Herbal Medicines Journal **2018; Vol. 3, No. 1:14-25**
DOI: 10.22087/hmj.v3i1.704 **ISSN:** 2538-2144

Original Article

An Investigation of the Secondary Metabolites and Antioxidant Capacity of Some Commercial Iranian Pomegranate (*Punica granatum* L.) Cultivars under Drought Stress

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Received: 17.2.2018; Accepted: 22.03.2018

Abstract

Background and Aim: This research was conducted in order to investigate secondary metabolite contents such as ellagic acid, total phenol, total flavonoid and antioxidant capacity in some commercial Iranian pomegranate (*Punica granatum* L.) cultivars under drought stress.

Materials and Methods: The experiment was a factorial arrangement based on a completely randomized design with three replications. Two factors, including pomegranate cultivars (Rabab Neyriz, Nadery badroud, Shyshah cap Ferdous, Ardestany Mahvelat, Malase Yazd and Shirinshavar Yazd) and irrigation levels (60% and 40% field capacity), the moderate and severe stresses respectively, and 80% field capacity as "control" were used and the plants were kept for six weeks. Subsequently, the alteration of some secondary metabolite contents, including ellagic acid content, total phenol, total flavonoid and antioxidant capacity in fully developed leaves were measured under above treatments.

Results: In this research, all the examined cultivars had similar responses to drought stress treatments, but the intensity of these responses was different in various cultivars. Drought stress caused an increase in ellagic acid content, total phenol, total flavonoid and antioxidant capacity in all cultivars.

Conclusion: According to the ultimate results, due to the high amount of ellagic acid, total phenol, total flavonoid and the consequent antioxidant capacity of the pomegranate leaf, it can be used in medicinal industry to produce herbals drugs.

Keywords: Drought stress, Secondary metabolite, Ellagic acid, Antioxidant capacity, Pomegranate

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Please cite this article as: Hassani Moghaddam E, Esna-Ashari M, Shaaban M. An Investigation of the Secondary Metabolites and Antioxidant Capacity of Some Commercial Iranian Pomegranate (*Punica granatum* L.) Cultivars under Drought Stress. Med. J. 2018;3(1):14-25.

Introduction

Iran has the most diverse and richest gene pool of pomegranate cultivars in the world. Likewise, more than 760 pomegranate cultivars have been collected from different provinces of Iran (1). Iran ranks first in the production of pomegranate and the land area allotted to the cultivation of this fruit with the

production of 990,000 ton of pomegranate per year in 68,000 hectares of land (1). Nowadays, apart from being considered as a kind of fruit, pomegranate has drawn the attention of researchers throughout the world for its medicinal properties (2).

There are several metabolites, including sugars, organic acids, alkaloids, polyphenols, flavonoids, anthocyanin, fatty acids and vitamins, in the fruit and other parts of pomegranate tree (3). Three kinds of yellow tannins called ellagitannin, granaten and punicalin have been separated from pomegranate fruit pericarp. About ten types of tannins have been found in different parts of the pomegranate tree, most of which are found in fruit skin and leaf. They are currently used for pharmaceutical and industrial purposes (4). Phenolic compounds are a large group of plant secondary metabolites that protect plants from biological and environmental stresses and are produced in response to an attack of fungal and bacterial pathogens or under prolonged exposure to ultraviolet radiation (5).

Plants produce a large group of secondary products that have a phenolic group. Each phenolic group has a hydroxyl group that is located on an aromatic ring. Some of them are soluble only in organic solvents, and others are soluble carboxylic acids and glycosides in water (6). Tannins, particularly punicalagin, anthocyanins and ellagic acid, are considered as categories of polyphenol group (7). Many plants contain these substances. Polyphenols, which are powerful compounds capable of neutralizing free radicals and the toxic effects of these invasive agents, play a significant role in human health.

Flavonoids and flavones are compounds that are found in plants either free or in combination with glycosides. There are about 4,000 types of flavonoids in higher plants, especially in the nondestructive plants, which are chemically related to phenols and are found in their leaves, fruits, vegetables, seeds, plants, stems and flowers (8). Flavonoids can be classified into various groups including flavonols, flavones, isoflavones, flavanones, proanthocyanidins and anthocyanins. Flavonoids, quercetin and Kaempferol are the most important antioxidants (9, 10).

Flavonoids are one of the most significant natural compounds that have drawn attentions due to their noticeable medicinal properties. Over the past 50 years, the pharmacological impacts of flavonoids and their derivatives have been studied and identified. One of the major causes of this problem is that the researchers believe that flavonoids, as drugs, can play a significant role in the treatment of diseases in near future. The antioxidant effects of flavonoids, which are polyphenol compounds found in all herbal foods, have been proven. Hence, the consumption of vegetables and plants prevents cancer and cardiovascular diseases. On the other hand, since today the use of artificial antioxidants is limited due to their toxicity, medical communities prefer to use natural antioxidants (11).

Plants are potential sources of natural antioxidants. Today, it has been revealed that the antioxidant properties of plants are related to phenolic compounds (such as phenolic acid, phenolic diterpins, tannins, and flavonoids), sulfur compounds and some vitamins such as tocopherol and ascorbic acid. According to various reports, the use of natural and herbal antioxidants has a great influence on body health. Compounds such as polyphenols and flavonoids have the potential to inhibit the activity of free radicals and delay lipids oxidation (7, 12, 13).

Today, free radicals and reactive oxygen species as well as their effects on biological systems are among the important subjects in medical sciences. Many of mutagenic and carcinogenic materials might be affected by free radicals production such as reactive oxygen. These molecules are potentially dangerous and harmful. The role of free radicals in many diseases or their exacerbation has been confirmed and is clarified ever more every day. These materials play major roles as analytical processes agents, such as the damage to biological membranes and macromolecules including DNA, RNA, and proteins (14).

