

Antioxidant Capacity of Some Turkish Pomegranate Genotypes Grown in Siirt Region

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ABSTRACT: Investigated antioxidant levels of 30 pomegranate genotypes via FRAP, DPPH, iron-chelating methods in this study. Pomegranate genotypes were collected from the several orchards located in Sarıdana, Kapılı, Pirinçli and Zivzik villages of Şirvan district, at Siirt province in Southeastern Anatolia of Turkey and were numbered from 1 to 30. 26 genotypes studied in the study are Zivzik pomegranate clones. Four of 30 genotypes, which have different fruit characteristics from Zivzik variety. These four genotypes are called as Asili (Genotype 21), sour pomegranate (Genotype 22 and 23) and red pomegranate (Genotype 24). The difference in antioxidant levels of genotypes, determined by FRAP method were assessed using one-way analysis of variance. The results of ANOVA indicated a statistically significant difference in absorbance values between the genotypes ($p < 0.001$). Therefore, the genotypes were grouped based on absorbance values by Tukey's multiple comparison test. Test results showed that genotypes 30, 16, 22 and 29 had the highest antioxidant level, the mean antioxidant levels of 13, 23, 28, 18, 14, 9, 6, 12, 2, 4, 5, 3, 17, 27 and 11 genotypes were similar, and genotypes 15, 1 and 7 had the lowest antioxidant levels.

Keywords: Pomegranate, *Punica granatum* L., antioxidant, FRAP, DPPH, iron chelating.

1. INTRODUCTION

Pomegranate (*Punica granatum* L.), a symbol of life and health throughout human history, is a perennial plant belonging to the genus *Punica* of the *Lythraceae* family (Ashton et al., 2006). Cultural history dates back to BC 3000 years and is among the oldest known fruit species. Since it has an important place in terms of human health benefits and industrial value (Viudo-Martos et al., 2010; Mohammad and Kashani, 2012) Turkey and the World production have increased over time. Today the World pomegranate production is about 6 million tons. Turkey has made 465 thousand tons of pomegranate production in 2016. 179.920 tons of this was exported as fresh pomegranate. Although pomegranate cultivation is carried out in almost every region of our country, the most intensive production areas are Mediterranean, Aegean and Southeastern Anatolia regions which have suitable ecological conditions for growing (Yılmaz, 2017).

Components that inhibit delay oxidation processes caused by free radicals in alive cells or foods are defined as antioxidant substances (Kris-Etherton et al., 2002; Fernandez-Pancho et al., 2008). Synthetic antioxidants have been used as food additives for many years in order to extend the storage times of foodstuffs. However, the use of these products in terms of toxic and carcinogenic effects may be restricted or prohibited (Decker et al., 2010). In recent years, studies on obtaining plant based, cheap, edible and healthy antioxidant and antimicrobial compounds have been increased (Dimitrios, 2006; Movilenau et al., 2013; Koolen et al., 2013). Pomegranate is also rich fruit in phenolic compounds that make up 92% of antioxidant activity such as flavanoids (anthocyanins, catechins and other complex flavanoids), hydrolyzable tannins (punicalin, pedunculgin, punicalagin, gallic and ellagic acid esters of glucose), polyphenols, fatty acids (conjugated and non-conjugated), aromatic compounds, amino acids, tocopherols, sterols, terpenoids, alkaloids. (Syed et al., 2007; Wang et al., 2010; Prakash and Prakash, 2011). Several factors may have significant impacts on antioxidant content of plants in addition to the geographical location, climatic conditions and soil characteristics.

Zivzik pomegranate is a variety registered in 2008 by the Pistachio Research Institute located in Gaziantep of Turkey. While Siirt region has a continental climate, Zivzik pomegranate is grown in the region where micro climate is seen in the foothills of Botan River Valley in Siirt. There are covered orchards in this region where commercial breeding is carried out. This variety is an important source of livelihood for the

regional producer. Zivzik pomegranate is a variety with medium size fruits, high in taste-aroma and fruit juice, and suitable for storage about 6 months. This study was carried out to investigate the antioxidant levels of 30 Turkish pomegranate genotypes via FRAP, DPPH, iron chelating methods.

