

ANTIOXIDANT ACTIVITY OF POMEGRANATE

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ABSTRACT

Natural fruits antioxidants play a significant role in inhibiting reactive oxygen species and scavenging free radicals, thus avoiding chronic, degenerative diseases such as cardiovascular disease, aging, cholesterol rates and cancer. The study covered six categories of each three tastes of different pomegranate fruits obtained or purchased in Erbil/Kurdistan-Iraq, a total of 54 samples (6x3x3) were analyzed. Each type was tested for ferric reduction of antioxidant power assay, reduced power method, ascorbic acid content, total phenol and total flavonoid. Our results have shown that the Sour Smilan cultivar has the highest antioxidant properties, ferric antioxidant power reduction (536.89 ± 14.65), ferric power reduction (0.965), ascorbic acid content (0.252 ± 0.012), total phenol (139.66 ± 3.49), total flavonoid (23.08 ± 2.23), and short comparative studies have been conducted to analyze the amount of iron (11) in pomegranate in order to analyze their interferences to the antioxidant activity reported in the ferric reduction of antioxidant power assay.

Key words: total antioxidants ferric reducing antioxidant power, reducing power method, total phenol, and ascorbic acid.

عمر

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نشاط مضاد للأكسدة من الرمان

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المستخلص

تلعب مضادات الأكسدة الطبيعية المنبثقة من الفاكهة دوراً مهماً في تثبيط أنواع الأكسجين التفاعلية وإزالة الجذور الحرة، وبالتالي تمنع الإنسان من الأمراض التنكسية المزمنة مثل أمراض القلب والأوعية الدموية والشيخوخة ومستويات الكوليسترول والسرطان. غطت الدراسة ست فئات من كل نوع من ثلاثة أذواق من ثمار الرمان المختلفة التي تم الحصول عليها أو شراؤها في مدينة أربيل ، وتم تحليل ما مجموعه 54 عينة (3x3x6). تم اختبار كل نوع من اختبار قوة مضادات الأكسدة الحديديك، وطريقة الطاقة المنخفضة ، ومحتوى حمض الأسكوربيك ، والفينول الكلي والفلافونويد الكلي. أظهرت نتائجنا أن الصنف Sour Smilan له أعلى خصائص مضادات الأكسدة ، تخفيض طاقة مضادات الأكسدة الحديديك (536.89 ± 14.65) ، تخفيض طاقة الحديديك (0.965) ، محتوى حمض الأسكوربيك (0.252 ± 0.012) ، إجمالي الفينول (139.66 ± 3.49) ، إجمالي الفلافونويد (23.08 ± 2.23) ، وقد أجريت دراسات مقارنة قصيرة لتحليل كمية الحديد (11) في الرمان من أجل تحليل تداخلاتها مع النشاط المضاد للأكسدة في التخفيض الحديدي لفحص القدرة المضادة للأكسدة. ، وتم إجراء عمل مقارنة قصيرة لتحليل كمية الحديد (11) في الرمان لتحليل تداخلاتها مع اختبار قوة مضادات الأكسدة الحديديك.

الكلمات المفتاحية: إجمالي مضادات الأكسدة الحديديك الحد من الطاقة المضادة للأكسدة، طريقة الحد من الطاقة، مجموع الفينول وحمض الأسكوربيك.

