the response.

VCs identification

The analyses of VCs were carried out with an Agilent model 7890-A series gas chromatography and Agilent model 5975-C mass spectrometry coupled with a multipurpose CTC Combi Pal sampler. Separations were performed using HP-5 MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm i.d × 25 µm). The GC oven temperature was programmed from 60 to 210°C at 3°C/min, and 210 to 240°C at 20°C/min (8.5 min isothermal). Helium was the carrier gas at 1.0 mL/min in constant flow mode. The injector temperature was 280°C, and the split ratio was 1:50. Over 50 to 550 amu with an ionizing voltage of 70 eV, the quadrupole mass spectrometer was scanned. According to the method using n-alkanes (C_8-C_{25}) as standard, the retention indices for the whole ingredients were characterized. The compounds were specified by retention indices (RI, HP-5) comparison with those shown in the literature and by comparison of their mass spectra with the Wiley Adams and Nist Library data published mass spectra data (Adams, 2007; Joulain et al., 2001; McLafferty and Stauffer, 1989).

Preparation of crude extract

The definite weight of powdered pomaces extracted using maceration technique by soaking them in methanol, distilled water and methanol 80% (the ratio of raw material/solvent was 1:10) for about 24 h and also were immersed in distilled water and placed in a water bath at 60°C for 1 h, then filtrating for injecting to HPLC for polyphenolic determination. The crude extracts were concentrated in vacuo at 40°C by rotary evaporator for antioxidant capacity analysis, total phenol and total flavonoid content.

HPLC analysis

To achieve maximum sensitivity and separation, gradient elution was selected. The elution was done with altering the proportion of solvent A (formic acid 1% in deionized water) to solvent B (methanol (v/v)) as follows: methanol: formic acid 1% (10:90), at zero minute; methanol: formic acid 1% (25:75), at ten minutes; methanol: formic acid 1% (60:40), at twenty minutes and methanol: formic acid 1% (70:30) at thirty minutes, which was retained isocratic up to forty minutes. HPLC analysis was performed on an Agilent 1200 series, provided with a Zorbax Eclipse XDB-C18 column (4.6 × 5 µm i.d.; × 150 mm film thickness, RP), and a photodiode array detector (PDA). Elution was monitored at 280 and 320 nm. The column temperature was 30°C. The injection volume was 20 µl and it was accomplished automatically by autosampler (Bahmanzadegan et al., 2017). To determine the quantitative content of polyphenol of *P. granatum* pomaces calibration curves were provided in the concentration range from 1 to 500 mg L^{-1}. Equations of calibration curves and their correlation coefficients are presented in Table 1. The R^2 quantities were in the span from 0.996 to 0.999 which confirmed the linearity of the method.

DPPH assay

According to our previous work (Bahmanzadegan et al., 2017), the antioxidant capacity of extracts and the standard antioxidant were assessed on the basis of radical scavenging effect of the stable DPPH free radical. 200 µl of a 40 mg/L solution of DPPH radical in methanol was mixed with 20 µl of 6.25 to 3200 µg/ml extracts and gallic acid respectively, solutions were left at room temperature for 30 minutes. The DPPH radical inhibition was measured at 515 nm by using a micro-plate reader model biotek ELx808. The IC_{50} of each sample (concentration in µg/ml required to inhibit DPPH radical formation by 50%) was calculated by Matlab software. The antioxidant caoacity is given by:

100- [(sample Absorbance - blank Absorbance) × 100/ control Absorbance].

Determination of total phenolic

Total phenolic content (TPC) was determined according to the Folin-Cicocalteu method. Phenols on reaction with an oxidizing agent phosphomolybdate in FCR under alkaline conditions, lead to the formation of a molybdenum blue colored complex, the intensity of which can be measured at 765 nm colorimetrically. For the preparation of calibration curve gallic acid solutions (0.0093, 0.0187, 0.0375, 0.075, 0.15 mg L^{-1}) were used. 500 µl of each extracts and standards were mixed with 2 ml sodium carbonate solution (7.5%) and 2.5 ml Folin- Cicocalteu's (10%) reagent (FCR). For sixty minutes, the mixture was kept at room temperature. Then, by spectrophotometer (Lambda 950, Perkin-Elmer, USA) the absorbance of the samples was read at 765 nm. For each analysis, the samples were belayed in triplicate. Milligram GA/g dry weight (dw) extract is used to express the TPC values (Moein et al., 2015).

Total flavonoid assay

The total flavonoid content (TFC) was determined with an aluminium chloride and colorimetric assay. The sample contained 1.5 ml of solution of the extract in the concentration of 100 mg/L and 1.5 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at 415 nm (Lambda 950, Perkin-Elmer, USA). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was construed. The TFC was expressed in terms of quercetin equivalent (mg of Qu/g of extract) (Stankovic, 2011).

Statistical analysis

Data were represented as the means ± standard error of the mean of four autonomous experiments accomplished in triplicate. The data was statistically analyzed using one-way ANOVA by the program SPSS (23.0). By Duncan's multiple range test, significant differences among means from a triplicate analysis at (P <0.01, 0.05) were assessed.

RESULTS AND DISCUSSION

VCs with static headspace

Using an Agilent Chemstation Integrator, the percentage of relative amounts was calculated from peak areas. Data gained from quantitative and qualitative assessment of the VCs from pomaces of pomegranate are displayed in Table 2. 96.6% of the volatile of *P. granatum* were a total of ten compounds which were recognized in the pomace samples by SHS. The main VCs of pomegranate were limonene (50.6%) followed by β-pinene (25.1%), γ-terpinene (6.7%), α-pinene (5.7%) and sabinene (2.5%), respectively. Similar to our results a study by Jung (2014) indicated that α-pinene, β-pinene, limonene and sabinene were the main VCs in the fruit peel, stem peel and root peel of pomegranate. In 9 Spanish cultivars of pomegranate, *(Z)*-3-hexenol, *(E)*-2-hexenal, limonene and hexanal were the most abundant compounds in fresh juices (Melgarejo et al., 2011). There were eleven VCs (guaiacol, *(E)*-2-hexenal, hexanal, β-caryophyllene, *(E)*-α-bergamotene, limonene, myrcene, α-terpineol, 1-octanol, *(Z)*-3-hexen-1-ol and 1-hexanol) prevalent to all 5 pomegranate seeds and juices in Turkey (Guler and Gul, 2017). Although, we studied the VCs by SHS method not hydrodistillation, our results showed similar compounds in comparison with other studies. Similar to previous reports limonene was the main constituent in this studied pomace. Our results showed that monoterpene hydrocarbons were the major constituents in this studied pomace. 96.6% of identified compounds in *P. granatum*

