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Antioxidant Activity, Total Phenols, Anthocyanin, Ascorbic Acid Content and Woody Portion Index (wpi) in Iranian Soft-Seed Pomegranate Fruits

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ABSTRACT

Twenty one pomegranate accessions collected as soft-seed genotypes from different parts of Iran and cultivated in Yazd pomegranate collection (center of Iran) were assayed for some of their nutritional traits, including antioxidant activity, phenols, anthocyanins and ascorbic acid content, and for woody portion index (wpi). Antioxidant activity ranged from 64.54 to 75.12% and ascorbic acid was variable between 16.50 to 22.66 mg/100 g fresh weight (fw) among accessions. Anthocyanin index (absorption of 25% diluted juice at 510 nm) varied between 0.83 to 1.94 while the phenol content ranged from 12.60 to 18.97 mg/100 g dry weight (dw) in the aril and from 50.73 to 103.83 mg/100 g dw in the peel. The wpi of accessions varied from 5.37 to 14.13%, lower values of this parameter implying a softer seed and easily available nutritionally valuable seed contents such as fatty acids for the consumer. This study demonstrates the beneficial nutritional properties of pomegranate as an extremely rich source of antioxidants as well as the presence of extremely soft seeded genotypes between Iranian pomegranate germplasm. Also highly soft-seeded genotypes are a valuable source for genetic improvement of commercial pomegranate cultivars.

Keywords: ANOVA, fatty acids, gallic acid, linear regression, *Punica granatum* L., titrable acidity, total soluble solids

INTRODUCTION

Pomegranate (*Punica granatum* L.) is a widely grown horticultural crop in many tropical and subtropical countries. It is one of the most tolerant fruit crops and thrives well under arid and semi-arid climatic conditions (La Rue 1980). It is one of the main fruits in Iran, and with a production of 700,000 tons/year, Iran is the world's leading producer. Historical evidence reveals that the primary origin of pomegranate is Iran and that it has been spread from this region to other areas (Levin 1996). Wild types of pomegranate grow in Northern and Western forests and other districts of Iran, and about 760 accessions, including genotypes, specimens and cultivars of pomegranate from different areas of Iran, have been identified in the Yazd pomegranate collection. Within this collection, there are 21 soft seed genotypes, but little information about them is available.

Pomegranate fruits are generally harvested when fully ripe because this fruit shows a non-climacteric respiratory pattern (Ben-Arie *et al.* 1984). Seeds are the edible portion of the pomegranate fruit, whereas the rind and the carpellary membranes are not. Pomegranate seeds, termed aril, consist of three main parts including the testa (fleshy portion), tegument (hard portion) and embryo (Melgarejo *et al.* 1996). The testa is the seed's outer layers with a fleshy or pulpy (watery) consistency and is very soft. Pomegranate is usually consumed for this edible, juicy part of the seed. Arils are rich in sugars, organic acids, anthocyanins, phenols and posses antioxidant activity (Melgarejo *et al.* 1996). This part of the seeds determines the pleasantness of pomegranate fruit, which can be evaluated by its TSS (total soluble solids), TA (titrable acidity) and TSS/TA ratio or with the help of a panel of testers, and by the color of the arils (determined by the anthocyanin content)(Melgarejo *et al.* 1996; Melgarejo and Artes 2000). The color of the aril is a sensory factor which influences consumer acceptance greatly.

Within the tegument resides an embryo with its cotyledons. The tegument is the hard part of the seed, usually with a woody consistency. It covers the embryo containing the nutritive substances for germination and initial plantlet development. Pomegranate seed contents such as fatty acids (palmitic acid ($C_{16:0}$), estearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), punicic acid ($C_{18:3}$)) (Melgarejo and Artes 2000) are useful for human nutrition. The tegument is rich in fiber, and actually determines the severity of hardness and the palatability of the seeds.

