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Original Article

Study of Biochemical Compounds from Extract of Peel, Seed and Fruit Juice of some Pomegranate Cultivars (*Punica granatum* L.)

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Abstract

Pomegranate (*Punica granatum* L.) is a native plant, which has many different cultivars in Iran. This plant consists of rich biochemical compounds that plays an important role in human health. This research aims to study some of biochemical compounds of this valuable fruit. Extract of pell and seeds and juice of fruits were prepared. According to the outcomes of this study, there is a direct relationship between increasing amount of phenol compounds and the colour of fruit peel. This increase showed significant difference. The anthocyanin measurement in three cultivars showed that the colour of the peel and seeds of the fruit has a direct relationship with the amount of anthocyanin. Study of antioxidant activity with the method of DPPH revealed that the highest amount of antioxidant activity between the peel and the seeds of the three cultivars of pomegranate is dedicated to the peel of the BSY. The outcomes of the FRAP method showed that the highest amount of antioxidant activity among seeds and the skin belongs to the seed of the WSR. With the use of the ABTS method the highest amount of antioxidant activity belongs to the peel of the BSY cultivar. The results of the studying vitamin C revealed that the amount of Vitamin C per 100ml of the pomegranate juice is 54.6±2.2mg for RSQ, 42.8±2.3mg for WSR and 65.7±2.2mg for BSY. Analysing the biochemical compounds from different cultivars helps to sufficient selecting, defusing and commercializing the pomegranate.

Keywords: Pomegranate, Phenolic compounds, Anthocyanin, Antioxidant activity, Vitamin C

Introduction

Pomegranate (*Punica granatum* L.2n=2x=16) is a small genus *Punica* belonging to the order of Myrtals and the family of Lythraceae, which consists of two species *Punica granatum* L. and *Punica protopunica* Balf. f.. it seems that pomegranate to be indigenous to Iran[1]. Pomegranate has a long history of cultivation. Based on historical evidences, its cultivation history dates back to 2500 BC. Its main origin is known as the Near East, especially Iran[2]. A very various compounds of different pomegranate tissues has been purified which has a variety of therapeutic, dietary, cosmetic, hygienic and industrial utilizations. Among its compounds, Ellagitannins, Galoutanins, Anthocyanins, Flavonoids, Sterols, Terpenoids, Tannin, Polyphenols, Alkaloids, Organic Acids, B1, B2, C vitamins can be mentioned[3]. Clinical researches conducted on some of the pomegranate drug and toxicological mechanisms has found that pomegranate has a wide healing attributes and can be widely used as a possible therapeutic method for the prevention and treatment of various diseases[4].Pomegranates are among the Iranian valuable native plants and there are many types of the speciesin Iran.In addition to being a delicious and popular fruit, it is rich in biochemical and mineral compounds that play an important role in

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human health. Identifying and introducing phytochemical compounds of pomegranate is important from a pharmaceutical perspective [5]. This study investigated onsome chemical compounds that have a medicinal property such as total anthocyanin, vitamin C, phenolic compounds, antioxidant properties, and fruit juice index in three pomegranate types, which are highly pharmacologically important. Studying and identifying various compounds in pomegranate tissues and evaluating the performance of these compounds can be an effective step in advancement of pomegranate pharmaceutical and industrial utilizations.

Material and Methods

Collecting Plant Samples

In order to extract the contents of the biochemical compounds of pomegranate fruits, samples from pomegranate collections were prepared by the Agricultural and Natural Resources Research Centre of Isfahan Province. Based on the skin colour of the fruits (white, red, and black) and the coincidence of growth periods, three varieties, Red Skin of Qom (RSQ), White Skin of Rijab (WSR) and, Black Skin of Yazd (BSY), were selected (Figure 1) and then skin and seeds of fruits were sampled. The specimens were frozen immediately by nitrogen liquid and kept frozen at -20 °C.