II. MATERIALS AND METHODS

2.1. Pomegranate Genotypes

Pomegranate genotypes belonging to Zivzik pomegranate variety were collected from the several orchards located in Sarıdana, Kapılı, Pirinçli and Zivzik villages of Şirvan district, at Siirt province in Southeastern Anatolia of Turkey. The genotypes studied were known with their superior yield and quality characteristics and were numbered from 1 to 30. Four of 30 genotypes, which have different fruit characteristics from Zivzik variety, were included in the study. These four genotypes are called as Asili (Genotype 21), sour pomegranate (Genotype 22 and 23) and red pomegranate (Genotype 24). Other 26 genotypes are Zivzik pomegranate clones. Leaf samples of these 30 pomegranate genotypes were collected in spring and samples were stored at -20 °C until biochemical analyses performed.

2.2. Extraction Methods

Fifty ml of methanol (80%) was added to 1 g of shredded leaf sample and extracted in the dark at room temperature with an orbital shaker for 2 hours. The mixture was filtered through Whatman No.1 filter paper and then dried under vacuum at 40 °C using a clear filtrate rotary evaporator. Dried crude extract was weighed to calculate the extraction yield. Stock solutions were then prepared to prepare 1 mg.L⁻¹ methanol for each pomegranate genotype.

2.2.1. FRAP - The ferric reducing ability of plasma

FRAP method was introduced to determine the total amount of antioxidants by the reduction capacity of iron (III) (Benzie and Strain, 1996). The oxidant in the FRAP assay was prepared by mixing 2.5 mL TPTZ dissolved in 10 mM 40 mM HCl, 25 mL acetate buffer, 20 mM 2.5 mL FeCl₃ and water. This mixture is called as FRAP reagent. The final solution contained 1.67 mM Fe (III) and 0.83 mM TPTZ. The FRAP results may differ greatly depending on the time of the analysis. Rapid reacting polyphenols are determined at a short period like 4 minutes. However, some polyphenols react relatively slow and require a longer time to complete the assessment (from 30 minutes to several hours). This method is suitable to determine hydrophilic and lipophilic antioxidants. The most valuable advantages of the FRAP method are simplicity, fastness, low cost and reliability. The method does not require special equipment and can be performed by automatic, semi-automatic and manual means (Prior et al., 2005).

2.2.2. DPPH - Free Radical Scavenging Activity

DPPH radical scavenging activities of plant extracts were determined using the method described by Blois (1958). For this test, a volume of 1 mL various solutions of 0.1 mM 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was added to 3 ml of samples containing different concentrations of plant extracts. After preparation of mixtures, tubes were rapidly shaken and allowed to incubate in the dark and at room temperature for 30 minutes. Absorbances were measured at 517 nm. A volume of 3 ml of ethyl alcohol was added on 1 mL of DPPH and used as a blank. The DPPH solution was used for the control. The principle of the experiment is as follows: the prepared DPPH solution produces a dark purple color at maximum absorbance of 517 nm. The dark purple color of the solution is lost over time by the addition of a solution containing antioxidant or substances to the DPPH solution. This is the indication of scavenging the DPPH radical by antioxidants. This process occurs either by removing a hydrogen atom or transferring electron. Thus, the solution become colorless and contains bleached molecules (2,2-diphenyl-1-hydrazine or different analogs of hydrazine), which leads to a decrease in the absorbance value at 517 nm. The fastest decrease in absorbance value is an indication of the best antioxidant potential. The DPPH free radical scavenging percentage was calculated as follows (Eq. 1):

$$\text{DPPH Activity (Incubation \%)} = ((A_c - A_1)/A_c) \times 100 \quad (1)$$

Where, A_c is the absorbance of the control and A_1 is the absorbance of the sample.