Table 1. Plolyphenol compounds from pomace of *Punica granatum.*

Polyphenol	Linear regression equation[a]	Correlation coefficient (R^2)	60°C water	distilled water	methanol	methanol 80%
Gallic acid	Y=40.507x-33.427	0.999	11.25±0.05 [b](A)[c]	3.24±0.0002 (B)	3.02±0.002 (C)	1.09±0.004 (D)
Chlorogenic acid	Y=24.112x-8.6696	0.999	0.12±0.0002 (A)	0.07±0.0001 (B)	0.05±0.0001 (C)	0.03±0.0001(D)
Sinapic acid	Y=12.843x-82.917	0.999	nd[d]	nd	nd	nd
Coumarin	Y=55.203x+186.22	0.999	nd	nd	nd	nd
Hesperetin	Y=30.574x-141.76	0.999	nd	nd	nd	nd
Carvacrol	Y=10.675x-12.921	0.999	nd	nd	nd	nd
Caffeic acid	Y=15.247x+152.14	0.997	nd	nd	nd	nd
Vanillin	Y=42.74x+59.464	0.999	nd	nd	nd	nd
trans-Ferulic acid	Y=30.718x-214.48	0.999	nd	nd	nd	nd
Quercetin	Y=14.927x+72.349	0.999	nd	nd	nd	nd
p-Coumaric acid	Y=82.241x+287.72	0.999	0.40±0.003 (A)	0.09±0.0003 (C)	0.12±0.0005 (B)	0.08±0.00001(D)
Rosmarinic acid	Y=24.232x-101	0.99	0.62±0.0003 (A)	0.21±0.0004(D)	0.45±0.0002 (B)	0.28±0.0003 (C)
Hesperidin	Y=16.849x+40.817	0.997	0.55±0.0004(A)	0.19±0.0004(D)	0.38±0.0002 (B)	0.26±0.0003(C)
Ellagic acid	Y=17.803x-185.06	0.992	0.68±0.002(A)	0.31±0.0001(D)	0.43±0.003(B)	0.38±0.0008(C)
Eugenol	Y=11.32x-147.17	0.994	nd	nd	nd	nd
Thymol	Y=8.7065x-68.159	0.998	nd	nd	nd	nd
Catechin	Y=9.2191x-72.022	0.997	3.57±0.0003(A)	1.33±0.0003(B)	nd	nd

[a]Y: Area; X: Concentration; [b]Calculated mean amount of the polyphenol (mg/g) based on the weight of the ground dry pomaces in three replicates ± SD; [c]Duncan's mean separation; [d] nd: not detected.

pomace were monoterpene hydrocarbons. Limonene is utilized in drug and food manufacturing, that is, as a fragrance material in hand cleaners, bath products, after shave lotions, perfumery, etc. and as a flavoring agent toconceal the bitter taste of alkaloids utilized in some medicines. Limonene has a piney, turpentine-like odor and can be used, as a botanical insecticide. In natural and alternative medicine, limonene is applied because of its ability to becalm heartburn and gastro esophageal reflux disease (Sun, 2007).

Phenolic composition

The content of seventeen studied polyphenols are displayed in Table 1.The variance analysis illustrated significant differences in polyphenol compounds among the different extracts in *P. granatum* ($P < 0.01$; Tables 1 and 3). As shown in Figure 1, the highest amount of polyphenols of *P. granatum* pomace were gallic acid (11.25, 3.24 mg/g), catechin (3.57, 1.33 mg/g), ellagic acid (0.68, 0.31 mg/g), rosmarinic acid (0.62, 0.21 mg/g), hesperidin (0.55, 0.19 mg/g), p-coumaric acid (0.40, 0.09 mg/g) and chlorogenic acid (0.12, 0.07 mg/g) in 60°C water and water extracts, respectively. Gallic acid (3.02 mg/g), rosmarinic acid (0.45 mg/g), ellagic acid (0.43 mg/g), hesperidin (0.38 mg/g), p-coumaric acid (0.12 mg/g) and chlorogenic acid (0.05 mg/g) were the main phenolic constituents in methanolic extract of *P. granatum* pomace. Gallic acid (1.09 mg/g), ellagic acid (0.38 mg/g), rosmarinic acid (0.28 mg/g), hesperidin (0.26 mg/g), p-coumaric acid (0.08 mg/g) and chlorogenic acid (0.03 mg/g) were the major phenolic compounds in 80% methanolic extract of *P. granatum* pomace. Sinapic acid, thymol, coumarin, quercetin, carvacrol, vanillin, trans-ferulic acid, eugenol and hesperetin were not detected in *P. granatum* marc extracts. Catechin was only identified in 60°C water and water extract of *P. granatum* pomace. Our findings illustrated that gallic acid was the abundant phenolic compound in all studied extracts of *P. granatum* and it was agree with other previous studies. As a result, gallic acid, illogic acid, punicalin, and punicalagin reported as major phenolic compounds in *P. granatum* cultivated in Oman (Al-Rawahi et al., 2014).

Table 2. Chemical composition of pomace of *Punica granatum* by SHS analysis.