INTRODUCTION

Pomegranate is one of the fastest growing and most grown wild plants in Kurdistan/Iraq. In addition to its fruit qualities, pomegranate is an excellent febrifuge and is used for skin tanning; it is also used in the manufacture of pomegranate molasses commonly used in salads and in many dishes. Pomegranate is well known for its antioxidant properties. Studies in chemical analysis have shown pomegranate juice to be a rich source of antioxidants. (14 , 19) and the bioavailability of these compounds has been investigated[4]. Studies have shown that pomegranate juice is potentially better than apple juice to improve the antioxidant capacity of 1-1.46 mmol. In addition, in elderly subjects, plasma carbonyl content decreased as a biomarker in various inflammatory diseases (9). Pomegranate can also prevent cardiovascular disease (16). Pomegranate, like many other fruits and vegetables, is a rich source of phenolic compounds, including tannins, flavonoids, alkaloids, and organic acids. (4 ,8) ., flavonoids and tannins, have also been individually determined in pomegranate juices (24). Pomegranate isolated flavonoids include flavones, flavonols, anthocyanidins and flavan-3-ols, the amount of which decreases or increases with the time of growth (26). The most effective phenolic antioxidants in plants are flavonoid compounds, and it appears that important health-promoting compounds, including six types of anthocyanins, ellagic acid, phytoestrogen. Ascorbic acid is another important component of pomegranate juice (22). The effects of flavonoids are most prominent on mortality from coronary heart disease and not morbidity (2), and are potent inhibitors of low-density lipoprotein (LDL), both in vivo and in vitro (3,13). These compounds are antimicrobials (6), antivirals (10) and, in addition, the in vitro and in vivo ability of pomegranate peel aqueous extract (PGE) against both fungal pathogens causing brown rot (1). The annual production of pomegranate in Iraq / Kurdistan is steadily growing and stands at 26,000 tons, according to the Ministry of Agriculture and Water Resources. The area has produced 90,000 tons of pomegranate over the last few years, most of the products are organic; the fruit

composition varies with the type, growing conditions, climate, maturity and storage conditions. The aim of this paper is to evaluate the antioxidant content of pomegranate arils (by hand pressing and diluting with water as we drink it), from different geographical areas and from different tastes (sweet, sour-sweet and sour), as well as to screen the amount of iron in pomegranate and to examine its interference with the antioxidant activity recorded in the ferric reducing antioxidant power assay (FRAP) study.

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in the study were of analytical grade and mostly purchased from Fluke chemicals.

Sample collection

Pomegranate fruit has been obtained from a local farmer or market in Erbil City. The study includes six groups Pomegranate from different Kurdistan Regions of Iraq; group 1-Smaqli, group 2-Rawanduz, group 3-Smilan, group 4-Shaqlawa, group 5-Halabja, and group 6-Miscellaneous fruits (available for sale in Erbil market)

Sample preparation

The fresh fruit of the pomegranate washed very well with water, washed three times with distilled water and peeled. The arils were separated, a portion of 0.5 g was weighed and the juice was obtained by hand pressing with 50 mL of distilled water. The homogenate has been centrifuged. The supernatant was filtered, the filtrate was stored in a dark closed container and used for various tests, each analysis achieved on duplicate weight, and all tests were triplicates within a week.

Detection of total antioxidant activity FRAP chemical analysis

Antioxidant activity was measured by mixing 300 mM acetate buffer (pH3.6), 10 mM TPTZ(2,4,6-tripyridyl -s- trizaine) solution in 40 mM HCl and 20 mM FeCl₃.6H₂O in a ratio of 10:1:1 at 37 ° C, using FRAP assay by Benzie and Strain[20].50 µL of the sample supernatant was added to the 1.5 mL of freshly prepared and pre-heated (37°C) FRAP reagent in the test tube and incubated at 37°C for 10 min. The Blue Colored Complex Absorption was read at λ593nm against a blank reagent. Quantitative analysis was performed using an

external standard method using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1000, 750, 500, 250, 125 micro mole/ L; $y = 0.0017x + 0.0001$; $R^2 = 0.9987$) as standard and corresponding absorbance to the concentration. The result obtained from the standard curve is micro molar $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, these values were represented as the antioxidant concentration having a ferric reduction potential.

