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Full Length Research Paper

Phytochemical screening and polyphenol constituents of pomegranate peels and leave juices

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The leave and peels of pomegranate plants were manually separated and mechanically pressed to obtain their crude juices. The phytochemical results indicated that pomegranate peel juice contained high quantities of polyphenols and flavonoids, being about 1.22 and 1.43 times as great as that of leave juice, respectively. Crude peel juice possessed powerful antioxidant activity than leave crude juice, being approximately 6.59 times as great as that induced by leave juice. HPLC was used to characterize the polyphenolic compounds in pomegranate leave and peel juices. Thirty and eight polyphenolic compounds were separated from pomegranate peel and leave, respectively and nearly 50% of these compounds were quantitatively determined. The basic compounds found in pomegranate peel and leave juices were gallic acid, protocatechuic acid and gallic acid, 3-hydroxy tyrosol, respectively.

Keywords: Pomegranate peels and leave juices, phytochemical screening analyses, polyphenols, flavonoids, HPLC analyzer.

INTRODUCTION

Pomegranate is one of the important and oldest edible fruits of tropical and subtropical regions, which originated in the Middle East. The plant is also cultivated in Iran, USA, Turkey, Egypt, Italy, India, Chile and Spain. The world pomegranate production amounts to approximately 1,500,000 tons (FAOSTAT-FAO, 2014) and the peel amounts to approximately 60% of the pomegranate fruit weight (Lansky & Newman, 2007). It is widely reported that pomegranate exhibits antivirus, antioxidant, anticancer, antiproliferative activities (Faria *et al*., 2006, 2007, Adhami & Mukhtar, 2006). The pomegranate is a symbol of life, longevity, health, femininity, fecundity, morality, immortality and spirituality (Mahdihassan, 1984). For centuries, the barks, leave, flowers, fruits, and seeds of this plant have been used to ameliorate some diseases (Dandekar *et al*., 2008). The fruits have been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases (Graciousross *et al*., 2001, Kim *et al*., 2002 & Murthy *et al*., 2004). In Cuban traditional medicine the pomegranate fruits have been used to treat acidosis, dysentery, microbial infections, diarrhea, helminthiasis, haemorrhage, and respiratory pathologies (Fuentes & Exp´osito, 1995).

Pomegranate peels are exploited in traditional medicine because of their strong astringency, making them a popular remedy throughout the world. In the form of an aqueous decoction (boiling the hulls in water for 10-40 min), it was used for dysentery and diarrhea and also for stomatitis. It can be drunk and used as a mouthwash, douche or enema (Lansky *et al*., 2004). The phytochemistry of pomegranate has also been widely studied by some researchers and this fruit is found to be a rich source of polyphenolic compounds (Dandekar *et al*., 2008). Both flavonoids and tannins are more abundant in the peels (Ozcal & Dinc, 1993). Peels of pomegranate contain a wide variety of phytochemical compounds like

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gallotannins, ellagic acid, gallic acid, punicalins, punicalagins, as it was previously stated by some researchers (Mavlyanov *et al*., 1997, Reddy *et al*., 2007). Phytochemicals are often referred to non-nutritive compounds thought to be produced by plants as means of protection against such dangers as harmful ultraviolet radiation, pathogens and herbivorous predators. Pomegranate is consumed fresh and in processed form as juices, wines, flavors, and extracts. Commercial pomegranate juice has the highest antioxidant activity compared to other fruit juices, red wine, and green tea and currently is a high value product in the agricultural market. Phenolic compounds, including flavonoids, anthocyanins and tannins, are the main group of antioxidant phytochemicals with interesting properties and have deeply value to their biological and free radical scavenging activities (El-falleh *et al*., 2012).

The main objective of this work was to reveal the presence of certain phytochemicals and to quantify the total phenols and flavonoids beside the antioxidant activity of pomegranate juices of peels and leave. In addition, HPLC was used to characterize qualitatively and quantitatively the polyphenolic compounds in pomegranate leave and peel juices.

MATERIALS AND METHODS

Plant samples

Ripe pomegranate fruits were collected in October, 2013 from pomegranate trees in El- Menia governorate, Egypt. Samples of ripe pomegranate fruits were harvested from different trees of Wonderful cultivar. The plant was authenticated by Dr. Abdalatif, A. M. Associate Prof. of Horticulture Department, Faculty of Agriculture, Cairo University. The English, scientific and family names of the plant under study are: Pomegranate*, Punica granatum L* and Lythraceae, respectively.

