



Phenolic Compounds and Antioxidant Activity of Dried Peel of Iranian Pomegranate

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HIGHLIGHTS

- HPLC analysis identified various phenolic compounds in Iranian pomegranate peel extract showing considerable antioxidant activities.
- The highest extraction yield was obtained from the ethanol comparing to the other solvents.
- Drying method showed no relationship with yield of extraction and antioxidant activity of pomegranate peel.

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Acronyms and abbreviations

PP=Pomegranate Peel
DPPH=1,1-Diphenyl-2-picrylhydrazyl
BHA=Butylated Hydroxyanisole
RSA=Radical Scavenging Activity
HPLC=High Performance Liquid Chromatography

ABSTRACT

Background: Literature review shows that there are not sufficient data about polyphenolic compounds of peel of Iranian pomegranate. So, this work was mainly undertaken to determine phenolic compounds and antioxidant activity of dried peel of Iranian pomegranate.

Methods: Pomegranate fruits were obtained from mature fruits grown in Saveh, Iran and the Pomegranate Peel (PP) were dried with three different methods. Powders of PP were extracted with four different solvents, using a soxhlet apparatus. The compounds of PP extracts were analyzed by High Performance Liquid Chromatography (HPLC). Then, yield percentage and Radical Scavenging Activity (RSA) were determined. Statistical analysis was performed using the SAS 9.1 software.

Results: Different ranges of tannic acids, testosterone and α -estradiol, estriol, estrone, cyanidin 3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3-glucoside, pelargonidin 3,5-diglucoside, and delphinidin 3-glucoside were identified. Both the highest yield percentage (18.820 ± 0.661) as well as the highest RSA percentage (63.862 ± 0.376) were obtained from the ethanol showing significant ($p < 0.05$) difference with the other solvents. Drying method of PP showed no significant ($p > 0.05$) relationship with yield of extraction and also antioxidant activity of the PP extracts.

Conclusion: HPLC analysis identified some various phenolic compounds in Iranian PP extract showing considerable antioxidant activities. Although drying method showed no relation with yield of extraction and also antioxidant activity of the PP extracts, but type of solvent was effective on yield of extraction and type of extracted compounds of PP.

Introduction

Pomegranate (*Punica granatum* L.) as a native fruit in Iran is one of the oldest known edible fruits and is extensively cultivated in some countries such as Iran, India,

China, Afghanistan, Japan, Russia, and USA (Cam and Hisil, 2010; Fadavi et al., 2006). Total world production of pomegranate is approximately 1500000 tons which

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47% of the total amounts are cultivated in Iran. The export of pomegranate from Iran has increased in the last years and therefore it is rational soaring demand for pomegranates and products derived therefrom (Fischer et al., 2011). There is a lot of wastage of Pomegranate Peel (PP) in pomegranate process factories. According to the recent investigations, PP involves some polyphenolic compounds, anthocyanins, tannins, etc. that may have antioxidant, antimutagenic, and anticarcinogenic activities (Guo et al., 2003; Kong et al., 2003; Kulkarni and Aradhya, 2005; Negi et al., 2003; Singh et al., 2002; Wu et al., 2004).

Literature review shows that there are not sufficient data about polyphenolic compounds of peel of Iranian pomegranate. So, this work was mainly undertaken to determine phenolic compounds and antioxidant activity of dried peel of Iranian pomegranate.

Materials and methods

Sample preparation

Twenty kg pomegranate fruits were obtained from mature fruits grown in the collection of the Agricultural Research Center of Saveh, Iran. Fruits were transferred to laboratory on the same day as they were harvested. Fruits with cracks, cuts, sunburn, and other defects in their peel were discarded and only healthy fruits of uniform size and appearance were selected. The peels were manually removed, dried with three different methods, including microwave, oven, and in shade; and then packed on the modified atmosphere. Because of prevention of change in their quality characteristics, the dried peels were powdered in the day of extraction. Milling was accomplished within 30 s in sequence of 10 s. The powder was passed from harp with mesh of 40 μm .