2.2.3. Iron Chelating Activity

Iron-chelating activities of plant extracts were performed using the method described by Dinis et al. (1994). A volume of 1 ml plant sample extracts at varying concentrations (2, 5 and 10 mg.mL⁻¹) was added to 0.05 mL 2 mM FeCl₂ solution. The reaction was initiated by adding 0.2 mL 5 mM ferrozine (C₂₀H₁₃N₄NaO₆S₂). After completing the total volume to 5 ml with the solvent used, the solution was stirred vigorously and kept at room temperature for 10 minutes. Absorbance values of solutions were recorded at 562 nm. For the blank

solution, solvent was added to 0.05 mL FeCl₂ to a total volume of 4 mL. The same procedures were applied to the standards (BHT and Trolox).

The measurements were performed at 562 nm by a spectrophotometer. The control solution was prepared by adding 50 µl FeCl₂ (2 mM) and 3.75 mL solvent to 200 µl ferrozine (5 mM) without the plant extract. Absorbance was spectrophotometrically measured at 562 nm. The principle of the analysis is as follows: ferrozine, an indicator of iron ions, complexes with iron ions, causing the solution to become magenta and this solution gives the maximum absorbance at 562 nm. Activities of antioxidant agents in the plant extracts are based on the inhibition of ferrozine-Fe²⁺ complex formation by binding iron ions or binding iron ions from the complexes and progressively bleaching of color yielded the maximum absorbance at 562 nm and gradual decreasing of the absorbance value. The lowest absorbance value indicates the highest binding of iron ions. The percentages of iron-chelating ions were calculated formulas follow (Eq. 2):

$$\text{Chelating Activity, \%} = [1 - (A_1/A_c)] \times 100 \quad (2)$$

Where, A_c is the absorbance of the control and A₁ is the absorbance of the sample.

2.3. Statistical Analysis

The difference between the antioxidant levels of genotypes determined by the FRAP method was assessed by one-way analysis of variance (ANOVA). The ANOVA analysis indicated a statistically significant (p<0.001) difference between the genotypes. Therefore, Tukey's multiple comparison test was applied (α= 0.05) to identify the genotypes with similar antioxidant levels. In addition, Orthogonal Fragmentation Method (Orthogonal Comparison) was used to determine which antioxidant contents of genotypes were significantly different.

III. RESULTS AND DISCUSSIONS

Antioxidant capacity of Zivzik pomegranate genotypes determined by FRAP, DPPH and Iron Chelating methods were given in Table 1-3.

3.1. FRAP Analysis

The FRAP method was used to determine the antioxidant level in the tissue by iron reduction technique. Trolox and BHT are the synthetic antioxidant solutions. Antioxidant levels, higher than that of the stock solutions show the naturally antioxidant genotypes. The antioxidant levels obtained by FRAP technique were not higher than the stock solutions. The highest antioxidant level (1.22±0.05) was recorded in genotype 16 and followed by genotype 30 (1.20±0.01). Genotype 15 had the lowest antioxidant level (0.50±0.05) (Table 1).

Table 1. Antioxidant levels determined by FRAP analysis (concentration 10 µg.mL⁻¹)

Sample No	Mean	Sample No	Mean	Sample No	Mean
Control	0.35±0.01	9	1.10±0.01	20	0.91±0.01
Trolox	1.74±0.39	10	0.83±0.06	21	0.91±0.01
BHT	1.24±0.02	11	0.95±0.03	22	1.16±0.02
1	0.67±0.02	12	1.04±0.01	23	1.04±0.16
2	1.09±0.13	13	1.13±0.09	24	0.87±0.00
3	1.01±0.04	14	1.10±0.01	25	0.87±0.02
4	1.00±0.09	15	0.50±0.05	26	0.94±0.00
5	1.04±0.03	16	1.22±0.05	27	0.98±0.02
6	1.08±0.03	17	0.99±0.01	28	1.08±0.06
7	0.75±0.01	18	1.12±0.04	29	1.15±0.01
8	0.86±0.01	19	0.92±0.02	30	1.20±0.01

3.2. DPPH Analysis

Antioxidant contents of 30 genotypes according to DPPH analysis were given in Table 2. DPPH scavenging activity is mainly based on the principle that protons given by antioxidants are captured and reduced by the DPPH radicals. The results are expressed in the trolox equivalent (mg.mL⁻¹) used as standard. The highest antioxidant levels determined by DPPH (inhibition %) method were obtained in genotypes 22 (43.22) and 6 (42.04). While the lowest value was recorded in genotype 15 (13.55) (Table 2).