Preparations of crude pomegranate leave and peel juices

Leave and peels of ripe pomegranate fruits were manually separated, cleaned from dust followed by seed removal then mechanically pressed by a Carver hydraulic laboratory press (Carver model C S/N 37000- 156; Fred S. Carver nc, Menomonee Falls, WI, USA). The resultant crude juices were concentrated using freeze- dryer (Labconco Corporation, Kansas City, M.O. USA) and kept in brown bottles at -5ºC until use.

Chemicals

Gallic acid and Folin-Ciocalteau phenol reagent were purchased from Sigma Chemical Co. (St Louis, MO, USA). Quercetin was purchased from Aldrich, Milwaukee, WI, USA. The analytical reagent grade methanol was obtained from Lab-Scan (Labscan Ltd, Dublin, Ireland). Authentic phenolic compounds: gallic acid, 3-hydroxy tyrosol, protocatechuic acid, catechin, catechol, chlorogenic acid, caffeic acid, vanillic acid, caffeine, ferulic acid, oleuropein and coumarin (1, 2- benzopyrone) were purchased from Sigma Chemical Company (St Louis, MO, USA). The purity of these compounds was checked by HPLC and each compound gave only one peak. All solvents were of analytical reagent grade and redistilled before use.

Qualitative phytochemical screening methods

Pomegranate leave and peels crude juices were screened for the presence of key families of phytochemicals according to the methods reported by Sawant and Godghate (2013). Carbohydrates and reducing sugars were detected by Molisch and Benedict tests, respectively. The presence of glycosides and sterols were detected by Keller- Kiliani and Salkowski tests, respectively. The saponins were revealed by Froth test. The occurrence of phenolic compounds, tannins and proteins were confirmed by ferric chloride and xanthoproteic tests, respectively. The occurrence of amino acids in the crude juices was assessed by ninhydrin test. The existence of alkaloids in the juices was evaluated by Wagner's test. The flavonoids were detected by lead acetate test, while the possibility of the presence of oils was indicated by saponification test.

Total phenolic content (TPP)

The total phenolic compounds in the crude juices were determined by the Folin- Ciocalteau method (El-falleh *et al*., 2012). An aliquot of juice sample (0.2 ml) was mixed with 0.5 ml Folin- Ciocalteau reagent then 4 ml of sodium carbonate (1M) and allowed to stand for 30 min at room temperature. The absorbance was measured at 750 nm using a spectrophotometer (Beckman, DU 7400 USA). TPP content in the juice was calculated and expressed as gallic acid equivalent per g dry weight (mg GAE/g DW) by reference to regression equation of standard curve ($Y=0.018 – 0.039$, $R^2=0.986$).

Flavonoid content

The colorimetric aluminum chloride method (El-falleh *et al*., 2012) was used for the determination of the total flavonoid content of the crude juices. An aliquot of the crude juice (0.5 ml) was mixed with sodium nitrite (0.3 ml, 0.5%) for 5 min then aluminum chloride (0.3 ml, 10%) was added. After 6 min the reaction was stopped by adding sodium

hydroxide (2 ml, 4%). The total volume was made up to 10 ml with distilled water. The absorbance was recorded at 510 nm using known concentrations of quercetin. The concentration of flavonoids in the juice samples were calculated from the regression equation of calibration plot (Y=0.010-0.143, R^2=0.989) and expressed as mg quercetin equivalent /g of dry weight sample.

Antioxidant activity

The scavenging activity on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) radical of pomegranate leave and peels crude juices was determined following the method of Rajan *et al.,* (2011). The crude juice of different concentrations was mixed with an aliquot of DPPH (1 ml, 0.004% w/v). The mixture was vigorously shaken and left to stand for 30 min in the dark at room temperature. The absorbance at 517 nm was recorded to determine the concentration of remaining DPPH. The radical- scavenging activity was calculated as % inhibition by the following formula:
Inhibition (%) = (A control – A test) / A control X 100.
Where;
A control = the absorbance of the control reaction.
A test = the absorbance of the pomegranate leave and peels crude juices.
Ascorbic acid was used as a reference compound.
Effective concentrations at 50% (EC_{50}) were calculated from regression equations of calibration plots (Y =98.6x +28.82, R^2=0.965 and Y=86.6x+41.27, R^2=0.966 for peels and leave juices, respectively) to denote the effective concentration of a sample required to decrease the absorbance at 517 nm by 50%.