Chemicals

The anthocyanin standards, including pelargonidin3-glucoside, cyanidin3-glucoside, delphinidin3-glucoside, pelargonidin3,5-diglucoside as well as cyanidin3,5-diglucoside standards were purchased from Apin Chemicals (Abingdon, UK). Phytoestrogen flavonoids, including kaempferol, estrone, estriol, luteolin, testosterone, α -estradiol, stigmastrol, ellagic acid, syringic acid standards, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and Butylated Hydroxyanisole (BHA) were purchased from Sigma Aldrich (Steinheim, Germany). All standards were High Performance Liquid Chromatography (HPLC)-grade. Spect-grade ethanol and HPLC-grade ethanol, methanol, ethyl acetate, acetone, and water were obtained from Merck Chemical Company (Darmstadt, Germany). Nitrogen was from Daga Co (Tehran, Iran).

Soxhlet Extraction (SE)

Using a soxhlet apparatus, powders of PP were extracted with four different solvents, including ethanol, ethyl acetate, acetone, and mix solvent (equal mixture of ethanol, acetone, ethyl acetate, and water). The extracts were concentrated by the vacuum oven in the low temperature (40 °C) and filtered through Whatman No. 41 filter paper and centrifuged at 4500 rpm within 3 min for removal of peel particles and turbidity. Fifty percent of concentrated and centrifuged extracts were separated for HPLC analysis and evaluation of Radical Scavenging Activity (RSA), transferred into brown glass bottles, and stored in a freezer until used. The rest of extract was used for specification of extraction yield. This process was performed by removal of solvent in a vacuum oven at 90 °C until constant weights of the samples were reached.

Samples cleanup and HPLC analysis

Before injection to HPLC, extracted samples from soxhlet extraction method were cleaned up with liquid-liquid separation method. In this method, samples were centrifuged in an eppendorf tube at 3000 rpm within 4 min and organic solvent (ethanol) of centrifuged samples was removed by nitrogen flow and added two ml water to dried samples. After that, noteworthy compounds were extracted using 0.5 ml ethyl acetate. Extraction was accomplished at room temperature and the contents were transferred to organic phase. Watery phase was separated and removed from organic phase. Cleaned up samples were returned in initial volume before cleanup using a suitable solvent such as methanol.

The chromatographic analysis was carried out by a Hewlett Packard (HP) 1100 Series HPLC system (Santa Clara, California, USA) equipped with a HP auto sampler (G1313A), a HP-1200 iso pump (G1310A) and a HP UV-Vis detector. For separation of individual substances in extracted samples from PP, a variety of compositions of mobile phase was studied. Before injection, each sample was allowed to pass through a 0.45 μm PTFE filter (Chromafil CA-45/25 S, Duren, Germany). Twenty μl of prepared samples at room temperature was injected onto the HPLC. A column $\mu\text{Bondapack}^{\text{TM}}$ C₁₈ (4.6 \times 200 mm, particle diameter (dp) 10 μm) from waters was used for the separation of ellagic acid, syringic acid, anthocyanins (cy3 - pg3 - dp3 - cy3,5 and pg3,5), and six phytoestrogenic compounds (kaempferol, estrone, estriol, luteolin, testosterone and α -estradiol), and a HP-Zorbax C₈ column (4.6 \times 200 mm, dp 10 μm) was used for the separation of stigmastrol from samples. In this study, there was a different analysis conditions for various components of samples. To analyze anthocyanins, the elution was carried out using 83% water (A) as well as 17% acetonitrile (B) with phosphate buffer

(phosphoric acid and sodium hydroxide) in pH 2.5 by an isocratic run for 10 min. The flow rate was 1.0 ml/min with UV-Vis detector at 510 nm. To analyze stigmastrol, the elution was carried out using 3% A and 97% B with phosphate buffer in pH 2.5 by an isocratic run for 15 min. The flow rate was 0.8 ml/min with UV-V as detector at 208 nm. To analyze six phytoestrogenic compounds, the elution was carried out using 55% A and 45% B with phosphate buffer in pH 2.5 by an isocratic run for 12 min. The flow rate was 1.2 ml/min with UV-V as detector at 200 nm. To analyze ellagic and syringic acid, the elution was carried out using 77% A and 23% B with phosphate buffer in pH 2.5 by an isocratic run for 10 min. The flow rate was 1.2 ml/min with UV-V as detector at 280 nm. Phosphoric acid was effective in increase peak resolution in each case.