The fresh juice of pomegranate contains 85% moisture and a considerable amount of total soluble solid (TSS), total sugars, reducing sugars, anthocyanins, phenolics, ascorbic acid and proteins (El-Nemr *et al.* 1990). It is also reported to be a rich source of antioxidant compounds (Kulkarni *et al.* 2004; Kulkarni and Aradhya 2005). Phenolic and antioxidant compounds may produce their beneficial effects by scavenging free radicals. In the past few years, there has been an increasing interest in determining relevant dietary sources with antioxidant phenolics in fruits. Thus, red fruit juices such as red grape and different berry juices have received special attention due to their antioxidant activity (Pantelidis *et al.* 2007). Pomegranate juice is becoming more popular due to its relevance in important biological properties (Lansky *et al.* 1998). The antioxidant and antimicrobial activity of pomegranate bark tannins (punicacortein) and antioxidant activity of fermented pomegranate juice (Schubert *et al.* 1999) have already been reported. Pomegranate juice is a rich source of anthocyanins such as 3-glucosides and 3, 5-diglucosides of delphinidin, cyanidin and pelargonidin (Du *et al.* 1975). In addition, pomegranate bark, leaf, and the fruit husk are very rich in ellagitannins and gallotannins (Nawwar *et al.* 1994). Several kinds of apigenin and luteolin glycosides in pomegranate leaves (Nawwar *et al.* 1994), and hydrolysable tannins punicalagin

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Research Note

Table 1 The studied pomegranate genotypes, their peel and aril color, taste and seed hardness.

№	Genotypes*	Peel color	Aril color	Taste	Seed hardness
1	Bihaste Neiriz	Yellow	White	Sweet	Semi-soft
2	Bihaste Najaf Abad	Yellow	White	Sweet	Semi-soft
3	Bihaste Ladiz	Yellow	White	Sweet	Semi-soft
4	Bihaste Dane Sefide Ravar	Yellow	White	Sweet	Soft
5	Behaste Sistan va Balochestan	Yellow	White	Sweet	Semi-soft
6	Bihaste Porbar Shirin	Yellow	White	Sweet	Semi-soft
7	Shirin Bibaste Najaf Abad	Yellow	White	Sweet	Semi-soft
8	Bitolf Dane Ghermez	Red	Pink	Sweet-sour	Semi-hard
9	Bihaste Khafre Jahrom	Yellow	White	Sour	Semi-soft
10	Bihaste Sangan	Yellow	White	Sweet	Soft
11	Bihaste Shirin Khabre Baft	Red	Red	Sweet	Hard
12	Bidane Kashmar	Red	Pink	Sweet-sour	Semi-hard
13	Bihaste Ghasrodasht	Yellow	White	Sweet	Semi-soft
14	Bihaste Shirin Kambar	Yellow	White	Sweet	Semi-soft
15	Bihaste Ardestan	Red	Red	Sweet-sour	Semi-hard
16	Bitolf Dane Sefid	Red	Pink	Sour	Semi-hard
17	Bihaste Shirin Saravan	Yellow	White	Sweet	Soft
18	Bidane Darjazin	Yellow	White	Sweet	Semi-soft
19	Bihaste Chenche	Yellow	White	Sweet	Semi-soft
20	Bihaste Dane Ghermez Kerman	Yellow	White	Sweet	Semi-hard
21	Bihaste Hajiabad	Yellow	White	Sweet	Soft

* Bihaste, Bidane and Bitolf all meaning soft-seeded in Persian language (Farsi) for pomegranate genotypes, according to the names at the original growing places given to them.

and punicalin in pomegranate husk have been previously identified (Tanaka *et al.* 1986). These compounds are all active antioxidant compounds, and due to these properties of pomegranate different parts, it has been widely used in traditional Iranian medicine.

To the best of our knowledge, this is the first evaluation of the antioxidant capacity, phenol, anthocyanin, ascorbic acid contents and woody portion index of some Iranian soft-seeded pomegranates.

MATERIALS AND METHODS

Plant material

The fruit samples of 21 pomegranate accessions registered as soft-seeded genotypes (**Table 1**) were collected from mature trees growing in the pomegranate collection at Agricultural Research Center of Yazd, a city in central Iran. Three trees per accession as replications and five fruit samples per replication, altogether 15 mature fruits for each accession, were evaluated.

Ascorbic acid content

Ascorbic acid was measured according to the protocol of Redox titration using an iodine solution (www.outreach.canterbury.ac.nz, University of Canterbury, Christchurch, New Zealand). Vitamin C concentration was determined by titration (redox titration using iodate solution), a method that determines the vitamin C concentration in a solution by a redox titration with potassium iodate in the presence of potassium iodide. When iodate ions (IO_3^-) are added to an acidic solution containing iodide ions (I^-), an oxidation-reduction reaction occurs and the iodate ions are reduced to form iodine:

$IO_3^- + 6\ H^+ + 5\ e^- \rightarrow ½\ I_2 + 3\ H_2O$

while the iodide ions are oxidised to form iodine.