Extraction

Samples of skin and seeds prepared in the sampling stage were powdered in the presence of liquid nitrogen in pestle and mortar and mixed in 50 ml of acid ethanol (volume ratio of 99:1 from absolute ethanol and chloric acid 36%, respectively) and then placed at 25 °C for 24 hours.The solution was filtered with a thin cotton cloth and finally, the extracts were centrifuged (Hettich universal 320 R, Germany) at 3000 g for 15 minutes and the supernatant was transferred to the sterile container for further experiments [6,7].

Preparing Juice

After washing and slicing the fruits into smaller pieces, the seeds were extracted from the tissue by hand and the juice was extracted by rubbing on a metal net.To avoid quality degradation, juice was stored in 4 ˚C.

Identification of phenolic compounds

To identify phenolic compounds the Folin–Ciocalteu method was used. For this purpose, two ml of sodium carbonate (2%), 2.8ml of distilled water and 100µl of Folin–Ciocalteu reagent (50%) were added to 100µl of the pomegranate extract prepared in the previous step. After half an hour, the absorbance of the resulting mixture was recorded by a UV- visible spectrop (Beckman DU 530, USA) at 720 nmin comparison to the control. Gallic acid was used as the standard for drawing a standard curve (Figure 2) and the total phenol content of the extracts was based on mg equivalent of Gallic acid per gram of dry weight of the plant was reported [8, 9].

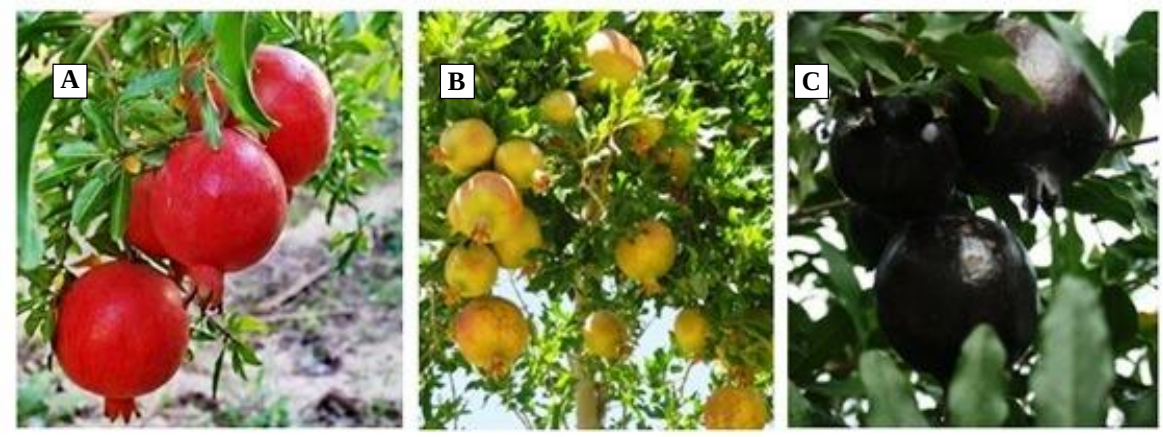

Fig. 1 Three varieties of pomegranate. A: Red Skin of Qom, B: White Skin of Rijab and C: Black Skin of Yazd.

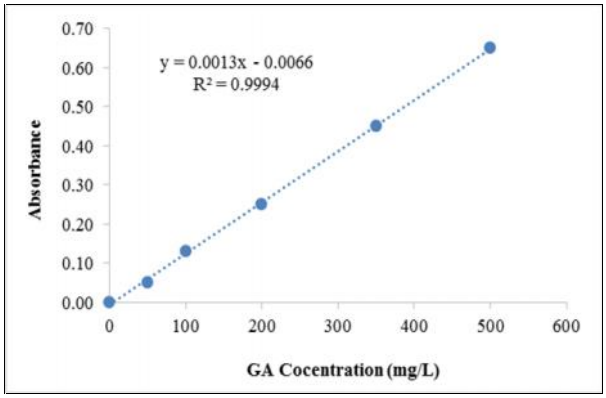

Fig. 2 Standard curve of gallic acid

Counting Total Anthocyanin Level

The prepared pomegranate extract was centrifuged at 3000 rpm for 15 minutes and supernatant was transferred to sterile containers. The absorbance of the solution was measured by a UV-visible spectrophotometer (Beckman DU 530, USA) at 530 and 657 nm wavelengths compared to the control. The ethanol chloride acid solution was used as a control by the ratio of 99:1. The anthocyanin level was calculated for each extract by using the following equation [10]:

anthocyanin's =A530 – (0.25×A657)

Antioxidant Capacity

Antioxidant Capacity was measured by DPPH, ABTS and FRAP methods.