No	Compounds	RI [a]	RI [b]	Amounts (%)[c]	RSD [e] (%)
1	n-Nonane	901	900	[d] -	-
2	Tricyclene	923	926	-	-
3	α-Thujene	927	930	1.1	3.6
4	**α–Pinene**	**934**	**935**	**5.7**	**2.8**
5	Camphene	952	950	1.5	4.7
6	Thuja-2,4(10)-diene	958	960	-	-
7	**Sabinene**	**974**	**972**	**2.5**	**7.3**
8	**β-Pinene**	**976**	**977**	**25.1**	**3.5**
9	6-methyl-5-Hepten-2-one	987	986	-	-
10	Myrcene	992	991	1.7	5.6
11	n-Decane	1000	999	-	-
12	α-Phellandrene	1002	1004	-	3.6
13	p-Mentha-1(7),8-diene	1004	1008	-	-
14	α-Terpinene	1017	1014	0.5	2.1
15	p-Cymene	1025	1024	1.2	3.4
16	**Limonene**	**1029**	**1031**	**50.6**	**1.5**
17	(Z)- β-Ocimene	1037	1036	-	5.1
18	(E)- β-Ocimene	1047	1050	-	3.4
19	**γ–Terpinene**	**1059**	**1060**	**6.7**	**4.7**
20	Terpinolene	1089	1090	-	3.1
21	Linalool	1099	1100	-	-
22	Citronellal	1153	1150	-	-
23	Terpinen-4-ol	1177	1179	-	1.2
24	α-Terpineol	1190	1189	-	-
25	n-Dodecane	1199	1200	-	-
26	n-Decanal	1206	1202	-	-
27	Citronellol	1229	1226	-	-
28	Neral	1241	1239	-	-
29	Geranial	1269	1268	-	-
30	n-Tridecane	1299	1300	-	-
31	δ-Elemene	1337	1338	-	-
32	Neryl acetate	1365	1362	-	-
33	Geranyl acetate	1385	1384	-	-
34	β-Elemene	1392	1390	-	-
35	(E)-Caryophyllene	1418	1417	-	2.4
36	trans-α-Bergamotene	1435	1436	-	-
37	β–Bisabolene	1509	1506	-	-
Total				**96.6**	

[a]Linear retention indices were calculated using a homologous series C_8-C_{25} n-alkanes; [b]Retention indices taken from literature;[c] Relative area percent (peak area relative to total peak area); [d]Not detected; [e]percentage relative standard deviation for SHS method (relative peak area).

Table 3. Compare of 4 different extracts of *Punica granatum* for extraction of polyphenol compounds using one-way ANOVA.

Polyphenol	MS	CV (%)	Polyphenol	MS	CV (%)
Gallic acid	60.90**	0.54	Quercetin	-	-
Chlorogenic acid	0.005**	0.16	p-Coumaric acid	0.07**	0.88
Sinapic acid	-	-	Rosmarinic acid	0.10**	0.08
Coumarin	-	-	Hesperidin	0.07**	0.3
Hesperetin	-	-	Ellagic acid	0.08**	0.41
Carvacrol	-	-	Eugenol	-	-
Caffeic acid	-	-	Thymol	-	-
Vanillin	-	-	Catechin	7.6**	0.01
trans-Ferulic acid	-	-			

**: significantly different at 1% level.

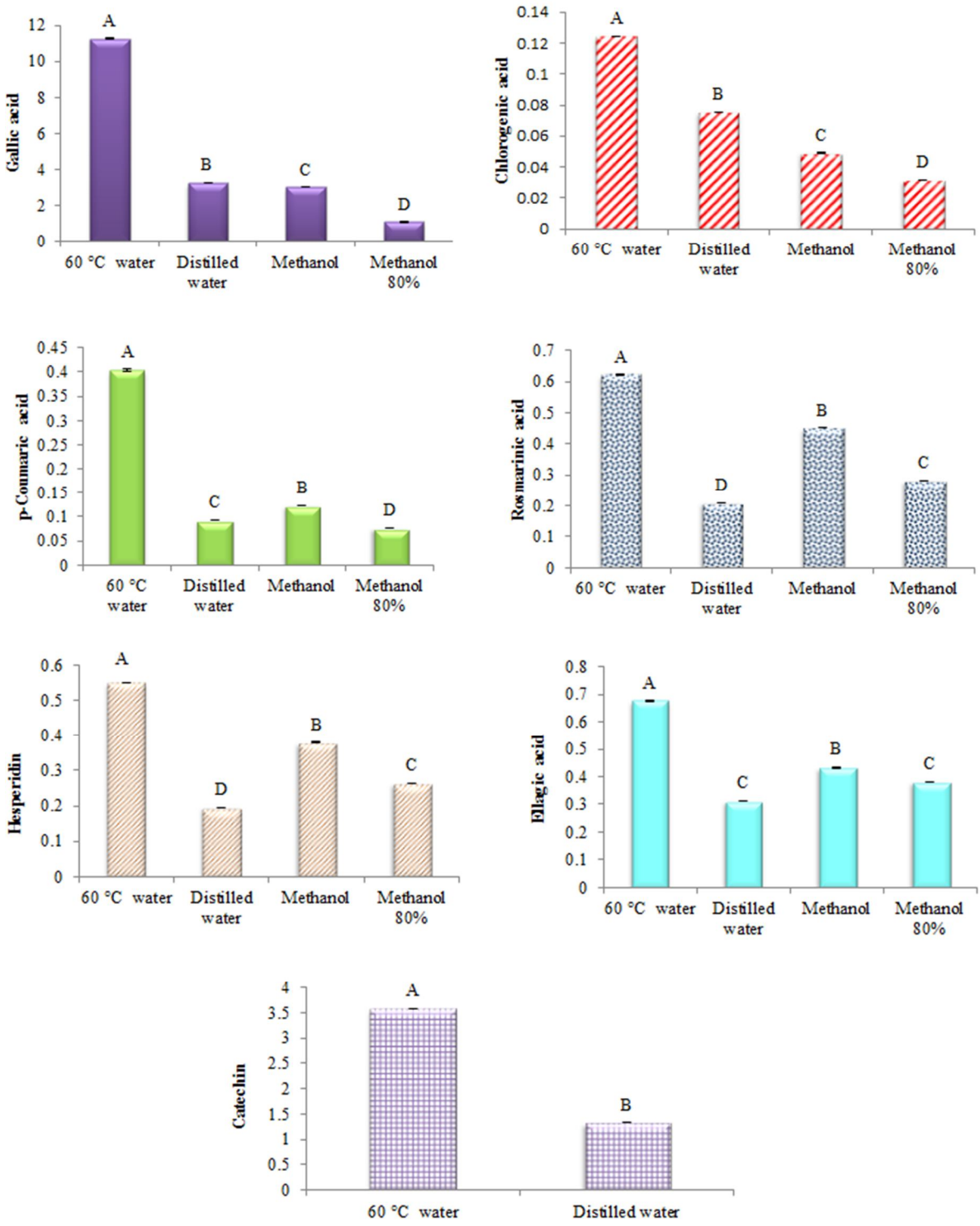

Figure 1. Comparison of the phenolic compounds of *P. granatum* in different extracts.