The calculation of concentrations was based on the external standard method and various compounds were identified by comparison of its retention time with those of pure standards. Linear calibration equations of standards for peel extracts are presented in Table 1. Linearity was evaluated by linear regression analysis. For each sampling point, there were three replicates. Data were recorded and processed using Agilent Chemstation A.10.01 software.

Evaluation of RSA by DPPH method

The antioxidant activity of PP extracts on DPPH radical was measured according to the method of Blois (1958) as well as Moon and Terao (1998). About 0.2 ml ethanolic solution of test sample at 100 µg/g (dry extract/dry material) concentration was mixed with 0.8 ml Tris-HCl buffer (pH 7.4) in which 1 ml DPPH (500 µM in ethanol) was added. The mixture was shaken vigorously and left to stand for 20 min. The absorbance rate of acquired solution was calculated at 517 nm in a UV-Vis spectrophotometer (CE 2502, 2000 series, Cecil co. England). All measurements were carried out in three replicates and RSA was expressed as the inhibition percentage which was measured by the following formula:

$$\%RSA = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Where $Abs_{control}$ is the initial absorbance with DPPH and Abs_{sample} is the value for added sample concentration. BHA as a positive control and ethanol was used for the baseline correction.

Equivalent density of BHA was calculated using the equation shown at Figure 1.

Statistical analysis

All analyses related to %RSA and yield of extraction were performed in triplicate. Results were expressed as means ± standard error. Descriptive statistical analysis, one-way analysis of variance (ANOVA), and Duncan test

were performed using the SAS 9.1 software and a p value of 0.05 or less was considered significant.

Results

Amount of the identified components (mg/100g dried peel) from the extracts obtained from PP dried via three methods by soxhlet method using four different solvents are shown in Table 2. Different ranges of tannic acids, testosterone and α -estradiol, estriol, estrone, cyanidin 3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3-glucoside, pelargonidin 3,5diglucoside, and delphinidin 3-glucoside were detected. Luteolin, kampferol, and stigmastrol were not detected in the extracts.

As shown in Table 3, both the highest yield percentage (18.820 ± 0.661) as well as the highest RSA percentage (63.862 ± 0.376) obtained from the ethanol showed significant ($p < 0.05$) difference with the other solvents. Also, the lowest yield as well as the lowest RSA percentages were belonged to ethyl acetate. Drying method of PP showed no significant ($p > 0.05$) relationship with yield of extraction and also antioxidant activity of the PP extracts (Table 4).

Discussion

In the current research, 11 compounds were identified in PP extracts. Comparison of organic solvents applied for extraction indicated that ethanol had the best effect on the extraction of evaluated phenolic compounds. There are some other studies that investigated amount of compounds of pomegranate fruit. Alighourchi et al. (2008) reported amounts of six anthocyanin in the pomegranate juice with the range of $0.01 \pm 0.0 - 166.32 \pm 1.94$ mg/L depend on variety of fruit and anthocyanin. Similarly, Mousavinejad et al. (2009) evaluated the total phenolic compounds in pomegranate juice with the range of $2380 \pm 0.0 - 9300 \pm 0.1$ mg/L. Similar with our finding, there are also some reports that the pomegranate seeds and peel extracts contain steroid hormones, including estrone (Dean et al., 1971; Moneam et al., 1988), estradiol (Abd El Wahab et al., 1998), and testosterone (Lau et al., 2003). However, Choi et al. (2006) were gainsaid presence of steroid hormones in pomegranate.