DPPH method

In the DPPH method, neutralization activity of extract on radical 2,2-diphenyl-1-picrylhydrazyl was determined by spectrophotometric method at 517 nm wavelength. The amount of neutralization activity radical of DPPH with several density of the extract was calculated by following equation: (DPPH = (100 (1-AS / AC)). In this equation, AC absorption radical of DPPH without any antioxidant was used as control, AS absorption of DPPH plus specimen and methanol were used as blank. With the percentage of inhibition of several concentrations and regression line plotting (Figure 3), the IC50 and anti-radical efficacy (AE=1/ IC50) were calculated for each sample [11].

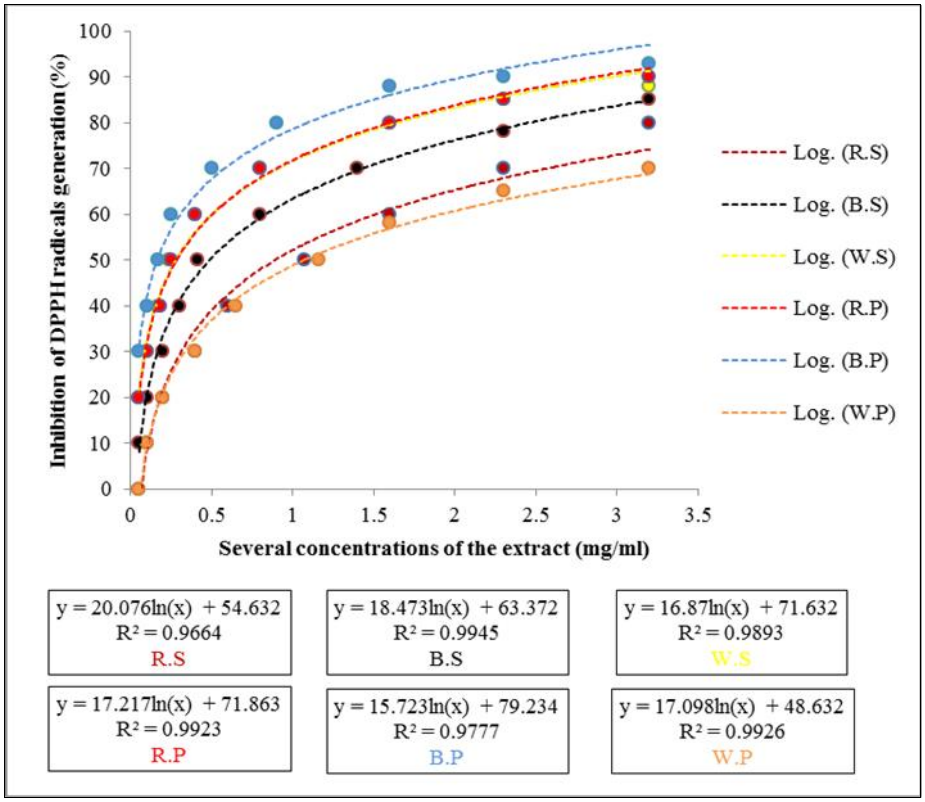

Fig. 3 Regression line plotting of several concentrations. R.S: seed of Red Skin of Qom, B.S: seed of Black Skin of Yazd, W.S: seed of White Skin of Rijab, R.P: peel of Red Skin of Qom, B.P: pell of Black Skin of Yazd, W.P: pell of White Skin of Rijab,