In peel extract of Tunisian pomegranate, gallic acid was the main compound, followed by ellagic acid, caffeic acid, p-coumaric acid, quercetin, and vanillic acid (Mansour et al., 2013). From one of the Egyptian variety ellagic acid, epicatechin, catechin, chlorogenic acid, p-cumaric acid and protocatechuic acid identified as the main phenolic compounds in *P. granatum* peel (Zaki et al., 2015). Phenolic compounds of pomegranates are graded on phenolic acids (ellagic acid, gallic acid, chlorogenic acid, caffeic acid, vanillic acid, ferulic acids, trans-2-

Hydrocinnamic acid, quercetin) (Bassiri-Jahromi and Doostkam, 2019). In our study the greatest content of gallic acid was determined in 60°C water extract. Phenolic compounds in *P. granatum* pomace in our study included 2 hydroxybenzoic acids (gallic and ellagic acids; and as hydrolyzable tannins), 3 hydroxycinnamic acids (rosmarinic, p-coumaric and chlorogenic acids), one flavanon glycoside (hesperidin) and one flavan-3-ol (catechin). Gallic acid and ellagic acid may be the compounds responsible for *P.granatum* anti-inflammatory effect (BenSaad et al., 2017). Hydrolyzable tannins have a polyhdric alcohol at their core, the hydroxyl groups of which are partially, or fully, esterified with either gallic or ellagic acid. They may have long chains of gallic acid coming from the central glucose core. On hydrolysis with acid or enzymes, the hydrolyzable tannins break down into their constituent phenolic acids and carbohydrates. The high antioxidant capacity of pomegranate juice is attributed mainly to the tannins, which pass to the juice during the industrial extraction process (Gardeli et al., 2019). At the present study gallic and ellagic acids, as the precursors of hydrolyzable tannins, were detected at four studied treatments. Caffeic acid was not found in our treatments but its ester form, rosmarinic acid, was detected at all treatments. Rosmarinic acid is naturally occurring in several plants of the Lamiaceae family. This compound exhibits antioxidant and anti-inflammatory effects and has recently been shown to protect neurons in vitro against oxygen-glucose deprivation (Alfieri and Mann, 2015).

Antioxidant capacity

IC_{50} was used to represent the radical-scavenging activity on DPPH. This quantity was the concentration in µg/ml involved to exclude DPPH radical formation by 50%. All of *P. granatum* pomace extracts showed a good antioxidant capacity but 60°C water extract gives higher capacity than other extracts (P <0.01). Mean values were 200.3, 216.9, 227.8 and 250.8 µg ml^{-1}, respectively for 60°C water, water, 100% methanolic and 80% methanolic extracts (Table 4, Figure 2a). Our research for antioxidant capacity of *P. granatum* extracts displayed different results as compared to other studies. Negi and Jayaprakasha (2003) discovered that water rendered the lowest antioxidant yield and methanol rendered the maximum one in pomegranate peel. Generally, when regular people belay traditional extract they use water. In fact, compare to methanol, water due to its high polarity is not an efficient solvent for the phenols extraction. According to some studies, pure water is not an efficient solvent to extract polyphenols because these compounds are more soluble in solvents less polar than water (Jakopic and Veberic, 2009; Sepahpour et al., 2018). Similar to our results (Table 4), the findings afforded with water represented appropriate amounts of natural antioxidants (Mansour et al., 2013). Although we considered the marcs of the studied samples, which were dumped as waste, they showed high to moderate antioxidant capacity. We found these marcs had bioactive compounds, although they were waste and useless. It means that the pomaces could be rich potent for antioxidant capacity. In these extracts, the phenolic and flavonoid compounds might be considered as great antiradical traits of them (Shiban et al., 2012; Sultana et al., 2015). The observed antioxidant activities of *P. granatum* pomace extracts in the present study might be attributed to the presence of polyphenols, such as gallic acid and ellagic acid (Gil et al., 2000). The data expressed that, in pomace of *P. granatum*, the total polyphenol and flavonoid play an important role in antioxidant capacity.

Total phenolic content (TPC)

Table 4 presents the amount of total phenol content from pomaces extracted by different solvents. The extracts of *P. granatum* which received from 60°C water followed by 80% methanol had the greatest TPC content (291.1 and 291.0 mg GA/g) (Table 4, Figure 2b). The results indicated that *P. granatum* exhibited significant TPC (P < 0.01; Table 4). TPC were ranged from 281.6 to 291.1 mg GA/g in the *P. granatum* samples. These values were higher than those obtained by Mansour et al. (2013), from 82.0 to 230.4 mg GA/g in the accession 3 from Gabes. The peel extract of Omani pomegranate was 64.2 mg GA/g dry solids (Al-Rawahi et al., 2014). The results revealed that the most efficient solvent for extraction of TPC for *P. granatum* pomace is 60°C water, followed by 80% methanol, distilled water and pure methanol. As proposed elsewhere, particularly with the use of mild heating (up to 60°C), the integrity of the cell wall is altered, and therefore there is a greater contact of the cellular components, among them the polyphenols, with the extraction solution (Yang et al., 2009). TPC plays a probable role in preventing different diseases related to oxidative stress such as cardiovascular, cancer and neurodegenerative diseases. Pomegranate has excellent antioxidant capacity and is beneficial for atherosclerosis prevention. In this regard, polyphenols are capable of moderating the broad range of enzymes activities and cell receptors (Bassiri-Jahromi and Doostkam, 2019).