The results of this study revealed that among the different solvent extracts, ethanol extract provided the highest DPPH free radical scavenging activity, followed by acetone, mix, and ethyl acetate extracts. Kulkarni et al. (2004) investigated the DPPH radical scavenging activity of punicalagin isolated from pith and carpellary membrane of pomegranate and reported a 49.6% yield for methanol extract. These researchers indicated that the methanol extract (more polar solvent extract) showed the

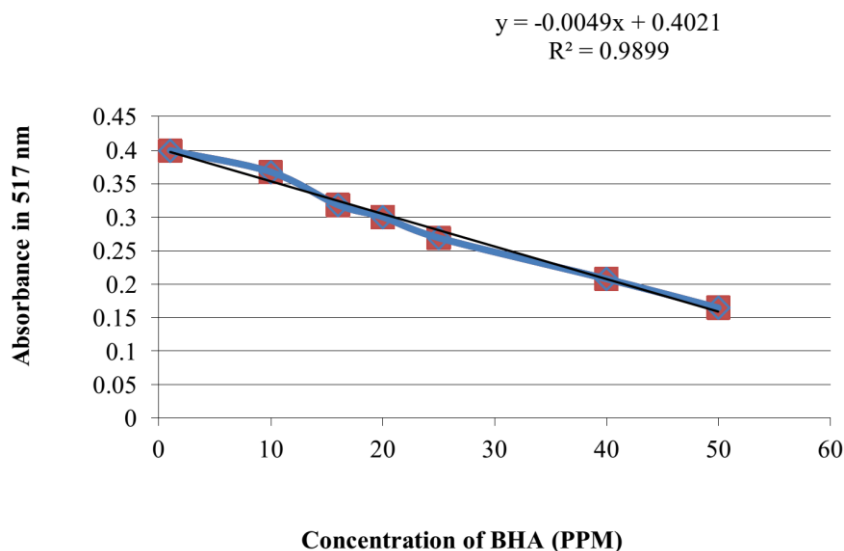


Figure 1: Absorbance of different concentrations of BHA solutions in 517 nm

Table 1: Linear calibration equations of individual compounds standards

Compound	t_R (min)	Linear range (mg/L)	Linear equation	R
cyanidin3,5-diglucoside	4.410	0.1-10.0	$A=19.66C-0.47$	0.9999
pelargonidin3,5-diglucoside	4.993	0.1-10.0	$A=19.007C-0.7$	0.9998
delphinidin 3-glucoside	5.702	0.1-10.0	$A=58.26C-0.79$	0.9999
cyanidin 3-glucoside	6.982	0.1-10.0	$A=60.45C+0.1$	1.0000
pelargonidin 3-glucoside	8.722	0.1-10.0	$A=54.36C+0.3$	0.9998
estriol	4.676	1.0-25.0	$A=70.01C-1.12$	0.9991
α -Estradiol	8.745	1.0-10.0	$A=48.96C+1.8002$	0.9991
testosterone	8.745	1.0-10.0	$A=48.96C+1.8002$	0.9991
estrone	9.670	0.3-2.0	$A=71.75C+0.91$	0.9986
syringic acid	5.918	0.5-5.0	$A=19.52C+0.52$	0.9965
ellagic acid	9.772	10.0-30.0	$A=10.86C+0.53$	0.9941

A: peak area; C: concentration (mg/L).