ABTS Method

In the measurement of ABTS, antioxidant capacity was measured using an extract for the radical digestion of $ABTS^{\bullet+}$ (2,2-Azinebis-3-ethylbenzothiazolin-6-sulfonic acid) radicals. Radical $ABTS^{\bullet+}$ was formed by adding potassium persulfate to ABTS and placed in dark for 16 hours. The base solution was then diluted with addition of ethanol to obtain 0.7 at wavelength of 734 nm, and the extract and radical samples were mixed at 5:100µl and its absorption at 734 nm was read after seven minutes. Inhibition trolox calculated by the formula (inhibition trolox=[(AC–AS)/AC]×100), in this equation, Ac is the absorption radical of ABTS without any antioxidant as control and as is the absorption of ABTS plus the extract of the specimen. The TEAC value was calculated using the standard trolox curve (Fig. 4) slope [12].

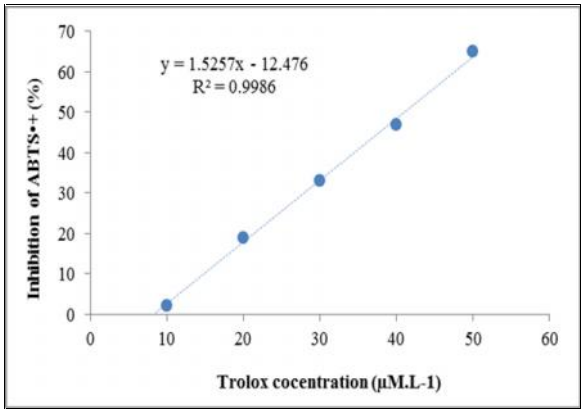

Fig. 4 Standard trolox curve

FRAP Method

Ferric reducing antioxidant power (FRAP), in this method, the antioxidant electroporation properties at low pH causes ferricion of ferric to ferrous. In order to measure this property, 0.1 g of frozen herbal tissue was homogenized with 5 ml of distilled water in a cold pestle and mortar in an ice bath. The resulting homogenate was filtered using No.1 Watman Filter paper and then, 5.1 ml of FRAP reagent (300 mM sodium acetate with pH 6.3, ferric-3-pyridyl-s-triazine and ferric chloride) was added to 50ml of the obtained extract. The resulting mixture was vortexed and incubated for four minutes at a temperature of 30 °C. The absorbance of solutions was read by a UV-visible spectrophotometer (Beckman DU 530, USA)at 593 nm compared to the control (containing 50µl of distilled water plus 5.1 ml of FRAP reagent). Ammonium ferrous sulphate (100-1000 µM) was used as a control (Figure 5)[13].

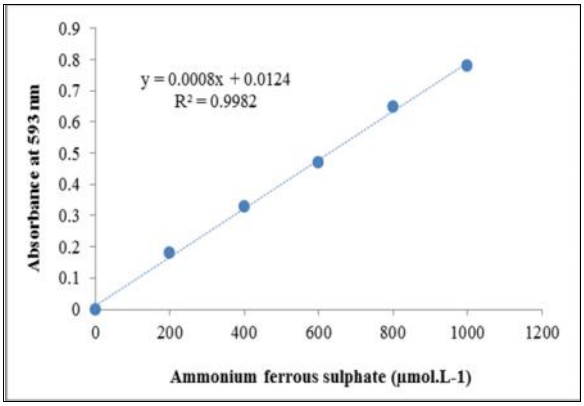

Fig. 5 Calibration curve for Ammonium ferrous sulphate

Ripening Index of the Fruits

It was determined by the ratio of sugar to acid in juice. The amount of total soluble solid in fruit juices was calculated using a refractometer by determining the brix number at laboratory temperature [14]. In order to measure the titratable acid of the fruit, 10 cc juice and 10 cc distilled water and a few drops of phenolphthalein were poured into the Erlenmeyer, and its contents were titrated with 0.1 N NaOH till the emergence of a slight pink colour. Then, considering the volume of NaOH consumed, and using the following formula, we calculated the titratable acid of fruit juice.