In normal conditions, there is often a balance between free radicals production and antioxidant defense system. The rise in the production of free radicals or reduction in the antioxidant defense results in damage caused by free radicals, which leads in turn to oxidative stress in these conditions (15). In addition to endogenous defense, the consumption of some nutrients such as antioxidants plays an important role against mutagenic or carcinogenic substances (16).

Oral antioxidants such as tocopherol, ascorbic acid, carotenoids, and phenolic compounds play a pivotal role against free radicals through the elimination of free radicals (17). Today, it is believed that the consumption of fruits and vegetables that are rich in antioxidant is more influential than the use of supplements to counter oxidative damage (18). The antioxidant activity of pomegranate is due to the presence of ascorbic acid and phenolic compounds such as punicalagin, punicalin, gallic acid, ellagic acid and anthocyanins. Moreover, the antioxidant activity, which is influenced by the amount of phenolic and ascorbic acid compounds, differs among different cultivars of pomegranate (19). The antioxidant and chemical properties of pomegranate cultivars depend on cultivar, growth region, weather, degree of fruit maturity and agricultural practices (20). Pomegranate peel is a rich source of natural antioxidants. The antioxidant capacity of the extracts of pomegranate peel is due to the presence of phenols such as ellagic tannin, acetic acid and gallic acid. This antioxidant capability is achieved thanks to the existence of phenols and their capacity to regulate free radicals (21).

Ellagic acid is a dimer derived from gallic acid that is found mainly in organic plants, such as fruits and dried fruits. Ellagic acid exists in the plant vacuole in its free form, namely, ellagic acid or ellagic acid derivatives (22). Ellagic acid exists in plants as hydrolysable tannins. Hydrolysable tannins consist of the two types of gallotannins and ellagic tannins. They are the components of complexes derived from ellagic acid such as glucose esters with ellagic acid, which produce ellagic acid when they are hydrolyzed. Ellagic tannins are the structural composition of plant cell wall and cell membranes. Ellagic tannins have an important role in human nutrition thanks to their beneficial properties including anti-oxidant, anti-cancer, anti-arterial, anti-inflammatory, anti-bacterial and anti-AIDS properties. In Japan, ellagic acid is added to food as an antioxidant (23). In human body ellagic tannins and ellagic acid are absorbed in daily diets by eating fruits, grains, dried fruits and beverages. Ellagic acid in high concentrations is found not only in pomegranate but also in different fruits including strawberries, raspberries, cranberries and grapes (24). The aim of this experiment is study on secondary metabolites in pomegranate cultivars under drought stress.

Materials and Methods

This research was conducted in the research greenhouse of Lorestan Agricultural Sciences and Natural Resources Center in 2013 from March to July. The required experiments were carried out in three laboratories, namely a) the laboratory of horticultural sciences department in the School of Agriculture, Lorestan University, b) the research laboratory of Lorestan Agricultural Sciences and Natural Resources Center, c) the analysis laboratory of Kharazmi University.

Plant Materials

The plant material used in this study consisted of annual seedlings of six commercial pomegranate cultivars with the same age, diameter and seedlings size. This study was conducted in factorial arrangement based on a randomized design with three replications. Factors were six completely pomegranate cultivars including: Rabab Neyriz, Nadery Badroud, Shyshah cap Ferdous, Ardestany Mahvelat, Malase Yazd and Shirinshavar Yazd and three drought levels as 40%, 60% and 80% (control) field capacity.

Each experimental unit was five potted seedlings. Seedlings were planted in 15-liters plastic pots (34 and 32 cm height and diameter respectively) containing 1: 1:3 mixtures of manure, sand and soil. Before seedlings were transplanted into the pot, and their roots were disinfected with a water and manure mixture containing the Mancozeb fungicide (2 in 1000). The experiments related to soil moisture at the field capacity and soil physicochemical properties were conducted in the water and soil laboratory of Agricultural Research and Natural Resources Center of Lorestan Province.

The average moisture content of six pots was used to calculate soil moisture content. For this purpose, the pots were irrigated on the first day until drainage water was removed. To prevent evaporation from the pots, their upper surface was covered with aluminum foil. After 24 hours, sampling from the pot soil started and continued for 10 days, and soil moisture content was calculated according to the following formula:

Soil moisture percentage = ((primary soil weight-secondary soil weight)/ secondary soil weight)) × 100

To apply different stress levels, after field capacity determination, other stress levels were considered as a field capacity percentage and the amount of moisture content was calculated to reach the desired moisture content in grams and was added to the pots. Seedlings were exposed to drought stress for 6 weeks (from May 20 to July 2), and then the amount of secondary metabolites was measured and evaluated.

Leaf Sampling

At the end of the experiment for each drought stress level, the mature and healthy leaves of each seedling were selected from the third to fifth nodes in the main stem and were sampled. The required samples were dried at room temperature in shade to measure secondary metabolites.

Secondary Metabolites Measurement

After the application of drought treatments, secondary metabolites were evaluated as follows.

A) Extraction

Powder obtained from leaves (5g) was mixed with 50 ml Erlen and then added 50 ml of 80% methanol with a 1 to 10 ratio. To complete the extraction process, the sample mixture and methanol were crossed from filter paper after 72 hours. To obtain methanol from the extract, the methanol was transferred to rotary machine under vacuum and finally, the pure extract was located in a small container to determine the total phenol, flavonoid and antioxidant activity.