Reducing power method (RP)

The potassium ferricyanid-ferric chloride method by Oyaizu 1986 (18) was used to measure the reducing power of the fruit extracts. In this assay, 0.5 mL of sample supernatant was combined with 0.5 mL of phosphate buffer (0.2 M, PH 6.6) and 0.5 mL of (1%) potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$]. The mixture was incubated at 50°C for 20 min, followed by an addition of 0.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm, in this method 0.5 mL of sample supernatant were mixed with 0.5 mL of phosphate buffer (0.2 M, PH 6.6) and 0.5 mL of (1%) potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$]. The mixture were incubated at 50°C for 20 min followed by addition of 0.5 mL of 10% trichloroacetic acid. Lastly 1 mL of the upper part was combined with 1 mL of 1 percent FeCl_3 ferric chloride. The absorbance was measured against blank at $\lambda 700$ nm.

Determination of ascorbic acid

The fruit extract's ascorbic acid content was calculated using the Suntornsuk et al. 2002 [22] test. 2 mL of the sample was transferred to 25 mL of conical flask. 2 mL of 2N H_2SO_4 was added, followed by 5 mL of distilled water and 0.5 mL of starch indicator. The solution was well mixed and titrated with 0.001 N iodine. One ml of iodine = 8.806 mg of ascorbic acid.

Determination of total phenolic

Folin-Ciocalteu method Velioglu et al (25), applied to determine the total phenolic content of the pomegranate. The supernatant (200 μL) was mixed with 1.5 mL of Folin-Ciocalteu reagent [previously diluted 10 times with double distilled water] and allowed to stand at room temperature for 5 min. 1.5 mL of 7.5% sodium bicarbonate solution was added to the mixture and incubated for 90 min at room temperature. The absorbance level has been measured at 725 nm. Total phenolic was quantified through the calibration curve

obtained by measuring the absorbance of known concentrations of gallic acid standard solutions [25-150 mg / L]. The standard gallic acid line equation $y = 0.0065x - 0.0214$; $R^2 = 0.9942$. The results were calculated and reported as mean \pm standard deviation (SD).

Total flavonoid content

Total flavonoid content was determined according to the method described by (25). 1 mL of plant extract was added to 10 mL volumetric flask containing 4 mL of double distilled water. Then 0.3 mL 5% NaNO_2 was added to the flask stand for 5 min, then 0.3 mL 10% AlCl_3 was added. At 6th min, 2 mL NaOH (1 M) was added and the total volume was made up to 10 mL with double distilled water. The absorbance of the reaction mixture was measured at 510 nm versus prepared reagent blank. Quercetin was used as standard and flavonoid content was determined as quercetin equivalent.

Quantitative iron analysis

Stock solution of ferrous chloride tetra hydrate 1.14 mg /mL ppm was prepared. This stock solution was then serially diluted to get the standard solutions of 1-20 ppm of element iron. The standard solutions were then analyzed using atomic absorption spectrometer. The recorded values were used to generate a standard curve of ferrous chloride. Then the fruit extract solutions were analyzed to quantitate the amount of iron in pomegranate samples.

Statistical analysis

Data were reported as mean \pm standard deviation. The mean values of antioxidant activity in different cultivars were compared using the variance analysis (ANOVA) test. When significant ($p < 0.05$) difference was detected by Graph Pad Prism in Windows, Version 7 (Graph Pad Software). Linear regression coefficient (R^2) for phenolic and flavonoid content with antioxidant activity was analyzed.

RESULTS AND DISCUSSION

FRAP assay

The FRAP test is based on the reduction of Fe^{3+} Tripyridyltriazine $\text{Fe}(\text{TPTZ})^{3+}$ complex, to the strongly blue Fe^{2+} complex $\text{Fe}(\text{TPTZ})^{2+}$ by antioxidants in the acidic medium. Results are obtained as absorbance rises at 593 nm and can be expressed as equivalents of micromolar

Fe²⁺ equivalents or relative to an antioxidant standard. However, that the measured capacity reduction does not necessarily indicate antioxidant activity. Instead it offers a very useful 'total' antioxidant concentration, without calculating and summing the concentration of all the antioxidants involved. Higher value means a better reduction of the sample's capacity. The current study was conducted to determine the antioxidant capacity of the pomegranate extract and to use it as a replacement for synthetic antioxidants. Table 1

summarizes the FRAP value and ascorbic acid antioxidant activity of six different pomegranate cultivars. (Mean FRAP value±SD : a systematic comparison was made between the antioxidant activity of six different cultivars with the ANOVA test (Fig. 1, 2, and 3). Tests suggested significant differences for the taste variables between cultivars figure 1. Furthermore, the results as shown in figure 2, 3 indicate that simelan–sour has the highest FRAP value (517.28), and Smaquli –sweet has the lowest value (261.65).