Total flavonoid content

Table 4 illustrates the TFCs in studied extract samples. Our results were correlated with antioxidant and the TPC reports. It resulted that the highest amounts of the TFC were available in 60°C water from *P. granatum* pomace extracts (P < 0.01, Table 4). In this study, the TFC were limited from 14.2 to 85.1 mg QU/g extract in *P. granatum*

Table 4. Comparison of 4 different extracts of *Punica granatum* for antioxidante capacity, total phenolic and flavonoid contents using one-way ANOVA.

	MS	CV (%)	60°C water	Distilled water	Methanol	Methanol 80%
Antioxidant capacity by DPPH (µg/ml)	1797.7**	1.8	200.3 ± 1.1 (A)[a]	216.9 ± 2.0 (B)	227.8 ± 2.6 (C)	250.8 ± 7.2 (D)
Total phenolic content (TPC) (mg/gr extract)	59.2**	1.5	291.1 ± 5.9(A)	288.1 ± 2.9 (B)	281.6 ± 2.9 (AB)	291.0 ± 4.4 (A)
Total flavonoid content (TFC) (mg QU/gr extract)	3508.2**	2.4	85.1 ± 0.7 (A)	18.1 ± 0.8 (B)	14.2 ± 0.9 (C)	18.1 ± 0.7 (B)

[a]Duncan's mean separation; **: significantly different at 1% level.

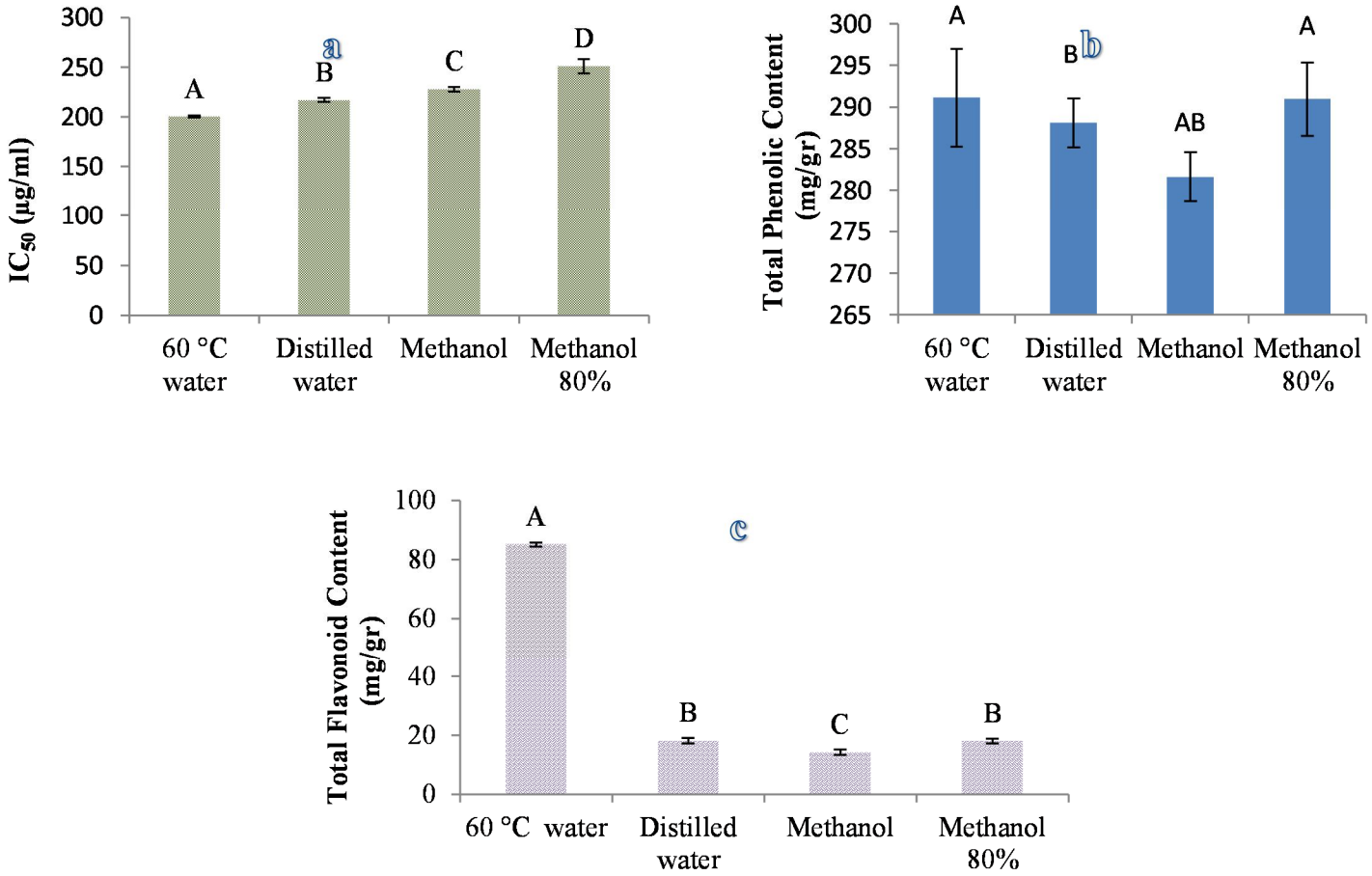

Figure 2. Antioxidant capacity (a), total phenolic (b) and flavonoid (c) contents of *P. granatum* in four different extracts.