B) Total Phenol Measurements

To make the calibration curve, primarily standard solutions in concentrations of 0, 40, 80, 160, 320 and 480 mg per ml gallic acid were prepared. Each of the concentrations was injected three times to UV-Vis spectrophotometer then their absorbance was read. In each sample with 2.5 ml Folin Ciocalteu (1 to 10 diluted with distilled water) and 2 ml sodium carbonate (75 g per liter) were added to 0.5 ml methanolic extract and were then mixed. Blank sample was methanol instead of methanolic extract in samples, and was used for spectrophotometer calibration. The above solution was placed in the dark for 15 minutes and then absorbed at 765 nm. The standard curve was plotted based on gallic acid (Fig. 1). The total phenol content was determined based on gallic acid (mg in 100 g leaf dry weight) based on the following equation:

Total phenol= (the number which has been read*(extract volume (ml)/sample weight (g))

C) Total Flavonoid Measurement

To prepare calibration curve and slope equation, standard solutions were prepared with concentrations of 0, 40, 80, 160, 320 and 480 mg per ml of quercetin. First, a preliminary mixture of quercetin with 1 mg concentration per liter was prepared, and then it was diluted to obtain all concentrations. Each concentration was injected three times to the UV-Vis spectrophotometer and the absorbance was determined. The total flavonoid content was measured by aluminum chloride chromatography. Based on this method, 0.5 ml methanolic extract was mixed with 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate (2.4 ml in 10 ml of distilled water) and 2.8 ml distilled water in a Falcon tube. The mixture was placed in darkness at room temperature for 0.5 hour and was absorbed at 415 nm. The standard curve was depicted based on quercetin concentration (Fig 2). The flavonoids content was calculated based on the following equation:

Total flavonoids= Extract volume (mg)/Sample weight (g)

D) Evaluation of Antioxidant Activity by DPPH Method

The extract antioxidant effect was evaluated by free radical inhibitory capacity measurement using 2 and 2-diphenyl-picyrilhydrazil (DPPH). Based on this method, 0.05g of extract was placed in a 50 ml balloon with methanol and then 0.1, 0.2, 0.4, 0.6, 0.8 was prepared at 1 $mg.ml^{-1}$ concentrations. 2.5 ml solution with above concentration was placed in each tube and was added to 1ml DPPH. Samples were placed at room temperature for 15 minutes in darkness and then absorbed the obtained solutions and control at 517 nm by UV-Vis spectrophotometer. The percentage of radical inhibitory percentage was calculated by the following formula:

Radical inhibitory percentage= (control absorbance-sample absorbance)/ control absorbance

E) Extraction for HPLC Injection

To prepare the sample for being injected to HPLC machine, primarily, 0.5g powdered sample was

dissolved in 5 ml extraction solution including methanol and acetic acid in 9 to 1 (v/v) ratio. After homogenization, the extract was filtered via 0.45 micron cellulose filter then 20 µl of extract was injected into HPLC.

F) Measurement of Ellagic Acid

Primarily, ellagic acid standard was purchased from Sigma Company. Ellagic acid was determined using, Diagel and Conkerton (25) method. The used HPLC device was the Unican-Crystal-2000 mole made in United Kingdom, with 25cm length Erospher-100-C18 column, 4mm internal diameter and 5µm particle diameter. The moving phase consisted of a mixture of methanol, water and acetic acid at 50, 45 and 5 ratio respectively with 1mm per minute in speed. Each material in sample was determined by comparing the inhibition time with the standard sample peak. Their amounts were determined based on the comparison of the below peak in the sample curve with calibration curve with different standard concentrations. The used detector was ultraviolet with 200 nm wavelength.

Data Analysis

All the obtained data from the experiments conducted in this research were categorized by SAS-9.1 software and mean comparison was done using Duncan's multiple range tests. Interaction effect mean comparison was done by MSTAT-C software using Duncan's multiple range tests. Charts were plotted using Microsoft Excel. To calculate the line slope equation, Minitab software was used to calculate the total phenol and flavonoid.

Results and Discussion

The results showed that the effect of cultivar and drought stress and also their interaction effect were significant on secondary metabolites such as total phenol, total flavonoid, and leaf ellagic acid in pomegranate cultivars (P≤0.01), but their interaction effect was not significant on leaf antioxidant capacity (Table 1).

Antioxidant Capacity

The results indicated that pomegranate cultivars had significant differences in antioxidant capacity (P≤0.01). The effect of drought stress treatment was significant on antioxidant capacity in 1% level (figures 7 and 8). But the interaction effect of cultivar and drought stress was not significant on it. The mean comparisons results showed that there were significant differences between pomegranate cultivars with regard to antioxidant capacity. The maximum antioxidant capacity was observed in Malase Yazd and Nadery Badroud cultivars with a mean of 54.7 and 40.2%, respectively, and no significant differences were observed between them. The mean antioxidant capacity of two Ardestany Mahvelat and Shyshah cap Ferdous, was significantly lower than other cultivars. The antioxidant capacity of pomegranate cultivars significantly increased under the influence of drought stress treatments and in this regard there was a significant difference between treatments (Table 2).

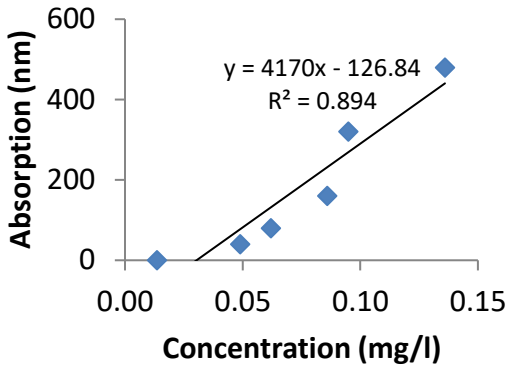

Figure 1. Standard curve of gallic acid and equation for total phenol measurement.