$2\ I^- \rightarrow I_2 + 2\ e^-$

Combining these half-equations demonstrates the reaction between iodate and iodide:

$2\ IO_3^- + 10\ I^- + 12\ H^+ \rightarrow 6\ I_2 + 6\ H_2O$

It is the iodine formed by this reaction that oxidises the ascorbic acid to dehydroascorbic acid as the iodine is reduced to iodide ions:

Ascorbic acid + $I_2 \rightarrow 2\ I^-$ + dehydroascorbic acid

Due to this reaction the iodine formed is immediately reduced to iodide as long as there is any ascorbic acid present. Once all the ascorbic acid has been oxidised, the excess iodine is free to react with the starch indicator, forming the blue-black starch-iodine complex. This is the endpoint of the titration. This method is more straightforward than the alternative method using potassium iodide.

Total phenol content of aril and peel

Aril and peel of samples were air-dried at 55°C and homogenized. Dry samples (1 g) were transferred to test tubes containing 10 ml of extraction solution (50% methanol/H_2O) according to Vinson *et al.* (2001) and the mixture was kept in the dark at 4°C for 24 h. The supernatant was collected and replaced with an equal quantity of extraction solution, then placed in the dark at 4°C for a further 48 h. The two supernatants were mixed and the volume was fixed at 25 ml by adding extraction solution and used for determination of phenol content.

The amount of total phenolic compounds was determined according to the procedure of Folin-Ciocalteu (Singleton and Rossi 1965). Briefly, 0.05 ml of extract and 0.45 ml water were added to 2.5 ml of 1:10 diluted Folin-Ciocalteu's phenol reagent, followed by the addition of 2 ml of 7.5% (w/v) sodium carbonate. After 5 min incubation at 50°C, absorbance was measured at 760 nm. Phenol content was estimated from a standard curve of gallic acid and results expressed as mg gallic acid equivalent (GAE)/100 g dry weight.

Antioxidant activity

Antioxidant activity of pomegranate juice (0.1 ml) was determined by the 2, 2-diphenylpicrylhydrazyl (DPPH) method described by Moon and Terao (1998). Fresh pomegranate juice (0.1 ml) was mixed with 0.9 ml of 100 mM Tris-HCl buffer (pH = 7.4) then 1 ml of DPPH (500 µM in ethanol) was added. The mixture was shaken gently and left for 30 min. Absorbance of the final solution was measured at 517 nm by a UV-Visible spectrophotometer (Perkin Elmer, Lambda EZ201, USA). The reaction mixture without DPPH was used as a background correction. The antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity (\%)} = (1 - A_{\text{sample (517 nm)}} / A_{\text{control (517 nm)}}) \times 100.$$

Anthocyanin index

For evaluating anthocyanin concentration, 1 ml of fruit juice was diluted in 3 ml distilled water, and the absorbance of the diluted solution was measured at 510 nm by spectrophotometer. The absorbance of diluted juice was used as the index for anthocyanin concentration.

Woody portion index (wpi)

A sample of 25 fresh seeds (fleshy portion removed) were taken at random, individually weighed (Ws) and also their length and width were recorded. The testa then was opened and the remaining interior parts (the embryo) and testa were weighed (woody part, Wwp). The woody portion index was defined as Wpi = (Wwp/Ws) ×100 (Melgarejo 1997), is related to seed hardiness and is useful for measuring palatability of arils.

Statistical analysis

At least three sets were run for each cultivar for measuring ascorbic acid, anthocyanin content, phenol and antioxidant activity. Each set consisted of three replicates (with three samples) and means over the three samples were used for ANOVA. Data run for analysis of variance, followed by means comparison using the Duncan's Multiple Range Test (DMRT) at significant level of 1 or 5% ($P \leq 0.01$ or 0.05). Data in percentage form were subjected to Arc Sin transformation prior to statistical analysis. Liner regression used for regression analyses by Microsoft office (Excel, Ver. 2003). Analysis of variance for all traits was performed with SAS software.