Table 1. The total antioxidant activity of pomegranate pulp extract as FRAP value, in comparison with vitamin C.

Cultivar	μMFeSO ₄ .7H ₂ O ± SD			mM vit.C ± SD		
	Sweet	Sweet-Sour	Sour	Sweet	Sweet-Sour	Sour
Group 1	261.56 ±13.23	274.83±20.37	348.09±16.53	0.134±0.012	0.153±0.013	0.187±0.011
Group 2	267.48±23.21	289.36±17.27	380.21±20.58	0.148±0.014	0.160±0.012	0.201±0.013
Group 3	349.64±25.44	460.96±22.73	536.89±14.65	0.198±0.008	0.235±0.012	0.252±0.012
Group 4	259.64±19.78	342.32±23.21	351.55±14.41	0.158±0.021	0.187±0.013	0.231±0.012
Group 5	338.25±15.46	354.33±11.54	355.11±20.60	0.172±0.015	0.203±0.020	0.198±0.020
Group 6	289.46±19.63	287.67±24.13	367.99±21.03	0.170±0.012	0.187±0.021	0.192±0.014

Values represent means ± standard deviations calculated from three replicates.

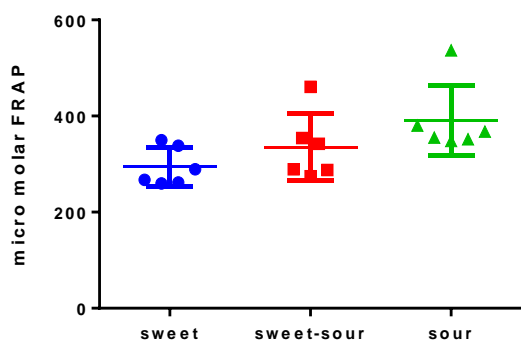


Fig. 1. The antioxidant activities of six different pomegranate (The ANOVA test shows the significant difference between the samples, p<0.05).

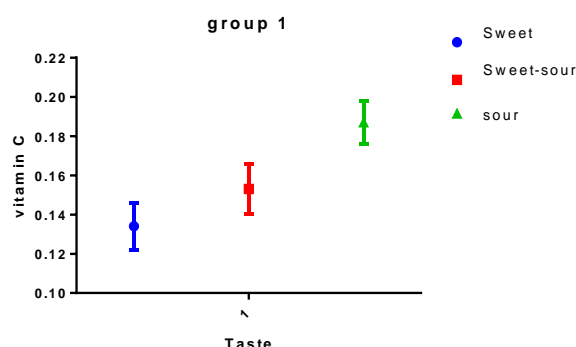


Fig. 3. The antioxidant activities (vitamin C) of six different pomegranate (The ANOVA test shows the significant difference between the samples, p<0.05).

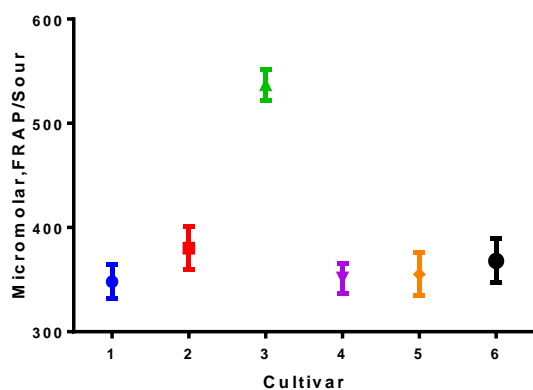


Fig. 2. The antioxidant activities of six sour of different pomegranate (The ANOVA test shows the significant difference between the samples, p<0.05).