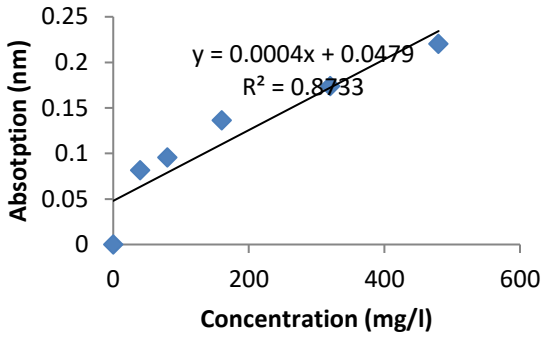

Figure 2. Standard curve of querestin and equation for flavonoid measurement.

Total Phenol

The results of the analysis of variance concerning the effect of drought stress and cultivar on total phenol content is shown in Table 1. The studied pomegranate cultivars indicated significant differences in terms of total phenol (P ≤ 0.01). Moreover, the effect of drought stress on total phenol content was significant

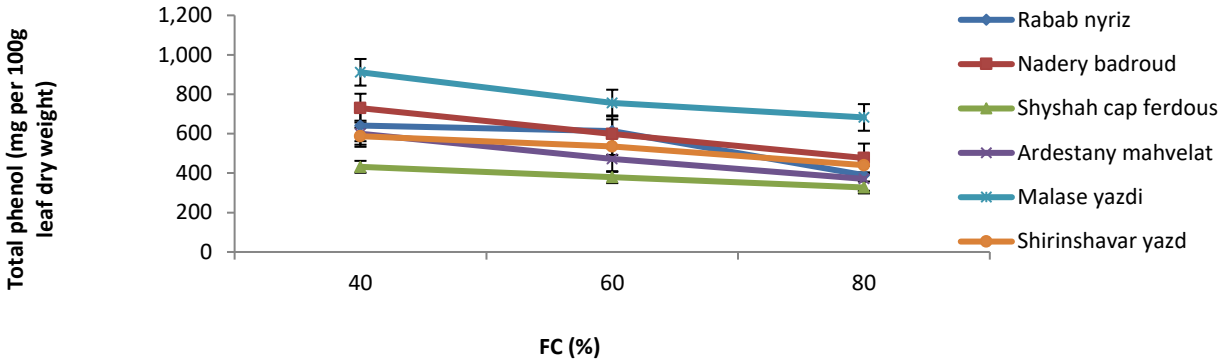

Figure 3. Effects of different drought stress levels on total phenol in six pomegranate cultivars. The results are indicating of the mean ± standard error (SE) for three replicates.

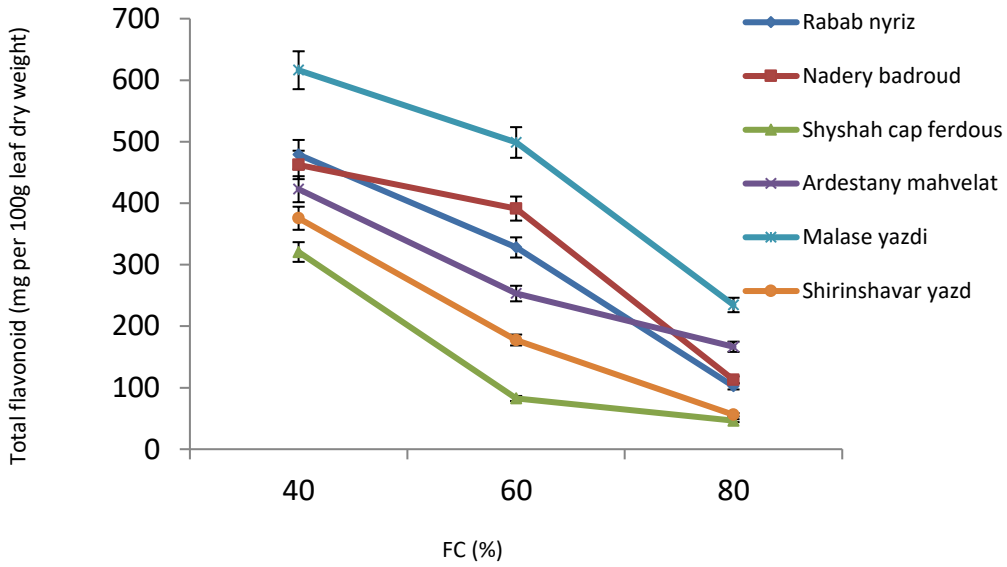

Figure 4. Effects of different drought stress levels on total flavonoid in six pomegranate cultivars. The results are indicating of the mean ± standard error (SE) for three replicates.

at 1% level. Furthermore, the interaction effect of cultivar and drought stress factors on this trait was significant ($P \leq 0.01$). With the rise of drought stress level, the total phenol content in all cultivars increased and there was a significant difference between different drought stress levels, so that the highest total phenol was observed in severe drought stress (Fig. 3). The highest total phenol content was measured in Malase Yazdi (783 mg / 100 g leaf dry matter) and the lowest (379 mg) was in Shyshah cap Ferdous cultivar. In other cultivars, the total Phenol is stated in the middle of these two cultivars (Table 2).