RESULTS AND DISCUSSION

Ascorbic acid content

Means comparison for ascorbic acid content of juice divided accessions into 4 groups (1: a, 2: b, bc, 3: c, cd, 4: d) (**Table 2**). The range of ascorbic acid was between 22.66 to 16.50 mg/100 g fw among genotypes. Genotype No. 5 had the highest ascorbic acid content. The loss of ascorbic acid has been reported during the development of fruits (Kulkarni and Aradhya 2005). Arils of pomegranate are reported to have a similar trend with rapid depletion in the ascorbic acid content during fruit development. Kulkarni and Aradhya (2005) reported the highest (36 mg/100 g) and lowest (15 mg/100 g) ascorbic acid content during different stages of fruit development in pomegranate. Compared to many fruits, pomegranate is relatively rich in ascorbic acid content (e.g. Pantelidis *et al.* 2007). Simple linear regression between ascorbic acid and antioxidant activity was $R^2 = 0.54$. Also a highly significant correlation was observed between ascorbic acid and phenol content of arils (r = 0.59) and with antioxidant activity (r = 0.74) (**Table 4**).

Antioxidant activity

Antioxidant activity of pomegranate juice was measured in terms of its radical scavenging potential. DPPH is a stable free radical and the assay can accommodate a large number of samples in a short period of time. It is also sensitive enough to detect active principles at low concentrations (Kulkarni *et al.* 2004; Kulkarni and Aradhya 2005). The pomegranate arils showed significant antioxidant activity. The highest and lowest recorded antioxidant activity was 92.1 and 64.54% in genotypes No. 15 and 19, respectively (**Table 2**). Anthocyanin, ascorbic acid and phenolic acids, either alone or in combination, are responsible for antioxidant activity of pomegranate arils (Iannoccari *et al.* 2004). The major types of phenolic acids reported in pomegranate fruit juice include punicalagin, punicalin, gallagic acid, ellagic acid and gallic acids (Kulkarni *et al.* 2004). The antioxidant activity of pomegranate fruit juice is even higher than wine and tea drinks (nearly three times) and it is also suggested that the industrial process to extract juice of pomegranate increased the antioxidants content or enhanced their activity (Poyrazoglu *et al.* 2002).

Anthocyanin

Significant differences in anthocyanin indexes (content) responsible for red color of fruit juice were recorded among accessions (**Table 2**). Genotype No. 20 contained the highest anthocyanin absorbance ($O.D._{510} = 1.94$) whereas genotype No. 12 showed the lowest absorbance ($O.D._{510} = 0.83$). Similar results for anthocyanin content were reported by other researchers (Kulkarni *et al.* 2004; Kulkarni and Aradhya 2005).

Total phenol content of aril and peel

Quantities of phenols expressed as gallic acid equivalents (GAE) in peel were between 50.73-103.83 mg/100 g dw, higher than in the aril (12.60-18.97 mg/100 g dw) (**Table 3**). A relatively high amount of phenolic content was recorded in the aril and it was highest in genotype No. 15 and lowest in genotype No. 19 (**Table 3**). Simple linear regression between phenol content of aril and antioxidant activity is shown in **Fig. 1** with a high correlation (r = 0.86) (**Table 4**). Total phenolic content from days 20 to 140 of pomegranate fruit development have been reported to decrease by about 75% (Kulkarni *et al.* 2004; Kulkarni and Aradhya 2005). Decreases in phenolic content in some pomegranate genotypes might coincide with an increase in anthocyanin pig-

Table 2 Means comparison of ascorbic acid content (mg/ 100 g fw), antioxidant activity (%) and anthocyanin index of the examined genotypes.