Table 2: Amount of the identified components (mg/100g dried peel) from the extracts obtained from PP dried via three methods by soxhlet method using four different solvents

Components	Shade dried				Oven dried				Microwave dried			
	Acetone	Ethanol	Ethyl acetate	Mix	Acetone	Ethanol	Ethyl acetate	Mix	Acetone	Ethanol	Ethyl acetate	Mix
syringic acid	0.00	1.04	0.17	0.00	0.79	0.00	0.00	0.00	0.00	1.92	0.00	2.37
ellagic acid	82.20	40.88	122.16	185.10	34.74	52.62	21.57	42.07	364.50	177.48	6.81	157.79
estriol	2.70	2.24	0.00	0.00	1.53	0.00	0.00	2.17	1.29	7.08	4.74	2.66
estrone	0.00	1.60	0.14	0.00	0.00	0.00	0.00	0.00	0.52	0.00	0.00	0.00
testosterone and α -Estradiol	3.47	3.41	0.00	0.00	2.21	0.27	0.00	4.60	2.94	3.61	1.76	5.66
cyanidin3,5-diglucoside	0.00	2.08	0.67	2.45	0.00	2.29	0.00	2.41	1.51	2.87	0.00	2.90
pelargonidin 3,5-diglucoside	0.13	0.55	0.00	0.49	0.33	0.00	0.00	0.59	2.79	3.66	1.36	0.55
delphinidin 3-glucoside	0.30	0.00	0.05	0.00	0.63	0.00	0.00	0.18	0.06	0.00	0.00	0.06
cyanidin 3-glucoside	1.74	2.98	0.27	0.00	2.07	4.35	0.00	0.48	0.46	0.69	0.00	0.42
pelargonidin 3-glucoside	2.44	2.74	0.00	0.00	2.77	3.65	0.00	0.25	1.03	0.67	0.00	0.10

Table 3: Antioxidant activity of different solvent extract (at 100µg/g concentration) of PP and their density equivalent of synthetic antioxidant BHA

Solvent	%Yield	Density equivalent of BHA	%RSA
Ethanol	18.820±0.661 ^a	51.086±0.322 ^a	63.862±0.376 ^a
Mix	15.511±0.162 ^b	49.839±0.423 ^{bc}	62.407±0.493 ^{bc}
Acetone	10.237±0.450 ^c	50.292±0.243 ^{ab}	62.937±0.283 ^{ab}
Ethyl acetate	1.190±0.086 ^d	48.864±0.565 ^c	61.270±0.659 ^c

* Values are means±standard error of 9 analysis

* Significant differences between values in the same column are indicated by different letters ($p<0.05$)**Table 4:** Antioxidant activity of extracts (at 100µg/g concentration) of PP dried via three drying method and their density equivalent of synthetic antioxidant BHA

Drying method	%Yield	Density equivalent of BHA	%RSA
Shade dried	11.271±2.166 ^a	50.514±0.412 ^a	63.194±0.480 ^a
Oven dried	11.409±1.937 ^a	49.561±0.411 ^a	62.083±0.479 ^a
Microwave dried	11.639±2.007 ^a	49.986±0.396 ^a	62.580±0.462 ^a

* Values are means±standard error of 12 analysis

* Significant differences between values in the same column are indicated by different letters ($p<0.05$)

highest %RSA (93.2%), followed by ethyl acetate extract (92.1%); and both extracts are very close to the radical scavenging ability of BHA (94.5%). Li et al. (2006) revealed that mixture of methanol, ethanol, acetone, and water was extracted the highest amount of antioxidant compounds from pulp and peel of pomegranate. Totally, some controversies are visible among the results of the previous studies which could be attributed to the differences in the analytical procedures. For example, Negi et al. (2003) announced that at 25 and 100 µg/ml concentrations, the methanol extract showed strong antioxidant capacities, whereas acetone and ethanol acetate extracts revealed strong antioxidant capacities at 50 and 75 µg/ml concentrations, respectively. In our research, it was shown that drying method had no relationship with the yield of extraction and also antioxidant activity of the PP extracts. It seems that there are probably some non-identified compounds in this study that influence the antioxidant activity of PP extracts.

Conclusion

HPLC analysis identified some various phenolic compounds in Iranian PP extract showing considerable antioxidant activities. According to the results of this research, although drying method showed no relation with the yield of extraction and also antioxidant activity of the PP extracts, but the type of solvent was effective on the yield of extraction and the type of extracted compounds of PP.

Conflicts of interest

There is not any conflict of interest.

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