Phenolic compounds analysis by high performance liquid chromatography (HPLC)

Phenolic compounds of pomegranate leave and peel juices were identified by HPLC system with a reversed phase column ZORBAX SB-C18 (250 x 4.6 mm i.d., 5 µm particle size (Agilent, USA) and UV detector set at 280 nm (Hewlett- Packard, Pale Alto, A). Elution was performed using mobile phase consists of water: acetic acid (98:2, v⁄v as solvent A) and methanol ⁄ acetonitril (50:50, v⁄v as solvent B), starting with 5% B increase to levels of 30 % for 25 min at a flow rate of 1.0 ml/min. Juice samples and mobile phase were filtered through 0.45 µm Millipore filter prior to HPLC analysis. Quantification of phenolic compounds was carried out at a wavelength of 280 nm using gallic acid, 3-hydroxy tyrosol, protocatchuic acid, catechin, catechol, chlorogenic acid, caffeic acid, vanillic acid, caffeine, ferulic acid, oleuropein, coumarin and quercetin. Retention time and peak area (%) were used to calculate the phenolic compound concentrations by Hewlett Packard data system. Each juice sample of pomegranate leave and peel juices was analyzed in triplicate and the mean values are presented in the text.

Statistical analysis

Statistical analysis was carried out on the total polyphenol and flavonoid contents as well as the antioxidant activity data. Three determinations of each of the measured compounds of pomegranate leave and peel juices were performed. Hence, the data are presented as means (n=3) ± standard error. Analysis of variance and least significant difference (LSD) tests were used to compare the mean values of the studied parameters of pomegranate leave and peel juices using SPSS 17.0 software. (SPSS Inc., Chicago, IL, USA). P values ≤ 0.01 were regarded as statistical significant.

RESULTS AND DISCUSSION

Several researchers have been studied the constituents and characteristics of internal sap of plant parts through extraction with different solvents of varied polarities (Tiwari *et al*., 2011, Miguel *et al*., 2004). In the present work, the internal plant sap was obtained by mechanical press without recourse to solvents. One has to point out that the pomegranate botanical parts are safe natural organs and obtained from annual pruning of pomegranate trees and are regarded as waste materials. It is well known that some solvents might possess side deleterious effects on human being organs. Therefore, the main target of the present work was to obtain the internal plant sap in its native form to study the phytochemicals as a primarily step. Further step is focused upon the constituents of pomegranate leave and peels crude juices responsible for the free-radical scavenging property. Consequently, the polyphenolic components were qualitatively and quantitatively studied by HPLC analyzer.

Qualitative phytochemical screening

The identification of phytochemicals in pomegranate peel and leave juices is a crucial starting point for assessing their nutritional, biological and technological aspects. Table 1 shows the qualitative phytochemical screenings of pomegranate leave and peel juices. Each juice was screened for the presence of key families of phytochemicals, i.e., carbohydrates, reducing sugars, glycosides, proteins, amino acids, phenolic compounds, tannins, alkaloids, flavonoids, saponins, sterols and oils. In general, there is great differences of the phytochemicals between the botanical parts (leave and peel). Pomegranate peel juice contained, carbohydrates, reducing sugars, phenolic compounds as major constituents. Proteins, amino acids, tannins and flavonoids were present in

Table 1. Qualitative phytochemical screenings of pomegranate leave and peel juices

Phytochemical test	Compound detected	Inference	
		Peel	Leave
Molisch's test	Carbohydrates	+++	++
Benedicts' test	Reducing sugars	+++	++
Keller -Kiliani's test	Glycosides	+	+
Xanthoproteic test	Proteins	++	+
Ninhydrin's test	Amino acids	++	++
Ferric chloride test	Phenolic compounds	+++	++
Ferric chloride test	Tannins	++	+
Wagner's test	Alkaloids	+	+
Lead acetate test	Flavonoids	++	+
Froth's test	Saponins	+	++
Salkowski's test	Sterols	+	+
Saponification test	Fixed oils	-	+

The notations, +++, ++, + and – refer to appreciable amounts (positive within 5 min); moderate amounts (positive after 5 min but within 10 min); trace amounts (positive after 10 min but within 15 min) and completely absent, respectively.