Titratable acid=(volume of NaOH consumed)× 100×0.0064×0.1

Calculating the Vitamin C Rate

The amount of vitamin C was determined by iodine titrimetry method. Solutions of Sodium thiosulfate 10 mM, potassium iodide 5 mM and potassium iodide 1 mM were prepared. Titration was performed using sodium thiosulphate solution in acidic environment in the presence of starch reagents. The ending point of the titration was determined by colourlessness (from the original dark purple colour). The amount of iodine reacted with vitamin C was determined by the different titration of iodine with thiosulfate solution and total iodine liberated in the reaction process, which can be calculated based on the volume of thiosulfate solution and its molar concentration. The number of vitamin C moles was determined from the number of iodine moles and according to the volume of initial juice, the number of molar ascorbic acid (vitamin C) was calculated in litters. The amount of this vitamin was calculated by

multiplying the number in 174.2 (molecular weight of vitamin C) in milligrams per 100 ml [15, 16]

Data analysis

The obtained data of pomegranate cultivars were analysed by SAS software. In discussing our results, whenever we compare different characteristics, we apply Duncan multiple range test at 5% probability level.

Results

Study of Phenolic Compounds and Anthocyanin

Based on the gained results, increasing the amount of phenolic compounds along with the increase in the skin colour of the fruit of pomegranate shows a significant increase of 5%.The fruit skin of BSY showed the highest amount of phenolic compounds among the three types of fruits studied. Among the seeds, the WSR seed, had the highest phenolic compounds, but the difference in the phenolic compounds between fruit seeds of the three pomegranate varieties was not significantly different at the 5%. The amount of total anthocyanin in the fruit skin of BSY is at highest level. Among the seeds of pomegranate fruit, the WSR seed had the highest total anthocyanin level. The difference between the total anthocyanin levels between the seeds and also between the skins was significant at 5%. The results of total anthocyanin measurements in these three cultivars showed that the colour of the skins and the seeds had a direct correlation with the total anthocyanin level.

Studying Antioxidant Properties

By DPPH method the BSY showed the highest and the WSR showed the lowest amount of antioxidant activity in the skin (Table.1).The highest amount of antioxidant activity in the seeds was related to WSR and the lowest amount was related to the RSQ (Table 2). In this method, the highest antioxidant activity between the skin and the seeds of three types was related to the skin of the BSY. The results of FRAP assay showed that the amount of antioxidant activity in the WSR seed was at the highest in comparison to the seeds and skins of other types of fruits. The analysis of antioxidant activity by ABTS method showed that the highest antioxidant activity between skin and seeds of three cultivars is related to fruit skin of the BSY. The order of antioxidant activity from the highest to the lowest in skin and seed tissues was similar in all three methods.

ABTS was calculated as the neutralizing percentage of $ABTS^{\bullet+}$. FRAP was calculated as µmol/litr FeII. DPPH was calculated as the anti-radical efficacy.The concentrations of total anthocyanin were calculated as µM/g FW. The concentrations of phenolic compounds were calculated as mg/kg DW.Different letters indicate statistically significant differences ($P \leq 0.05$).

Fruit Ripening Index

The amount of dissolved solids in fruit juice of different cultivars was measured between 14.43 to 17.82 and titratable acidity was also found between 1.2 to 4.1 mg of acid in 100 grams of fruit juice.

Vitamin C

The results of the studying vitamin C revealed that the amount of Vitamin C per 100ml of the pomegranate juice is 54.6±2.2mg for RSQ, 42.8±2.3mg for WSR and 65.7±2.2mg for BSY .The results showed no significant difference between vitamin C in WSR and RSQ but this difference was significant between the WSR and BSY. Also, there was no significant difference between RSQ and BSY.