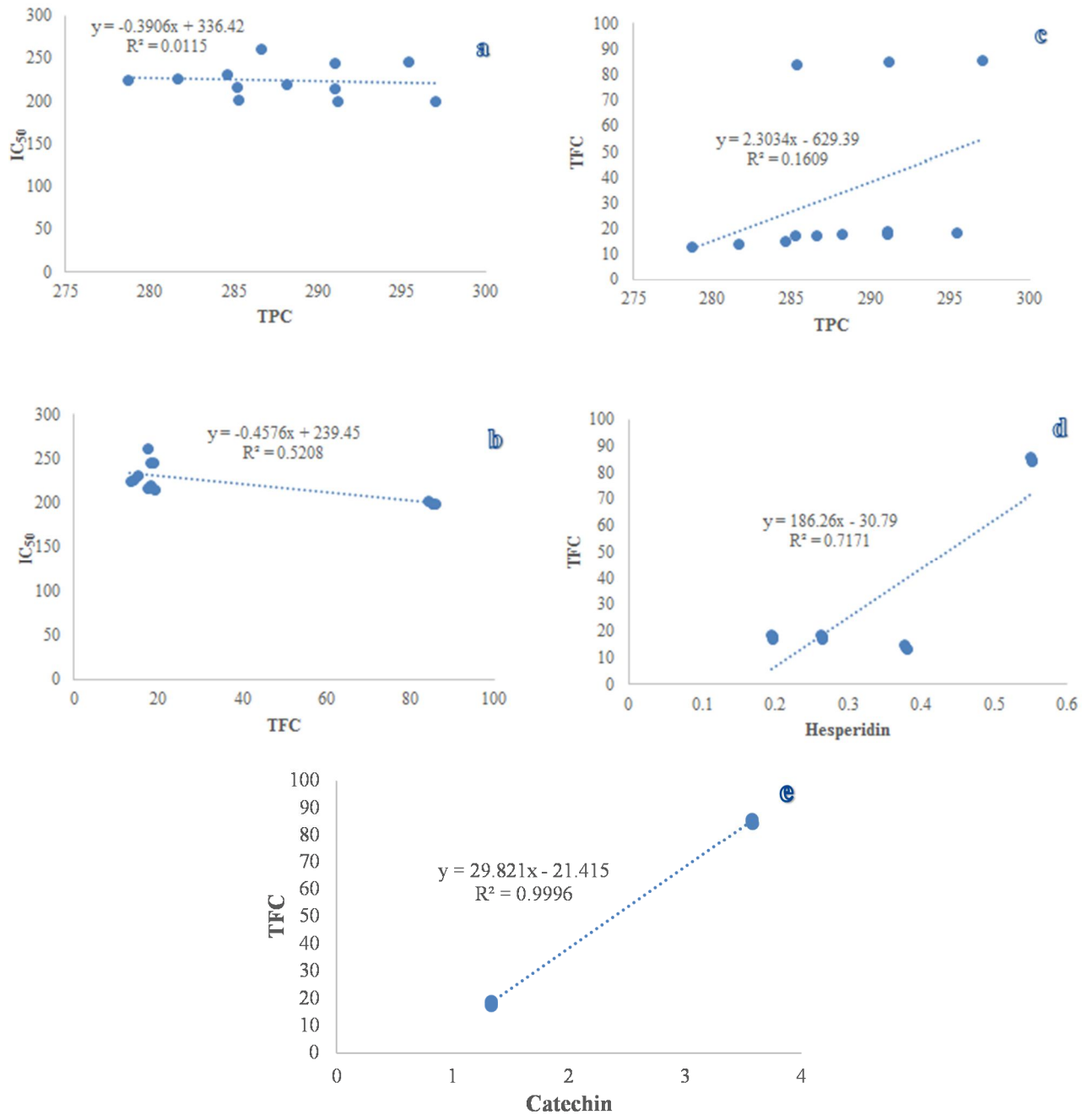

Figure 3. The correlation between antioxidant capacity (DPPH free radical-scavenging activity), phenolic compound, total phenolic and flavonoid contents of *P. granatum* extracts.

samples (Table 4, Figure 2c). All the extracts analyzed in this research showed high levels of phenol contents. One of the greatest groups of polyphenols are flavonoids. They are thought to illustrate advantageous health effects for their chelating and antioxidant attributes, and are the main participant to the plants antioxidant valence. They function either by breaking lipid peroxidation chain reactions, by scavenging free radicals, or by blocking the generation of hypervalent metal forms (Zaragoza et al., 2008). The TFC was 1.4 mg Catechin equivalent/g dry solids in P. *granatum* cultivated in Oman (Al-Rawahi et al., 2014). The TFC were 72.52, 26.08, 6.79 and 51.52 mg (mg Rutin/g DW) for flowers, leaves, seeds and peel, respectively from pomegranate trees in Gabe sprovince (Elfalleh et al., 2012). The TFCs (49.1 to 59.4 mg Catechin/g dry solids) in pomegranate fruits were reported (Kulkarni et al., 2004). Among investigated phenolic constituents, hesperidin, quercetin, hesperetin

and catechin were classified as flavonoid compounds. So, the TFC of *P. granatum* marc was resulted from catechin and hesperidin as flavonoid compounds in our study. Various investigations (Shan et al., 2005; Wong et al., 2006; Wu et al., 2006) found that phenolic compounds in herbs and spices considerably brought about their antioxidant attributes.

Correlation between TPC, antioxidant capacity, phenolic compound, and TFC

Figure 3 displays the correlation between DPPH assay, TPC, TFC and phenolic constituents of studied extracts. Finding display a positive correlation coefficient between TFC and catechin (in which only detected in two extracts) of *P. granatum* extracts ($P < 0.01$; $R^2 = 0.99$; Figure 3e). There were no positive correlations between antioxidante capacity, TFC and TPC of *P. granatum* (Figures 3a, b, c). There was a minor positive correlation coefficient between TFC and hesperidin of *P. granatum* ($R^2 = 0.72$, Figure 3f). There were no positive correlations between antioxidant capacity, TFC and TPC of *P. granatum* with other phenolic compounds (Figure S1). Similar to our study some authors have demonstrated poor linear correlation or report total antioxidant capacity and phenolic content with no comment (Czapecka et al., 2005), while others illustrate a linear correlation of flavonoid quantity and total phenolic with antioxidant content (Aryal et al., 2019). By comparing the correlations, we can be proposed that another flavonoids (flavonols: luteolin, kaempferol; anthocyanins: pelargonidin-3-glucoside, cyanidin-3-glucosid; flavanols) and phenolic groups (condensed tannins) are extremely accountable the antioxidant capacity of the *P. granatum* extracts in pomaces.