Genotype №	Ascorbic acid content (mg/100 g fw)	Genotype №	Antioxidant activity (%)	Genotype №	Anthocyanin index ($O.D._{510}$)
5	22.66 ± 0.1436 a	15	75.120 ± 0.1819 a	12	1.940 ± 0.0850 a
4	22.48 ± 0.1650 a	7	74.776 ± 0.1848 a	11	1.656 ± 0.1568 b
18	21.88 ± 0.1955 b	6	73.046 ± 0.1417 b	1	1.453 ± 0.1117 bc
15	21.84 ± 0.2084 b	3	72.573 ± 0.2426 bc	19	1.450 ± 0.1350 bc
6	21.64 ± 0.1313 b	1	72.376 ± 0.2325 cd	14	1.416 ± 0.0888 bcd
9	21.64 ± 0.1940 b	18	72.343 ± 0.2536 cd	6	1.410 ± 0.1249 bcd
21	21.22 ± 0.1834 b	21	72.106 ± 0.1707 cd	15	1.410 ± 0.1480 bcd
7	21.16 ± 0.1651 b	2	72.040 ± 0.1343 cd	2	1.406 ± 0.1122 bcd
1	20.92 ± 0.1320 b	4	71.920 ± 0.1484 d	7	1.223 ± 0.1146 cde
12	20.88 ± 0.2088 b	12	71.800 ± 0.1361 d	3	1.190 ± 0.1155 cdef
2	20.76 ± 0.1819bc	9	71.116 ± 0.1618 e	16	1.156 ± 0.0717 def
10	19.4 ± 0.1991 bc	20	70.943 ± 0.1521 e	18	1.103 ± 0.0617 efg
3	19.16 ± 0.1518 c	5	70.676 ± 0.2171 e	4	1.050 ± 0.1301 fg
13	18.68 ± 0.1633 c	16	69.963 ± 0.1978 f	17	0.996 ± 0.0884 fg
20	18.64 ± 0.1418 c	17	69.903 ± 0.1690 f	21	0.996 ± 0.0623 fg
8	18.4 ± 0.1359 c	10	69.896 ± 0.2000f	9	0.983 ± 0.0684 fg
14	18.36 ± 0.0994 c	14	69.790 ± 0.1778 f	13	0.963 ± 0.0570 fg
16	17.84 ± 0.1433 cd	11	68.410 ± 0.2517 g	8	0.916 ± 0.0463 fg
17	16.85 ± 0.2224 d	13	68.160 ± 0.2307 g	10	0.913 ± 0.0491 fg
11	16.64 ± 0.1899 d	8	67.390 ± 0.2194 h	5	0.883 ± 0.0731 g
19	16.5 ± 0.1899 d	19	64.546 ± 0.2128 i	20	0.830 ± 0.0802 h

* Data are means of three replications ± standard error. Values within each column followed by the same letter are not significantly different at $p \leq 0.05$ (DMRT).

Table 3 Means comparison of total phenol content of aril and peel (mg/ 100 g dw) and Wpi (%) of the examined genotypes.

Genotype №	Phenol content of aril (mg/100 g dw)	Genotype №	Phenol content of peel (mg/100 g dw)	Genotype №	Wpi (%)
15	18.971 ± 0.1729 a	3	103.830 ± 0.1852 a	11	14.136 ± 0.1359 a
7	18.623 ± 0.2241 a	1	97.486 ± 0.1924 b	15	9.130 ± 0.1308 b
6	17.640 ± 0.2438 b	17	92.960 ± 0.1704 c	16	9.016 ± 0.1598 b
3	17.603 ± 0.1997 b	18	90.376 ± 0.1981 d	2	8.910 ± 0.1617 b
1	17.416 ± 0.2167 b	14	87.980 ± 0.1808 e	8	8.766 ± 0.1601 bc
2	16.796 ± 0.2018 b	5	81.730 ± 0.2380 f	12	8.406 ± 0.1568 cd
9	15.776 ± 0.1867 c	2	79.730 ± 0.1890 g	18	8.070 ± 0.1480 de
12	15.576 ± 0.1963 c	10	78.920 ± 0.1709 h	3	7.696 ± 0.1617 ef
18	15.426 ± 0.1627 c	19	77.563 ± 0.2179 i	20	7.683 ± 0.1934 ef
21	15.223 ± 0.1737 cd	13	76.470 ± 0.2318 j	1	7.436 ± 0.1968 f
20	14.920 ± 0.1550 cd	11	75.430 ± 0.2219 k	5	6.806 ± 0.1891 g
4	14.733 ± 0.1560 cd	6	74.933 ± 0.1910 k	19	6.720 ± 0.2030 hg
5	14.523 ± 0.2234 cd	8	70.866 ± 0.1220 l	14	6.626 ± 0.1856 hg
8	14.423 ± 0.1841 d	12	64.866 ± 0.1613 m	6	6.540 ± 0.1721 hg
14	14.320 ± 0.0981 d	21	63.776 ± 0.1633 n	7	6.363 ± 0.1738 hg
16	14.253 ± 0.1646 d	9	63.736 ± 0.2534 n	9	6.286 ± 0.2126 hg
11	14.246 ± 0.1965 d	4	63.606 ± 0.1794 n	10	6.230 ± 0.1779 h
13	14.166 ± 0.1568 de	15	56.223 ± 0.1749 o	17	5.730 ± 0.0850 i
10	14.140 ± 0.2466 de	20	55.953 ± 0.1332 op	13	5.566 ± 0.1798 i
17	13.323 ± 0.1425 e	7	55.540 ± 0.2113 p	21	5.480 ± 0.1950 i
19	12.600 ± 0.1332 e	16	50.736 ± 0.2291 q	4	5.376 ± 0.1819 i

* Data are means of three replication ± standard error. Values within each column followed by the same letter are not significantly different at p≤ 0.05 (DMRT).