Correlation between Various Measured Parameters

Studying the correlation between the antioxidant activity values of the extracts with other measured factors (Table 3) indicates that antioxidant activity in DPPH method has a significant positive correlation of 1% with vitamin C content and significant negative correlation with phenol content. This means that by increasing the amount of vitamin C, the antioxidant activity of the fruit extract also increases and by increasing the phenolic content, the antioxidant activity calculated by the DPPH method is decreased. In this method, antioxidant activity with anthocyanin content is not significantly correlated. Antioxidant activity measurement by FRAP method has a significant positive correlation with phenolic and anthocyanin levels, contrary to DPPH, and does not have a significant correlation with vitamin C content. Correlation analysis of antioxidant activity by ABTS method also showed a significant positive correlation with vitamin C content but did not show a significant correlation with phenol and total anthocyanin content.

Table 1. Comparison of the mean of compounds measured in pomegranate peel

	Antioxidant properties				
Cultivar	ABTS	FRAP	DPPH	Total anthocyanin	Phenolic compounds
WSR	66.82 b	215.12 b	0.86 c	3.82 c	33.26 b
BSY	78.38 a	425.21 a	5.84 a	17.78 a	39.68 a
RSQ	75.46 a	349.32 a	3.95 b	11.43 b	37.88 a

Table 2. Comparison of the mean of compounds measured in pomegranate seed

	Antioxidant properties				
Cultivar	ABTS	FRAP	DPPH	Total anthocyanin	Phenolic compounds
WSR	79.53 a	557.38 a	4.32 a	14.86 a	37.89 a
BSY	73.23 b	432.46 b	2.43 b	10.84 b	35.88 a
RSQ	69.86 c	265.23 c	0.93 c	4.65 c	30.18 b

ABTS was calculated as the neutralizing percentage of $ABTS^{\bullet+}$. FRAP was calculated as µmol/litr FeII. DPPH was calculated as the anti-radical efficacy.The concentrations of total anthocyanin were calculated as µM/g FW. The concentrations of phenolic compounds were calculated as mg/kg DW. Different letters indicate statistically significant differences ($P \leq 0.05$).

Table 3. Correlation between various measured parameters

		Antioxidant properties				
ABTS	FRAP	DPPH	Vitamin C	Phenolic compounds	Total anthocyanin	-
1	0.136 ^{ns}	0.536**	0.650**	- 0.081 ^{ns}	0.245^{ns}	ABTS
-	1	0.376 ^{ns}	- 0.291 ^{ns}	0.845**	0.728**	FRAP
-	-	1	0.912**	- 0.624**	- 0.345^{ns}	DPPH
-	-	-	1	**- 0.568	0.205^{ns}	Vitamin C
-	-	-	-	1	0.789**	Phenolic compounds
-	-	-	-	-	1	Total anthocyanin

The results shows that different methods of calculating antioxidant activity results in different data. As in DPPH, the phenolic content has an inverse relationship with antioxidant activity while this relationship is direct in FRAP method and also, in the DPPH method, the level of vitamin C has a direct correlation with antioxidant activity, while in the FRAP method, this relationship was not significant.

Discussion

Phenolic compounds are important herbal ones because of their antioxidant characteristics that play main roles to omitting free radicals and to prevent transformation of hydroperoxides into free radicals [17,18]. Since past researches revealed a direct relationship between antioxidant influence and amount of phenolic compounds [19,20], the amounts of phenolic compounds and anthocyanin's in acidic ethanol extract of pomegranate's fruit was determined. In fact, identifying and investigating amounts of sufficient compounds of the plant is essential considering medical effects and pharmaceutical characteristics. On the base of the results, phenolic compounds increase significantly by more darkening of pomegranate fruit's skin colour in level 5%, but the difference phenolic compounds between seeds of three cultivars of pomegranate wasn't significant; and the results of measuring total anthocyanin's of cultivars showed that skin colour and seed have a direct relationship with total anthocyanin. Because the amounts of compounds in different parts of the plant in different weather conditions, so investigating the compounds in different regions is necessary.