CONCLUSIONS

According to the findings of this study, the marcs of *P. granatum* are natural sources of phenolic compounds. We concluded that amid the solvent systems investigated, the extracts received from 60°C water for *P. granatum* had the highest values of antioxidant capacity, TPC and TFC as compared with the other extracts. Moreover, these results encourage using water and avoid organic solvent to determine of antioxidant assay, TPC and TFC for *P. granatum* extract. The antioxidant evaluation proposes that the verified *P. granatum* marc and its related bioactive components like flavonoid and phenolic compounds can have a powerful potential as a novel devices for inhibiting different human diseases and a chemo prohibitive. As there was no correlation between DPPH assay, TPC and TFC of *P. granatum* extracts, we concluded that the high level of antioxidant capacity of *P. granatum* could be due to other compounds such as the high level of polyphenolic tannins and anthocyanins. TFC in *P. granatum* extracts could be correlated to another flavonoids (flavonols and flavones such as apigenin, rutin, kaempferol and nobiletin) in which we have not studied in this research. This marc with high antioxidant capacity might be proposed for preventing toxic oxidation in nutraceuticals, drugs for the treatment of diseases or in chemical industries and also useful as a substitute for synthetic antioxidants or as additives to prevent food deterioration. Whereas desirable utilization of agricultural waste such as pomaces of *P. granatum* will reduce costs and environmental hazards in which results from their disposal and remaining in the environment, our studies are continuing to investigate and introduce marcs of *P. granatum* as strong natural resources and we are going to use these marcs for other goals.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Fars Research Center for Agriculture and Natural Resources for providing the financial support to carry out the study.

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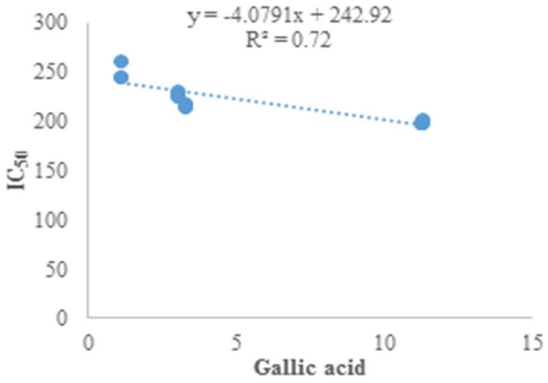
Citation: Tavallali H, Bahmanzadegan A, Tavallali V, Rowshan V, 2020. Phytochemical investigation of *Punica granatum* pomace: As source of bioactive and medicinal natural products. Adv Med Plant Res, 8(1): 1-13.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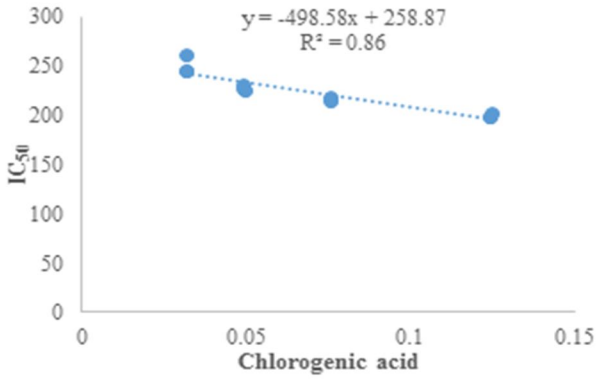
IC50
Gallic acid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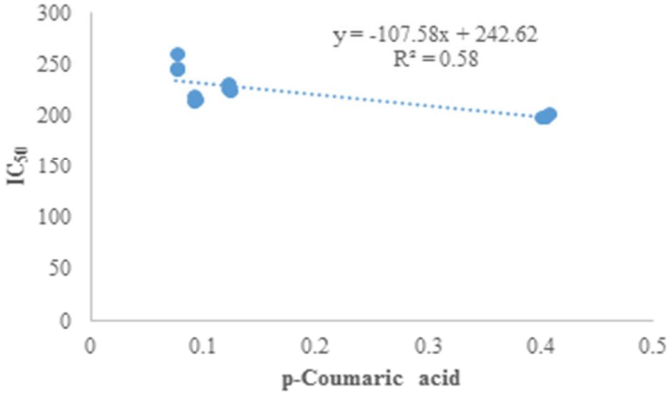
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IC50
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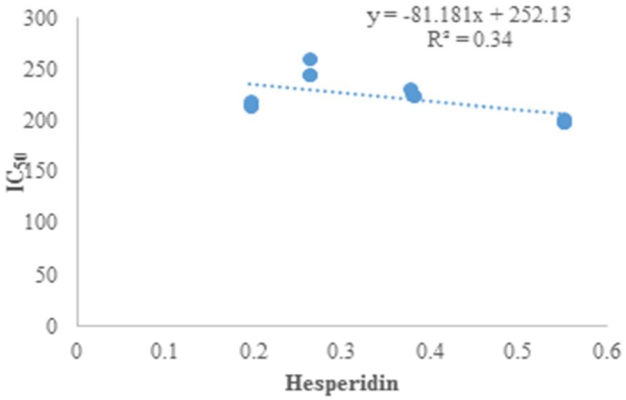
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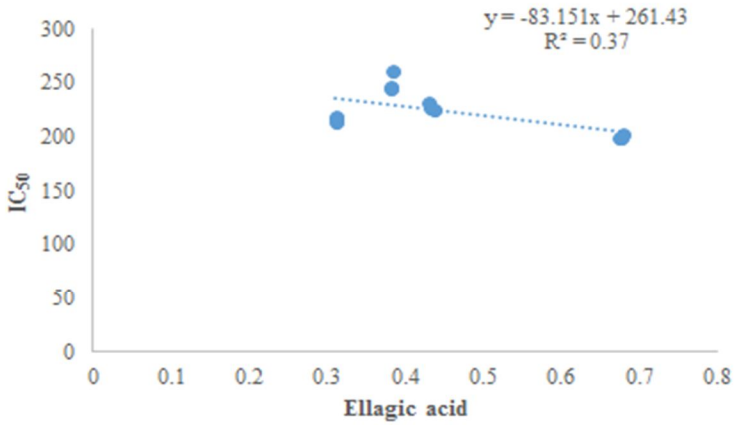
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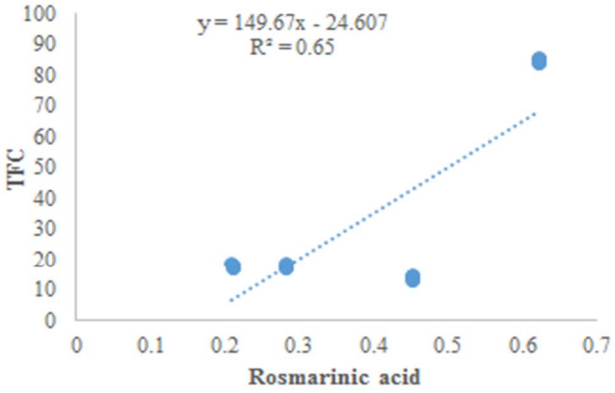
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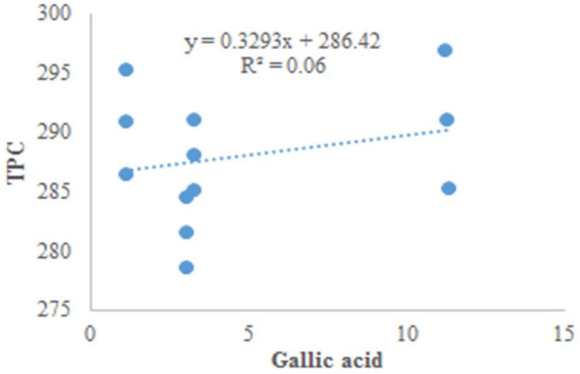
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TPC
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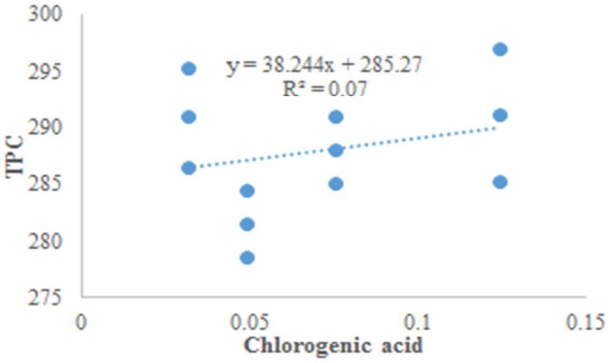
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Chlorogenic acid