Table 2. Inhibition percentage determined by DPPH analysis (concentration 25 µg.mL⁻¹)

Sample No	Inhibition %	Sample No	Inhibition %	Sample No	Inhibition %
Trolox	69.80	10	26.48	21	28.91

BHT	51.20	11	30.85	22	43.22
1	19.46	12	34.53	23	37.01
2	34.90	13	31.11	24	28.91
3	37.58	14	16.39	25	25.53
4	36.23	15	13.55	26	30.30
5	39.17	16	35.47	27	33.92
6	42.04	17	32.81	28	35.49
7	24.41	18	31.69	29	37.50
8	24.93	19	28.48	30	38.44
9	39.36	20	22.53	--	--

3.3. Iron-Chelation Analysis

The iron-chelating analysis was used to determine the antioxidant levels of genotypes investigated. The antioxidant levels of genotypes obtained were generally higher than the stock solutions. The results show that genotypes with high values have natural antioxidants. Genotype 26 had the highest level of antioxidant content (6.74 ± 0.80) followed by genotype 11 (6.49 ± 0.36), whereas the genotype 24 had the lowest antioxidant level (1.26 ± 0.32) (Table 3).

Table 3. The results of iron chelating analysis (concentration $164 \mu\text{g.mL}^{-1}$)

Sample No	Mean	Sample No	Mean	Sample No	Mean
Trolox	1.86 ± 0.78	10	3.63 ± 0.64	21	1.52 ± 0.22
BHT	3.50 ± 0.78	11	6.49 ± 0.36	22	2.81 ± 1.95
1	3.69 ± 1.11	12	3.16 ± 0.20	23	1.62 ± 0.13
2	3.41 ± 0.59	13	3.69 ± 0.89	24	1.26 ± 0.32
3	5.62 ± 2.40	14	4.15 ± 3.30	25	5.16 ± 1.93
4	3.14 ± 1.17	15	5.50 ± 1.07	26	6.74 ± 0.80
5	2.03 ± 0.94	16	3.75 ± 0.66	27	3.29 ± 0.29
6	2.53 ± 0.82	17	2.39 ± 0.34	28	3.52 ± 1.26
7	5.29 ± 1.97	18	4.41 ± 0.68	29	3.57 ± 1.42
8	2.38 ± 0.79	19	2.79 ± 0.04	30	1.44 ± 0.43
9	3.15 ± 0.62	20	2.61 ± 0.39	-	-

3.4. Statistical Evaluation of the Results

The difference in antioxidant levels of genotypes, determined by FRAP method were assessed using one-way analysis of variance (ANOVA). The results of ANOVA indicated a statistically significant difference in absorbance values between the genotypes ($p < 0.001$). Therefore, the genotypes were grouped based on absorbance values by Tukey's multiple comparison test ($\alpha = 0.05$) (Table 4). The results confirmed a significant difference between the antioxidant contents of the genotypes determined by three different methods. The Tukey's test showed that genotypes having the highest antioxidant content were genotypes 30, 16, 22 and 29, respectively. The mean antioxidant levels of genotypes 13, 23, 28, 18, 14, 9, 6, 12, 2, 4, 5, 3, 17, 27, and 11 were not significantly different from each other and placed in the same group. Genotypes 15, 1 and 7, were grouped in the lowest antioxidant level.

Orthogonal Fragmentation Method was applied for genotypes 21, 22, 23 and 24, which differed from other 26 genotypes in terms of fruit characteristics. Orthogonal Fragmentation Method is used to determine which of the mean differs significantly. The F test statistics and related p significance levels of orthogonal fragmentation method indicated that the difference between genotype 21 (Asili) and others was significant ($p < 0.01$). Statistically significant difference was not obtained ($p > 0.05$) between the genotypes 22, 23, and 24 compared with the other genotypes.