Zarezadeh *et al*. (2016) identified qualitatively and quantitatively anthocyanin's in pomegranate's skin extract. The results of chromatography show that pomegranate's skin has more monogluciside anthocyanins against diglucoside anthocyanins [21]. Alighourchi *et al*. (2013) investigated the influence of ultrasound on the amount of anthocyanins, total phenolic compounds and antioxidant capacity of pomegranate juice. The results show that intensity and different times of ultrasound hadn't considerable influences on pH, acidity and bricks. Amount of anthocyanins and amount of total phenolic compounds increased respectively in some range levels and some tested juices. Moreover, antioxidant activity of all tested juices showed significant differences against test sample [22]. Fool investigated influence maturity on biochemistry content, poly-phenol compound and antioxidant capacity of pomegranate's fruit. The results show that when the fruit completely rises, there is a significant increase of percent of sugar and total anthocyanin's, whereas there are considerable decrease in titration acidity (TA), organic acids and total phenol contents (TPCs). Total antioxidant capacity (DPPH, FRAP) decreased in the period of maturation that it shows decreasing antioxidant power of the juice. The information provided by evaluation and optimization of the juice and its antioxidant value can help to product high-grade pomegranate [23]. Tehranifar et al. (2011) investigated the relationship between antioxidant activities of different parts of the fruit and phenolic contents. The results show that phenolic contents of fruit skin are 1.8 times more than phenolic contents of the leaves and antioxidant capacities of fruit skins, seeds and leaves were 55.3%, 35.7% and 16.4% respectively more. So, it seems that high contents of phenols of fruit skin and seed can make strong antioxidant capacities for the extractions [24]. In the study, antioxidant capacity was investigated using DPPH, FRAP and ABTS methods. The results show that different methods of measuring antioxidant activities reveal different data. In DPPH method, phenolic content has a diverse relationship with antioxidant activity whereas there is direct relationship between them in FRAP; also, in DPPH, there is direct relationship between vitamin C content and antioxidant activity whereas there isn't significant relationship between them in FRAP. Results of ABTS method have a positive correlation with vitamin C content, but they haven't significant correlation with phenolic content and total anthocyanins. DPPH is a simple method but because of stability of nitrogen radical synthetic increase of antioxidant reaction by DPPH and even sometimes no reaction of some antioxidants with free radicals, the above mentioned method is been challenged [25]. Such different factors as reversibility of reactions of free radicals and DPPH, concentration of DPPH and different sample volume, such environmental circumstances as light, oxygen and pH, dependence of harness capacity of most of antioxidants to OH groups, chemical structure of antioxidant and polarity of the environment, type of environment of reaction, time of harness reaction of DPPH and type of solver (protic or aprotic), cause to use different methods for the test, so results of the abovementioned method aren't comparable [26-28]. FRAP is a simple and cheap method in which there isn't any free radicals and antioxidant capacity is investigated with reduction of Fe; each compound that has the potential of oxidation of Fe 3-valenct to Fe 2-valency can interfere in antioxidant level of the sample [29]. Results of FRAP mainly depends on analyse time [30]. TPTZ reacts with poly-phenolic solutions [31]and on the other hand, it doesn't react with thiol-based antioxidants[32].Bilirubin slowly takes a reaction with FRAP and on the other hand it has the characteristic of abstraction in wavelength of 593 nm and it has interference in the method. Reaction of FRAP is sensitive to pH and in order to gain corrector results, acidic and neutral pH must be used [33].In ABST method, the produced radical resist against pH and therefore it can be used to investigate influence of pH on antioxidant capacity. Also, reaction between free radical and antioxidant is done rapidly. On the other hand, producing free radical needs to extra stage in the method and it hasn't long resistance; because of diversity of the results, comparing the testers' results with other researchers will be difficult [34, 35].

Conclusion

The result of the study show that products of pomegranate i.e. fruit's skin and seed, are variety of high-valued rich resources and potential physiological activities. Rich environmental characteristics of pomegranate cause that its fruit is a nutrient and desirable one. Analysing the cultivars on the base of antioxidant capacity,

phenolic contents and total anthocyanin's helps to sufficient selecting, defusing and commercializing the pomegranate. The results encourage specialists of food and drug industries to use pomegranate's fruit to produce pomegranate oil or to formulize nutrients for feeding the human.

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