Total Flavonoid

The results concerning the impact of drought stress and cultivar on total flavonoid content are shown in Table 1. The studied pomegranate cultivars showed a significant difference in flavonoid content ($P \leq 0.01$). Furthermore, the effect of drought stress on total flavonoid content was significant at 1% level. Moreover, the interaction effect of drought stress and cultivar on this trait was significant ($P \leq 0.01$). As seen in Fig. 4, an increasing trend of leaf flavonoids under draught stress was observed in the studied cultivar. The average amount of flavonoids was 406.4 mg / g of dry leaf tissue. It should be noted that a significant difference was observed between the cultivars in terms of total flavonoid content. The highest total flavonoid content was observed in MalaseYazdi (616 mg / 100 g leaf dry tissue) in severe drought stress. The mean

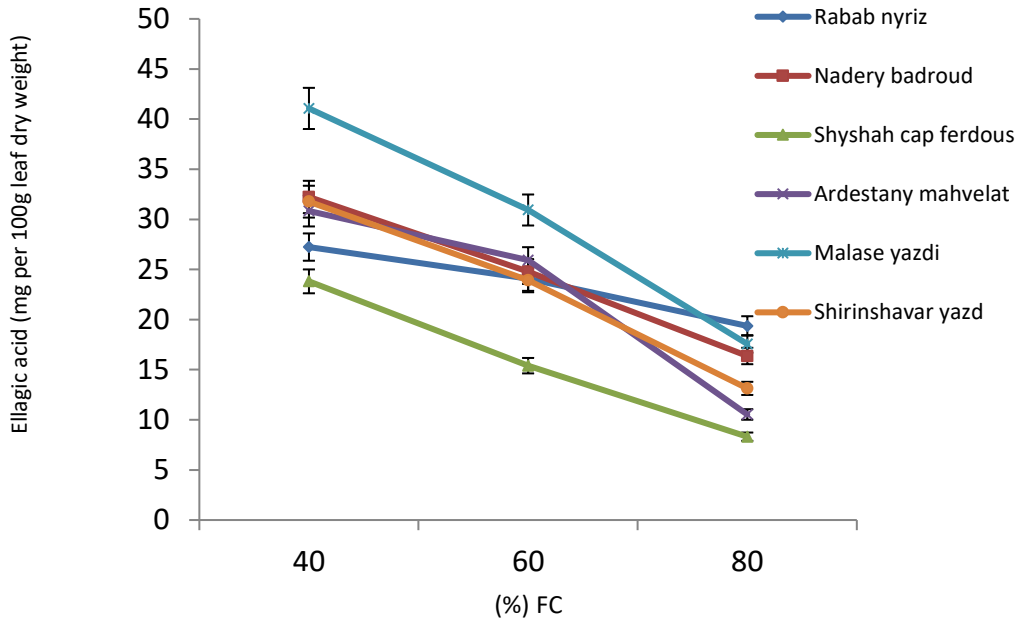

Figure 5. Effects of different drought stress levels on ellagic acid in six pomegranate cultivars. The results are indicating of the mean ± standard error (SE) for three replicates.

Table 1: Analysis of variance for effects of different drought stress levels on some secondary metabolites in leaf of six pomegranate cultivars.

S.O.V	DF	Antioxidant capacity	Total phenol	Total flavonoid	Ellagic acid
Cultivar	5	1123**	164971**	88778**	443**
Stress	2	1820**	184688**	693955**	1223**
Cultivar*stress	10	30^{ns}	5088**	7538**	5561**
Error	36	19	48	1416	15
CV (%)		12.41	1.25	4.59	11.02

** Significant at 1% level and ns: not significant.

total flavonoids in Shyshah cap Ferdous cultivar were significantly lower than other cultivars (Table 2).

Ellagic Acid

The results of the analysis of variance for the effect of drought stress and cultivar on the amount of ellagic acid are shown in Table 1. The pomegranate cultivars showed a significant difference in the amount of ellagic acid ($P \leq 0.01$). The effect of drought stress on ellagic acid was significant at 1% level. In addition, the interaction effect of drought stress and cultivar on this trait was significant ($P \leq 0.01$). Based on the mean comparison, drought stress significantly increased the amount of ellagic acid in the leaves and there was a significant difference between the cultivars under moderate and severe drought stress conditions. In non-stress condition, there was no significant difference between cultivars in the amount of leaf ellagic acid (Fig. 5). The highest amount of ellagic acid was observed in Malase Yazdi cultivar under severe drought stress (41 mg / 100 g leaf dry tissue). The mean of ellagic acid in Shyshah cap Ferdous cultivar (8.3 mg / g leaf dry tissue) was significantly lower than other cultivars. There was a remarkable difference between other cultivars and the stated intermediate (Table 2). It should be noted that the chromatogram of pomegranate leaves ellagic acid combination and its other derivatives is shown in Fig. 6.