Table 4. The results of Tukey's multiple comparison test

Sample Name	Result	Sample Name	Result	Sample Name	Result	Sample Name	Result	Sample Name	Result
Trolox	A	23	B-G	2	B-H	26	C-J	10	H-J
BHT	B	28	B-G	4	B-H	19	D-J	7	I-K
30	BC	18	B-G	5	B-H	21	E-J	1	J-K
16	B-D	14	B-H	3	B-I	20	E-J	15	K-L

22	B-E	9	B-H	17	B-I	24	F-J	Control	L
29	B-E	6	B-H	27	B-I	8	F-J	---	---
13	B-F	12	B-H	11	B-I	25	G-J	---	---

All pomegranate genotypes investigated have high antioxidant capacities determined FRAP, DPPH, and iron chelating methods. The highest value among the genotypes was obtained in genotype 16 (1.22 ± 0.05) and followed by genotype 30 (1.20 ± 0.01). The lowest antioxidant value (0.50 ± 0.05) was obtained in genotype 15. The highest antioxidant capacity of pomegranate genotypes determined by DPPH (inhibition %) method was in genotypes 22 (43.22) and 6 (42.04), respectively. The lowest value was obtained from genotype 15 (13.55). Similar studies have been conducted to determine the antioxidant levels of various fruits including the pomegranate using FRAP, DPPH, and Iron Chelating methods. In contrast to values obtained for the genotypes studied, Özgen et al. (2008) reported higher values (between 5.60 and 7.35 mmol TE 100 mL⁻¹) for six pomegranate varieties grown in the Mediterranean region. Antioxidant capacity in fruit juice of Hicaz pomegranate determined by DPPH method was reported as 6.49 (AEx10-3) (Kelebek and Canbaş, 2010). In our study, the closest value to the stock solution (43.22%), obtained by the DPPH method, was recorded in genotype 22 (Sour pomegranate).

Hmid et al. (2017) studied the antioxidant capacity of 10 local and eight foreign pomegranate varieties by the scavenging activity against 1,1-diphenyl-2-picrylhydrazine (DPPH) method. The inhibition percentage was reported ranging from 31.16 to 66.82% for local cultivars, from 45.65 to 76.3% for foreign cultivars, which were mostly higher compared to those obtained from Zivzik pomegranate genotypes. Fawole and Opara (2016) used FRAP, DPPH and ABTS methods to determine the antioxidant levels of pomegranate. Significant levels of phenolic compounds and antioxidants were found in pomegranate peel, juiced seed pulp (Marc) and fruit juice, respectively. Gil et al. (2000) compared the antioxidant levels, determined by ABTS, DPPH, DMPD and FRAP techniques, of red wine, green tea and pomegranate. The researchers reported three times higher antioxidant level in pomegranate (18-20 TEAC) than red wine and green tea (6-8 TEAC). Eghdami and Sadeghi (2011) investigated the antioxidant content levels of 10 different pomegranate varieties by using FRAP and SAR methods, which both yielded statistically similar results. Çam et al. (2009) investigated the antioxidant capacity of seven Izmir pomegranate varieties and Zivzik pomegranate using the DPPH method. The highest antioxidant capacity was obtained from variety İzmir-8.

IV. CONCLUSION

The results revealed that all pomegranate genotypes have high antioxidant contents. The FRAP technique showed that the antioxidant capacity of pomegranate genotypes were lower than the stock solutions. Similar to the FRAP technique, the results obtained by the iron chelating method were generally higher than that of the stock solution values. Genotypes that have higher values than stock solution values have natural antioxidants. This emphasizes that pomegranate genotypes have a very strong antioxidant capacity compared to the iron chelating method. Genotypes 22 and 23, which are also sour in fruit taste, have high antioxidant content in terms of DPPH% inhibition values. These genotypes are suitable for pomegranate sour production rather than table consumption. Asili (Genotype 21) is a very large and stone seed genotype; thus, it is not suitable for table consumption. Asili fruit juice can be used in the juice industry because of sweeter taste compared to Zivzik pomegranate genotypes. Red pomegranate (Genotype 24) and other 26 Zivzik pomegranate clones are suitable for use in both table and juice industry due to their sweet tart taste.

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