Ferric reducing antioxidant power (FRAP) assay: The reduction power of Fe²⁺ by the selected pomegranate was evaluated. The highest antioxidant-reducing power was recorded for simelan sour. The conversion ability of compounds from Fe³⁺ /ferricyanide complex to Fe²⁺/ferrous form acts as a potential indicator of antioxidant activity. For this experiment, depending on the reduction power of extracts or compounds, the yellow color test solution changes to green and blue . The presence of reductants in the test solution reduces Fe³⁺ to Fe²⁺, which can be monitored by measuring the Prussian blue color at 700 nm . The increase in reaction mixture

absorbance indicates the increased sample activity reduction (Figure 4). Lower reaction mixture absorbance indicates greater reduction strength.

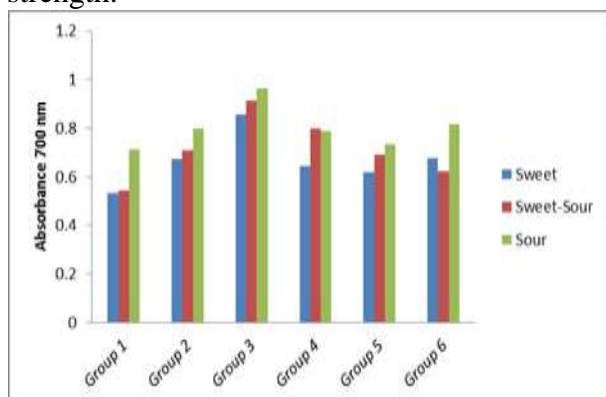


Fig. 4. Ferric reducing antioxidant power of selected pomegranate fruits. Results expressed as the mean \pm SD (n = 3) Of absorbance at 700nm

Table 2. The phenolic content and flavonoid of selected pomegranate

Cultivar	Total phenol mg/L galic acid \pm RSD			Total flavonoid mg /L Quercetin		
	Sweet	Sweet-Sour	Sour	Sweet	Sweet-Sour	Sour
Group 1	35.94 \pm 7.43	47.67 \pm 2.19	68.29 \pm 3.44	16.23 \pm 1.35	17.34 \pm 1.45	17.17 \pm 2.16
Group 2	39.75 \pm 3.20	51.73 \pm 1.19	52.69 \pm 4.80	16.77 \pm 2.15	16.67 \pm 2.17	17.40 \pm 4.13
Group 3	70.14 \pm 2.02	90.69 \pm 2.84	139.66 \pm 3.49	19.23 \pm 4.56	19.24 \pm 2.56	23.08 \pm 2.23
Group 4	33.20 \pm 5.28	55.14 \pm 5.49	53.97 \pm 2.10	17.45 \pm 2.17	16.32 \pm 2.42	16.54 \pm 3.18
Group 5	38.60 \pm 5.14	44.84 \pm 3.06	62.94 \pm 2.93	18.21 \pm 4.45	11.45 \pm 3.31	14.32 \pm 2.37
Group 6	37.38 \pm 3.53	49.54 \pm 2.80	83.57 \pm 6.97	11.76 \pm 1.76	15.54 \pm 1.23	12.76 \pm 2.31

Correlation between the total phenolic and flavonoid content, and the antioxidant activity Phenolic and flavonoid molecules are important antioxidant components that are responsible for the deactivation of free radicals based on their ability to donate hydrogen atoms to free radicals. They also have ideal structural features for free radical scavenging[41]. Different literature reports indicate a linear correlation between total phenolic and flavonoid content and antioxidant capacity[25]. The correlation between the total phenolic and flavonoid content and the antioxidant capacity is shown in Figure 5 a, b. High correlations between antioxidant capacity and total phenols ($R^2 = 0.8537$) and total flavonoids ($R^2 = 0.7106$) were observed. By comparing the correlation coefficients (R-values), it is possible to suggest that the phenolic and flavonoid groups are highly responsible for the antioxidant activity of the selected pomegranate extracts.