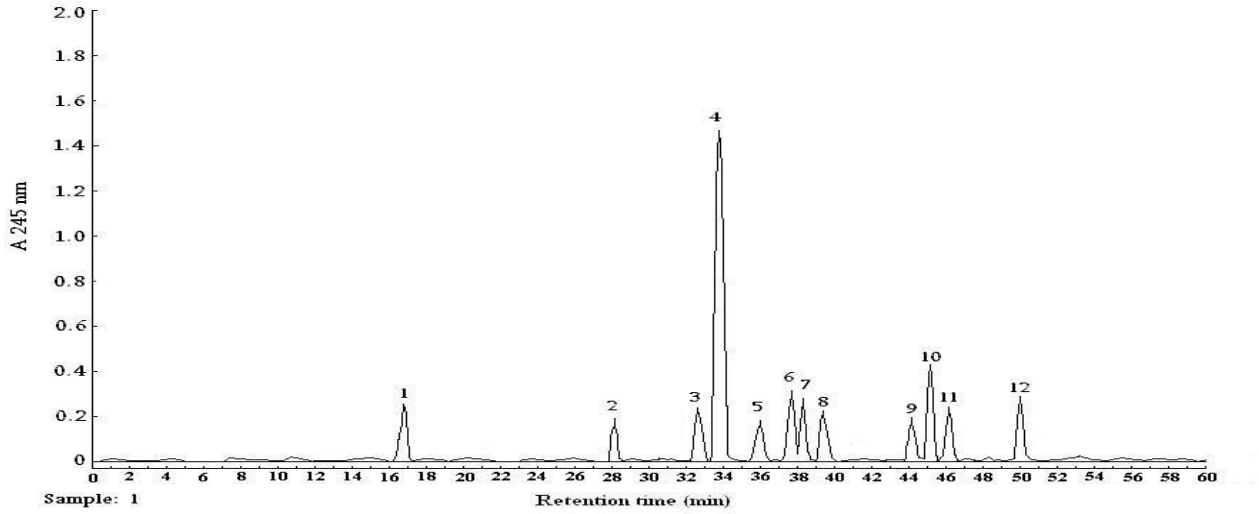

Figure 6. HPLC Chlamogram for ellagic acid measurement.

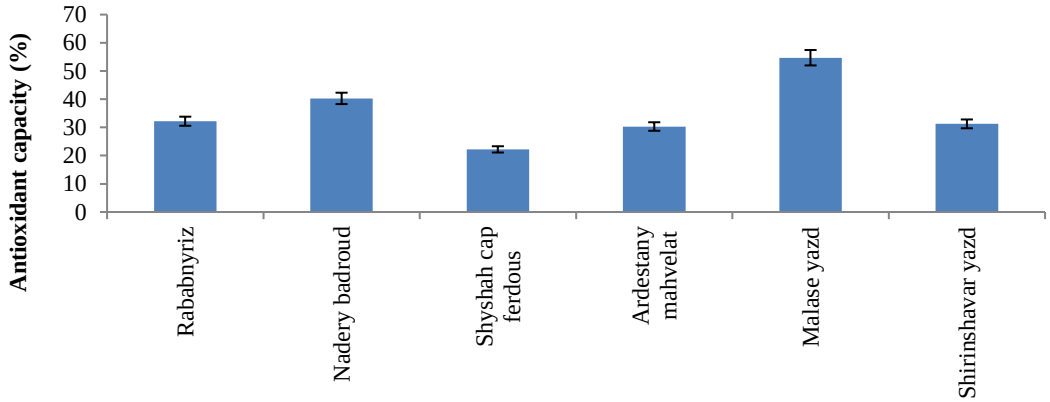

Figure 7. Effect of cultivar on antioxidant capacity in leaf of six pomegranate cultivars.

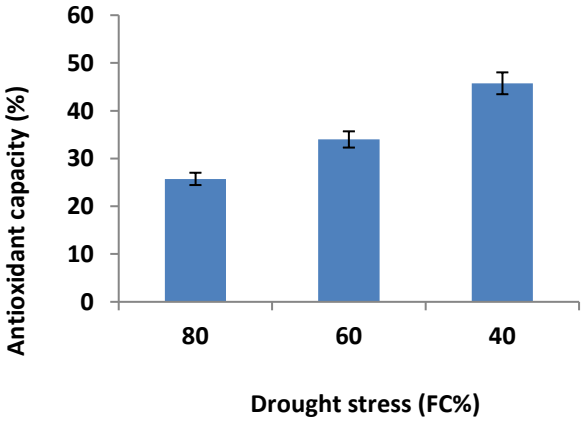

Figure 8. Effect of drought stress on antioxidant capacity in six pomegranate cultivars.

The Correlation between Traits

The results of the correlation analysis between different traits (Table 3) indicated that there was a positive and significant correlation between the total phenol content with flavonoid and leaf ellagic acid (r = 0.84) and (r = 0.71) respectively. Moreover, there was a positive and significant relationship between the amount of ellagic acid and flavonoid (r = 0.79).

In the present study, as shown in figures 3, 4 and 5, there was an increasing trend in total phenol, total flavonoid and ellagic acid in all pomegranate cultivars as drought stress increased, indicating a positive relationship between the severity of drought stress and the amount of these metabolites. It should be noted that total phenol, total flavonoid and ellagic acid dramatically increased with the rise of drought stress severity. Furthermore, the alteration trend of secondary metabolites changes was different with other parameters that were measured. This phenomenon indicates that the presence of secondary

metabolites could not be considered as an evaluation index for tolerance in the pomegranate tree.

Ellagic acid is one of the most important compounds in pomegranate the phenolic nature of which causes its

Table 2: Results of mean comparison for some secondary metabolites in leaf of six pomegranate cultivars under drought stress.

treatments	Antioxidant capacity (%)	Total phenol (mg/100 g DW)	Total flavonoid (mg/100 g DW)	Ellagic acid (mg/100 g DW)
C1	32.1c	547c	303c	23.5b
C2	40.2a	601b	322b	24.4b
C3	22.2f	397f	149e	15.8c
C4	31e	472e	267d	21.3b
C5	54.7a	783a	449a	29.8a
C6	35.2d	520d	203d	22.9b
MSE	1.47	3.13	6.43	1.24
S1	25.7c	447c	119c	14.2c
S2	33.9b	559b	288b	24.1b
S3	45.7a	650a	445a	31.1a
MSE	1.76	4.33	5.55	0.86
C1×S1	22	389k	102i-k	19.3c-f
C1×S2	32	612f	328ef	24b-e
C1×S3	43.4	641e	478bc	27.2bc
C2×S1	33.2	476i	112i-k	16.3d-g
C2×S2	38.5	599fg	391c-e	24.7b-e
C2×S3	49	729c	462b-d	32.2b-e
C3×S1	16.2	327m	46.4k	8.3g
C3×S2	19.5	379kl	82.4jk	15.4e-g
C3×S3	30.8	431j	320e-g	23.81b-e
C4×S1	21.3	370l	166h-j	10.5fg
C4×S2	30.9	472i	253fgh	25.9b-d
C4×S3	38.5	599fg	422bcd	30.8b
C5×S1	41.5	682d	234gh	17.5c-g
C5×S2	56.9	755b	498b	30.9b
C5×S3	65.7	911a	616a	41a
C6×S1	20	440j	56k	13.1fg
C6×S2	26.8	535h	177.5hi	23.9b-e
C6×S3	46.7	587g	375.4de	31.7b
MSE	0.89	1.3	1.37	0.43