Table 4 Simple correlations among anthocyanin index, ascorbic acid content, phenol content of aril, phenol content of peel and antioxidant activity of pomegranate accessions.

Character	Anthocyanin index	Ascorbic acid content	Phenol content of aril	Phenol content of peel	Antioxidant activity
Anthocyanin index	1				
Ascorbic acid content	-0.11	1			
Phenol content of aril	0.24	0.59*	1		
phenol content of peel	0.06	-0.15	-0.08	1	
Antioxidant activity	0.07	0.74**	0.86**	-0.15	1

* Significance level (0.05)
** Significance level (0.01)

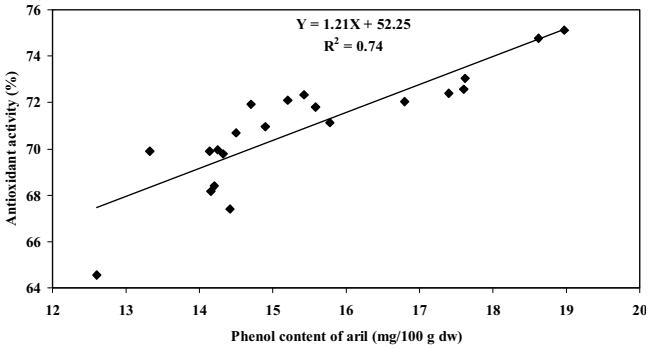

Fig. 1 Simple linear regression between phenol content mg/100 g dw of aril and antioxidant activity (%) recorded in 21 pomegranate accessions.

ment content due to the contribution in the biosynthesis of the flavylium ring of anthocyanin (Pantelidis *et al.* 2007). However, there was no significant correlation between anthocyanin and the phenol content of aril (**Table 4**). The major phenolic compounds in the peel and aril of pomegranate have been reported to be gallic acid and quercetin, respectively (Kulkarni and Aradhya 2005; Pantelidis *et al.* 2007).

Woody portion index (wpi)

The wpi varied greatly between accessions ranging from 5.37% in accession No. 18 to 14.13% in accession No. 11 (**Table 3**). Based on a panel test (we used about 20 student of horticulture science for measuring this character), 21 pomegranate accessions were divided into four groups: soft-seed, semi-soft seed, semi-hard seed and hard-seed (**Table 1**). Four accessions (Bihaste Dane Sefide Ravar, Bihaste Sangan, Bihaste Shirin Saravan, Bihaste Hajiabad) were determined as soft-seeded, one accession (Bihaste Shirin Khabre Baft) as hard-seeded, and the remaining accessions were classified as semi-soft or semi-hard seeded ones by panel test. High simple linear regression (R^2 = 0.71) observed between woody portion index and seed hardness was determined by the panel test. In some Spanish pomegranate genotypes the ranges of wpi varied between 6.145 to 9.68% (Martinez *et al.* 2006). This study represented even lower wpi genotypes in Iranian pomegranates. Six of 21 accessions were hard or semi-hard seed, indicating mislabeling or the effect of environmental conditions on seed hardness. There are some fatty acid compounds in pomegranate seed oil with useful effects on treating prostate, breast and skin cancer (Justin *et al.* 2003). Hence a less wpi is important because of better digestion of seeds and higher availability of the interior contents for human nutrition.

CONCLUSION

In this study, 21 pomegranate accessions were analyzed for their antioxidant activity, phenol, anthocyanin and ascorbic acid contents and seed hardness. Antioxidant activity varied among the studied pomegranate accessions and was high in all of them. The present study indicated that pomegranate is an extremely rich source of phenolic compounds, anthocyanin and antioxidants, demonstrating its potential use as a fresh fruit or food additive. Seed hardness, a determinant for consumer acception or rejection, is another important trait in pomegranate. So, the wpi which is useful in measuring the relationship between the woody portion and the whole seed, can reflect the hardness of seeds and based on results some accessions were highly soft-seeded.

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