Total phenolic content

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. The hydroxyl groups in fruit extracts are responsible for facilitating free radical scavenging. As a basis, phenolic content was measured using the Folin–Ciocalteu reagent in each extract. The results were derived from a calibration curve ($y = 0.0065x - 0.0214; R^2 = 0.9942$) of gallic acid (0–250 μ g/mL) and expressed in gallic acid equivalents (Table 2). The content of phenolic compounds in pomegranate extracts ranged from 33.20 \pm 5.28 to 139.66 \pm 3.49 mg.

Total flavonoid content

As a basis quantitative determination, flavonoid contents in selected pomegranate extracts were determined using aluminum chloride in a colorimetric method. (Table 2) Smilan sour had the greatest flavonoid content (23.08 \pm 2.23)

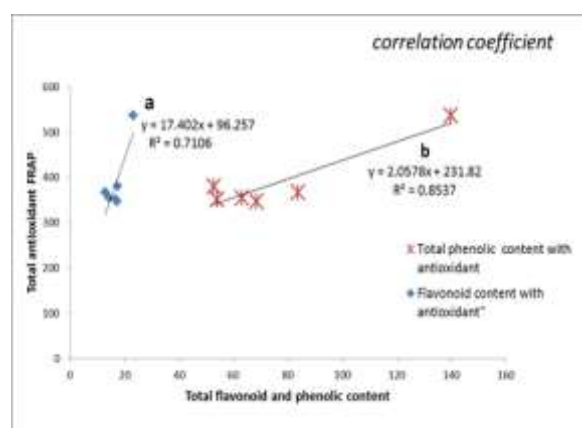


Figure 5. Graphs represent the mean value of antioxidant activity vs. (a) total phenolic content, and (b) total flavonoid content and antioxidant activity.

Calculation of the correlation coefficient includes the expectation that the relationship between the two variables is linear. There is a basic rule for measuring the strength of a relationship based on its R value: the value of R reflects the strength of the relationship if R

< 0.3 None or very weak, $0.3 < R < 0.7$ Normal, and $R > 0.7$ Strong. In the current study, the relationship between total phenolic, flavonoid and antioxidant activity is generally considered strong due to r values greater than 0.7.

Quantitative iron analysis

The most common antioxidant assays use the ability of antioxidants to produce colored complexes, such as ferric antioxidant FRAP, by transferring electrons to reduce ions. The total antioxidant activity of phytochemicals and their antioxidant mechanisms may vary with changes in the total antioxidant concentration, the presence of competing metals such as iron and copper, the different temperatures, the solvent used to dissolve antioxidant samples, the antioxidant position in different phases, the pH sample, the oxidation stage and the presence of oxygen during testing (11). Of all the factors mentioned above, whole plant extracts often contain significant amounts of iron and are sources of minerals as part of their nutraceutical significance. Fe(II) in samples may therefore interfere with the antioxidant activity reported throughout the FRAP and RP assays. In the current study, the amount of iron was calculated using atomic absorption spectroscopy, the findings indicate that a negligible amount of iron in the pomegranate samples has been observed, or that the amount of iron in the pomegranate extract is too small to influence the overall antioxidant activity, and this amount may be ignored when estimating the antioxidant activity of these extracts.

Conclusion

Antioxidants play an essential role in the oxidizing of oxidative stress in body cells caused by free radicals. This study illustrated the antioxidant analysis of various pomegranate fruits from different geographical and climatic regions in Kurdistan/Iraq. This research has been applied to a limited number of samples of a few varieties and our recommendation is to carry out further studies to check each variety separately and to consider the harvesting season, as this time was not taken into account in this analysis. The analysis was carried out at different seasons and a section of the groups was

analyzed with samples that were completely immature.

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