The same letters along with the numbers of each column in each section indicate a significant statistical difference between them at the 0.01 level of 0.01. MSE represents a standard error among the means. The C1 to C6 represent the pomegranate cultivars namely Rababnyriz, Naderybadroud, Shyshah cap ferdous, Ardestanymahvelat, Malaseyazd and Shirinshavaryazd. The S1, S2 and S3 indicate 3 drought levels as 40%, 60% and 80% (control) field capacity respectively.

strong antioxidant activity. Pomegranate leaves like fruit, fruit juice and fruit skin is rich in phenolic compounds, such as ellagic and gallic tannins. Among these compounds, ellagic acid has high antioxidant and anti-cancer properties (26). Xiang (26) evaluated the effect of season, variety and preparation of extract method on the amount of ellagic acid in pomegranate leaves, and stated that the amount of ellagic acid increased significantly during the growing season, but the effect of different cultivars was not significant on pomegranate ellagic acid. The chemical composition of the pomegranate leaf varies depending on the cultivar, growth season, climate, and planting practices (27).

Lihua and Zhang (21) measured the changes in phenolic compounds, flavonoids, alkaloids and antioxidant capacity in pomegranate leaves during the growing season. They stated that the amounts of these compounds would increase during the growing season, so that the maximum amounts of them were observed in late growing season. Corroborating our results, some researchers also have reported that pomegranate leaves antioxidant capacity positively correlates with total phenol and flavonoid content in leaves (20). Recently, many reports have been published in terms of the high antioxidant capacity of pomegranate fruits and leaves extract (7, 17, 20).

Tehranifard (20) reported that the measured total phenol in different pomegranate cultivars ranged between 295 to 985 $mg.100^{-1}$ g. The fact that the antioxidant capacity of pomegranate juice is higher than other fruits juice could be related to higher phenolic compounds in pomegranate. It was indicated that the various pomegranate organs contain significant phenolic compounds (17).

Wang et al. (28) reported that pomegranate leaves as a sub-product of this main tree are a rich source of phenolic compounds responsible for its high antioxidant capacity. They determined the relationship between the total phenol and ellagic acid content of pomegranate leaves with their antioxidant capacity using regression analysis, and stated that the leaf total phenol had a significant relationship with antioxidant capacity of leaf (R^2 = 0.8). However, there was no linear relationship between the amount of ellagic acid and antioxidant capacity.

Jamshidi et al. (29) indicated that there was a direct relation between the antioxidant activity in medicinal plants and the amount of phenolic and flavonoids in all organs. Their results, are in line with these findings. In the present study, the antioxidant activity had a direct correlation with total phenol and flavonoid. Moreover, like other studies, it was observed that pomegranate cultivars with higher phenolic and flavonoids had a higher anti-radical activity.

Flavonoids are one of the polyphenol groups that are influenced by environmental conditions. Flavonoids compounds had high medical and biological properties such as blood purification, immune system enhancement, blood cholesterol regulation, blood pressure regulation, cancer prevention, strong antioxidant effects, anti-radicals, anti-inflammatory and cardiac protection. Plants are potential sources of antioxidant compounds. In recent years, various studies have been conducted in order to investigate the potential of plant products as antioxidant to be used against diseases caused by free radicals. It has been reported that the consumption of natural and herbal antioxidants has a significant impact on human health (12, 30). Compounds such as polyphenols and

Table 3: Correlation coefficient (r) between total phenol, total flavonoid and ellagic acid.

	Antioxidant capacity	**Total phenol**	**Total flavonoide**	**Ellagic acid**
Antioxidant capacity	1			
Total phenol	0.9**	1		
Total flavonoid	0.8**	0.84**	1	
Ellagic acid	0.65**	0.71**	0.79**	1

** Significant at the level of 0.01.

flavonoids have the potential of inhibiting free radicals and are capable of delaying the lipid oxidation (12, 31).

Conclusion

In the present study, antioxidant activity had a direct relation with total phenol and flavonoid. Moreover, confirming the results of other studies, this research indicated that since pomegranate cultivar contains phenolic compounds and flavonoids, it could have higher anti-radical activity. According to the present results, due to the high total phenol and flavonoid content of the leaves and their high antioxidant capacity, they could possibly be used in pharmaceutical industry to produce drugs.