Table 2. Total polyphenolic and flavonoid contents and antioxidant activity of pomegranate leave and peel juices

Parameter	**Peel juice**	**Leave juice**
Total polyphenolic (TPP) (GAE mg/g dry weight)	58.63±0.129a	48.02±0.071b
Total flavonoids (TF) (Q E mg/g dry weight)	47.32±0.032a	33.02±0.009b
Antioxidant activity DPPH method(EC_{50}, µg/ml)	20.296±0.005a	3.081±0.009b

Values are means of three replicates of each parameter ± standard error.
Means within each row followed by the same letter are not significantly different at $p > 0.01$.
GAE and QE refer to gallic acid and quercetin, respectively.

pomegranate peel juice as minor components. Whilst, glycosides, alkaloids, saponins and sterols were occurred as trace substances. It is of interest to note that the pomegranate peel juice of Wonderful cultivar contained higher amounts of carbohydrates, proteins, phenolics and tannins than that of leave juice. On the other hand, pomegranate botanical parts (leave and peel) contained nearly equal quantities of glycosides, amino acids, alkaloids, sterols and fixed oils. Saponins quantity of leave juice was higher than that of peel juice. In this respect, Elfalleh *et al*., (2012) indicated that the levels of pomegranate phytochemicals differed according to the solvents used to extract these compounds. In addition, the composition of pomegranate juice depends on cultivar type, environmental, post harvest and processing factors (Houston, 2005). It is worth mentioning that the data of the present work suggest that pomegranate peel juice can be applied practically as food supplement to retard oil oxidation and to cure from certain diseases through its free-radicals scavenging property.

Total phenolics and flavonoids of pomegranate leave and peel juices

Table 2 presents the quantities of total polyphenols and flavonoids of pomegranate leave and peel juices. The data demonstrated that the levels of polyphenols and flavonoids varied according to the pomegranate botanical part. Peel juice contained higher amounts of total polyphenols and flavonoids, being about 1.22 and 1.43 times as great as that in leave juice, respectively. Similar results were obtained by Elfalleh *et al*., (2012). One has to pint out that phenolic compounds are important components since

Table 3. Retention times of the authentic polyphenols by HPLC

Retention time (min)	Phenolic compound	Peak No.
6.403	Gallic acid	1
7.381	3-hydroxy tyrosol	2
7.619	Protocatechuic acid	3
8.001	Catechin	4
8.1633	Catechol	5
8.354	Chlorogenic acid	6
9.424	Caffeic acid	7
10.372	Vanillic acid	8
10.758	Caffeine	9
11.222	Ferulic acid	10
12.333	Oleuropein	11
13.166	Coumarin	12
14.481	Quercetin	13

phenol-rich food retards the progression of arteriosclerosis and reduces the incidence of heart disease (Miguel *et al*, 2004, Houston, 2005, Gil *et al*., 2000).

Antioxidant activity

Some evidence suggests that the biological actions of the polyphenols possess antioxidant activity (Farag *et al*., 2003). In the present investigation, phytochemical screening of leave and peel juices of pomegranate revealed the presence of phenolic compounds. Hence, the present study was designed to evaluate the antioxidant activity of leave and peel juices. The free radical scavenging activity determined by DPPH was expressed as the EC_{50} value (the effective concentration of the juice required to inhibit 50% of the initial DPPH free radical). The EC_{50} values of leave and peel crude juices are shown in Table 2. Crude peel juice possessed powerful antioxidant activity than leave crude juice, being approximately 6.59 times as great as the induced by leave juice. On the contrary, Elfalleh *et al*., (2012) data indicated that the water extract of pomegranate leave exhibit higher antioxidant activity than that of peel extract. On the other hand, Singh *et al*., (2001) reported that peel is a good source of antioxidants. Furthermore, Ardekani *et al*., (2011) found that the antioxidant capacity of pomegranate peel extract was 10 times higher than the pulp extract. These results add weight to our findings.

Synthetic antioxidants BHA, BHT and gallic esters have been suspected to be carcinogenic. In addition, BHT at 200 ppm induced significant increase in the enzyme activities of rat's liver and kidney and severely altered the features of these organ tissues (Farag *et al*., 2006). Furthermore, WHO recommends the use of natural antioxidants that can delay or inhibit the lipids or other molecules by inhibiting the initiation or propagation steps of oxidative chain reaction (Velioglu *et al*., 1998). Consequently, strong limitations have been placed on the use of synthetic antioxidants and the trend nowadays is to replace them with naturally occurring antioxidants. Hence, the data of the present work suggest that pomegranate peel juice can be used adequately as food supplement to retard or prevent lipid oxidation and to cure from some diseases that induce through free radicals. It is worth noting that the effects of some phenolics are related to the increase in the activity of antioxidant enzymes (Chiang *et al*., 2006) and the induction of the synthesis of antioxidant proteins (Chung *et al*., 2006)