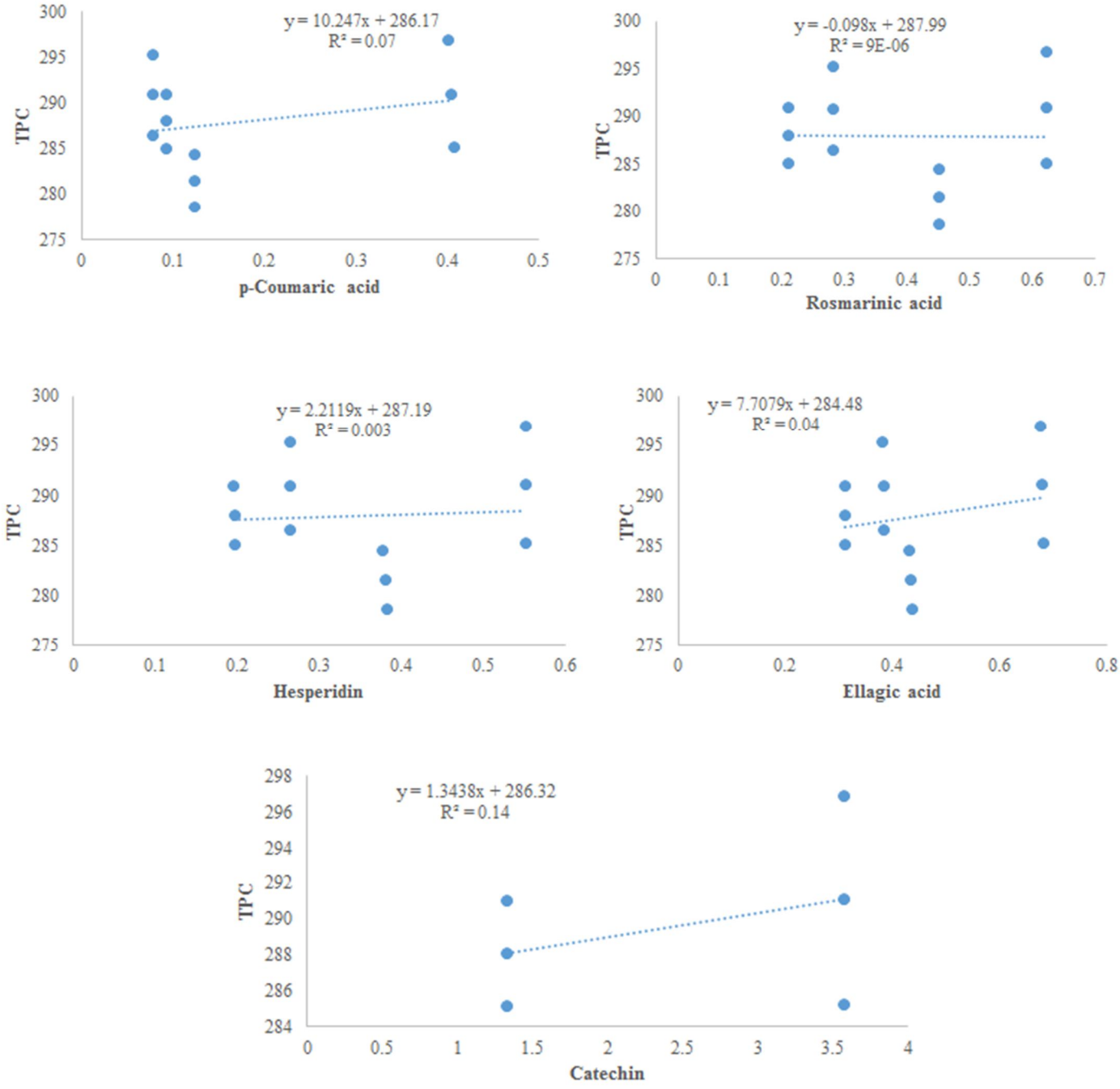

Figure S1. The correlation between antioxidant capacity, phenolic compound, total phenolic and flavonoid contents of *P. granatum* extracts.