Acknowledgment

We gratefully acknowledge Dr. Masoud Boojar of biochemistry at Department of Cell and Molecular Biology, Faculty of Biological sciences, University of Kharazmi for measurement of ellagic Acid using HPLC device.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Behzadi-Babbaki H. [Distribution and variety of pomegranate cultivars in Iran. Publishing of Farmer's Education in Karaj. 1998; P 265] Persian.
2. Seeram NP, Schulman RN, Heber D. [Pomegranates: Ancient Roots to Modern Medicine. Medicinal and Aromatic Plants". Industrial Profiles 43.CRC Press, Taylor and Francis Group. 2006; P 244].
3. Melgarejo P, Martinez JJ, Hernandez FMR. Legua P, Oncina R, Martinez-Murcia A. Cultivar identification using 18S–18S RDNA intergenic spacer-RFLP in pomegranate (*Punica granatum* L.). Sci. Horti. 2000; 120:500–503.
4. Mirjalili A. [Pomegranate knowing" .Agricultural Education Publishing. 2000; P 235] Persian.
5. Shukla S, Mehta A, Bajpai VK, Shukla S. In vitro antioxidant activity and total phenolic content of ethanolic leaf extract of *Stevia rebaudiana* Bert. Food Chem. Toxic. 2009; 47: 2338-2343.
6. Crozier A, Burns J, Aziz AA, Stewart AJ, Rabiasz HS. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. Biol. Res. 2000; 33:79-88.
7. Verrmeris W, Nicholson R. [Phenolic compounds biochemistry. 2006; Springer 1212].
8. Wang R, Wang, W, Wang L, Liu R, Ding Y, Du L. Constituents of the flower of *punica granatum*. Fitoter. 2006; 77:534-537.
9. Omidibi R. [Production and processing of medicinal plants. First volume. Third edition. Astan Quds Razavi Publishing. 2007; P 347].
10. Bozin B, Mimica-Dukic N, Samojlik I, Goran A, Igic R. Phenolic as antioxidants in garlic (*A. sativum* L., *Alliaceae*)". Food Chem. 2008; 111: 925–929.
11. Wang Y, Ma F, Li M, Liang D, Zou J. Physiological responses of kiwifruit plants to exogenous ABA under drought conditions. Plant Grow. Reg. 2011; 64: 63-74.
12. Heydari-Sharif-Abad H. [Plant, Dryness and Drought". Research Institute of Forests and Rangelands press, Tehran. 2000; No. 250, P 200] Persian.
13. Tachakittirungrod S, Okonogi S, Chowwanapoonpohn S. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. Food Chem. 2007; 103: 381-388.
14. Negi PS, Jayaprasha GK, Jena BS. Antioxidant and anti-mutagenic activities of pomegranate peel. Food chem. 2003; 80: 393-397 .
15. Scheibmeir HD, Christensen K, Whitaker SH, Jegaethesan J, Clancy R, Pierce JD. A review of free radicals and antioxidants for critical care nurses. Int. Crit. Care. 2005; 21: 24-28.
16. Mehrabian Sedigheh Majd A, Sotoudeh S. Effect of Physical and Chemical Factors on Weight Loss and Antioxidant Properties of Pomegranate Pomegranate Pellets (*Punica granatum* L.). J. Islamic Azad Univ. 2008; 1(70): 79-71.
17. Gill M, Itomas-Barberan FA, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J. Agric. Food Chem. 2002; 48: 4581-4589.
18. Zarban A, Malekaneh M, Baghratami MR. Antioxidant properties of pomegranate juice and its ability to neutralize free radicals. J. Birjand Univ. Med. Sci. 2007; 3: 27-19.
19. Sarkhosh A, Zamie Z, Fattahi Moghadam MR, Ghorbani G, Hadian J. Review of pharmacological and pharmacological properties of pomegranate. Quart. J. Med. Plants. 2007; 3(24): 13-22. (In Persian)
20. Tehranifar A, Zarei M, Nemati Z, Esfandiyari B, Vazifeshenas MR. Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (*Punica granatum* L.) cultivars. Sci. Hort. 2010; 126: 180–185.
21. Lihu Z, Yujiae G, Yuanhu Z. Jing L, Junwei Y. Change in bioactive compounds and antioxidant activities in pomegranate leaves. Sci, Horti. 2010; 123: 543-556.
22. Hakkinen SH, Karenlampi SO, Mykkanen HM, Heinonen IM, Torronen AR. Ellagic acid content in berries: influence of domestic processing and storage. Eur. food Res. Tech. 2000; 212: 75-80.
23. Elliott G, Crozier A, Burns J, Aziz AA, Stewart AJ, Rabiasz HS. Application of antioxidant vitamins in foods and beverages. Food Tech. 2000; 53:46-48.
24. Vattem DA, Shetty K. Biological functionality of ellagic acid: review. J. Food Biochem. 2005; 29:234-266.
25. Daigel DJ, Conkerton EJ. High-performance liquid chromatography of 34 selected flavonoid. J. Chroma. 1982; 240: 202-205.
26. Xiang L, Xing D, Lei F, Wang W, Xu L, Nie L, Du L. Effects of season, variety and processing method on ellagic acid content in pomegranate leaves. Tsin. Sci. Tech. 2008 13(4): 460-465.
27. Lee-C-Chen LG, Liang WL, Wang CC. Anti-inflammatory effects of *Punica granatum* Linne in vitro and in vivo. J. Food Chem. 2010; 118: 315-322.
28. Li Y, Guo, C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem. 2006. 96: 254- 260.
29. Jamshidi M, Ahmadi Ashtiani HR, Rezazadeh S, Fathi Azad F, Mazandarani M, Khaki A. Study and comparison of phenolic compounds and antioxidant activity of some native plant species of Mazandaran. Quarterly J. Med. Plants. 2010; 9(34): 178-183.

30. Mirjalili A. [Plants in stressful environments". Nourbakhsh press. 2007; P 234] Persian.
31. Zhang Y, Li S, Wu X. Pressurized liquid extraction of flavonoids from Houttuy acordata Thunb. Sep. Pur. Tech. 2008; 58: 305-310.