Qualitative and quantitative analysis of polyphenols

High- performance liquid chromatography (HPLC) was used for the identification and quantitative analysis of polyphenolic compounds of pomegranate peel and leave juices. Table 3 shows the retention times of the available authentic phenolic components and used to characterize the phenolic components in pomegranate juices under study. Tables 4 and 5 present the composition of polyphenolic compounds of juice samples of pomegranate leave and peel. The phenolics of pomegranate peel and leave juices were fractionated into 30 and 8 different components, respectively by HPLC of which 42.809 % and 50.339% were characterized. The lack of certain equipments, i.e., mass spectrometer,

Table 4. Composition (%) of the polyphenolic compounds of pomegranate leave juice.

Composition (%)	Phenolic compound	Peak No.
12.792	Gallic acid	1
37.098	Unknown	2
16.818	3-hydroxy tyrosol	3
6.454	Catechin	4
4.674	Chlorogenic acid	5
5.340	Caffeic acid	6
3.584	Coumarin	7
13.241	Unknown	8

Table 5. Composition (%) of the polyphenolic compounds of pomegranate peel juice

Composition (%)	Phenolic compound	Peak No.
14.147	Gallic acid	1
1.855	Unknown 1	2
2.618	Unknown 2	3
1.571	Unknown 3	4
1.740	Unknown 4	5
7.887	Unknown 5	6
14.512	Protocatechuic acid	7
3.311	Catechin	8
2.949	Catechol	9
2.355	Chlorogenic acid	10
1.128	Unknown 6	11
2.285	Unknown 7	12
1.326	Unknown 8	13
1.961	Unknown 9	14
2.748	Caffeic acid	15
3.088	Unknown 10	16
4.972	Unknown 11	17
3.851	Vanillic acid	18
1.888	Unknown 12	19
6.420	Caffeine	20
1.857	Ferulic acid	21
1.186	Unknown 13	22
1.536	Unknown 14	23
0.590	Oleuropein	24
1.982	Unknown 15	25
3.534	Coumarin	26
2.453	Unknown 16	27
0.949	Quercetin	28
1.404	Unknown 17	29
1.929	Unknown 18	30

prevented the complete identification the components of pomegranate peel and leave juices. In general, the composition of polyphenolic substances in leave juice was quite simpler than that of peel juice. For simplicity, the concentration levels of the polyphenolic components can be divided into 3 categories, i.e., major (>10%), minor (<10% - >1%) and trace component (<1%). Therefore, the leave juice of Wonderful cultivar contained 3-hydroxy tyrosol and gallic acid as major constituents. The quantity of the former compound was about 1.31 times as high as the second one. Catechin, chlorogenic acid, caffeic acid and coumarin were present as minor constituents. The concentration order of these substances can be arranged as follows: Catechin (6.454%)> caffeic acid (5.340%) > chlorogenic acid (4.674%) > coumarin (3.584 %).

Dealing with the crude juice of pomegranate peel, it contained gallic acid and protocatechuic as major substances. The phenolic compounds: catechin, catechol, chlorogenic acid, caffeic acid, vanillic, caffeine, ferulic acid and coumarin were present as minor constituents. Whilst, oleuropein and quercetin occurred as trace materials. Looking at the chemical phenolic constituents of peel and leave juices of pomegranate, one can deduce the following points. Gallic acid and coumarin were present nearly in equal quantities in both juices. Leave juice contained catechin, chlorogenic acid and caffeic acid about 2 times as high as that in peel juice. The following compounds: protocatechuic acid, catechol, vanillic acid, caffeine, ferulic acid, oleuropein and quercetin were present in peel juice and not in leave juice. These findings demonstrate that there were vast differences between the phenolic structures of both peel and leave juices of pomegranate plant. It appears that there is a relationship between the chemical structures of phenolic moieties in peel and leave juices of pomegranate plant and its antioxidant activity. The numbers of OH group and location at the aromatic ring have a profound effect on the antioxidant phenomenon. It is worth noting that some researches established that chlorogenic acid and flavonoids particularly quercetin and its glycoside derivatives are the main compounds responsible for the antioxidant properties (Silvia *et al*., 2011). These classes of compounds possess a broad spectrum of biological activities including radical scavenging properties (Balasnram *et al*., 2006). In general, this point needs further research to elucidate the effect of individual phenolic compound and its concentration on the antioxidant phenomenon.

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