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Antioxidant Activity, Polyphenol Content, and Related Compounds in Different Fruit Juices and Homogenates Prepared from 29 Different Pomegranate Accessions

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Pomegranate juice is well known for its health beneficial compounds, which can be attributed to its high level of antioxidant activity and total polyphenol content. Our objective was to study the relationships between antioxidant activity, total polyphenol content, total anthocyanins content, and the levels of four major hydrolyzable tannins in four different juices/homogenates prepared from different sections of the fruit. To this end, 29 different accessions were tested. The results showed that the antioxidant activity in aril juice correlated significantly to the total polyphenol and anthocyanin contents. However, the homogenates prepared from the whole fruit exhibited an approximately 20-fold higher antioxidant activity than the level found in the aril juice. Unlike the arils, the antioxidant level in the homogenates correlated significantly to the content of the four hydrolyzable tannins in which punicalagin is predominant, while no correlation was found to the level of anthocyanins.

KEYWORDS: Pomegranate; *Punica granatum* L.; antioxidant activity; polyphenol; anthocyanin; hydrolyzable tannins

INTRODUCTION

The pomegranate tree and its fruit have been employed extensively in the folk medicine remedies of many cultures (*1*). The traditional importance of pomegranate as a medicinal plant is now backed by data obtained from modern science. Recent research indeed shows that compounds in the fruit are anticarcinogenic (*2–7*), inhibit tumor initiation and development (*8, 9*), have antioxidant activity (*10–14*), and possess antimicrobial (*15, 16*) and antiviral activities (*17, 18*). Recent biological studies have proven that certain compounds contained in pomegranate juice (PJ), which has been shown to reduce blood pressure, are also antiatherosclerotic and significantly reduce low density lipoprotein (LDL) oxidation (*10, 12, 19, 20*). These activities are attributed to the pomegranate's high levels of antioxidant activity and its high total polyphenols content (*10, 14, 21*). Chemical analyses have shown that the phenol fraction of PJ contains a significantly high level of hydrolyzable tannins as well as anthocyanins, which exhibited high antioxidant activities (*14, 21*). Indeed, PJ was shown to possess a 3-fold higher antioxidant activity than that of red wine or green tea (*14*), and two-, six- and eight-fold higher levels than those detected in grape/cranberry, grapefruit, and orange juice, respectively (*22, 23*).

Because of the extensive knowledge about pomegranate's health attributes and increasing public awareness about nutritional food, the demand for the pomegranate fruit and its by-products has increased tremendously in the Western world. As a result of this trend, the extent of pomegranate growth was increased significantly in many regions throughout the world. Consequently, industries producing PJ were developed, as well as pharmaceutical companies, which extracted health beneficial compounds from the fruit (*24, 25*). However, most of the data on the health beneficial compounds of PJ were derived from one pomegranate accession, called Wonderful (*26*). Therefore, the main objective of this study was to compare the levels of antioxidants, total polyphenols, total anthocyanins, and the four major hydrolyzable tannins, which were previously suggested as being dominant in their contribution to the antioxidant activity of PJ (*14, 21*), in more than 20 pomegranates accessions. These accessions were chosen from a large collection present in Newe Ya'ar located in northern Israel, and they include many local and domestic types. Since pomegranates are consumed in our region in different ways, our further objective was to study

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Figure 1. The pomegranate accessions used in this study as photographed in the season of 2006.

polyphenol content and antioxidant activity in juices prepared from arils, from arils and the inner part of the fruit prepared in a juice extractor, from homogenates prepared from the whole fruit, and from the peel銼s homogenate.

Using statistical tools, we wanted to gain more knowledge about the relationships between the levels of antioxidants, total polyphenols, total anthocyanins, and the content of four major hydrolyzable tannins in different components of the fruit. In addition, we studied natural variations within the different pomegranate accessions. The data indicate that the homogenates of whole fruit have an approximately 20-fold higher antioxidant activity than that of aril juice. While the antioxidant activity of the homogenates correlated significantly with the level of the four major hydrolyzable tannins, the antioxidant activity of aril juice correlated with the total anthocyanins level, suggesting that the major component contributing to antioxidant activity in aril juice are the anthocyanins compounds.

MATERIALS AND METHODS

Plant Materials and Fruit Processing. Twenty-three (in the 2005 season) and 29 (in the 2006 season) pomegranate accessions were chosen from a collection in the Newe Ya'ar research center, ARO [registered in the Israel Gene Bank for Agriculture Crops (IBG, Web site: http://igb.agri.gov.il)] (*27*). These accessions differed in their peel and aril colors (**Figure 1**) as well as in their taste. The different accessions were harvested during the period of August to November 2005 and 2006. The trees were at least five years old and planted at a 3×5 m distance in 3–5 replicate trees per accession. Ten fruits from each accession (3–4 fruits from each tree) were harvested when fully matured according to commercial practice. The fruits were transported via a ventilated car to the laboratory, where they were characterized by physical (fruit weight, peel, and aril weights) and chemical parameters, as described below.

Table 1. LC-MS Analysis of the Four Hydrolyzable Tannins

compound	gradient number	cone voltage (V)	collision energy (eV)	mother ion (*m*/*z*)	daughter ion (*m*/*z*)
punicalagin	1	190	57	1083.7	601.0
punicalin	2	160	40	781.5	601.2
gallagic acid	3	260	34	601.3	299.2
ellagic acid	4	135	32	301.2	284.2

In the 2005 season, every fruit from the 10 fruits obtained for each pomegranate accession was divided in half. One-half was squeezed using a juice extractor; in this way, the inner part of the peel, the white membrane, and the arils were squeezed. The arils from the second half were hand-separated, weighed, and squeezed using a nylon sieve to produce the arils juice. The two different juices were centrifuged (15 min at 4000 rpm), and five pools (each containing juices prepared from two fruits) were prepared for each juice type (the arils juice and the squeezed juice). The juices were then frozen at −20 °C for further analysis.

In the 2006 season, 150 g from each fruit of each accession was homogenized (for 2 min) using a blender (Aghetto, model FC/7) with 150 mL of distilled water to prepare the fruit homogenate. The arils and peels from the other parts of the fruit were separated and weighed. Juice was prepared from the arils as described above, while 150 g of peels were homogenized (for 2 min) with 300 mL of distilled water. The two kinds of homogenates and the arils juice were then centrifuged (4000 rpm for 15 min). Five pools were prepared from the 10 fruits of each accession, each pool containing the homogenates or the juice prepared from two fruits. The aril juice and the homogenates were then frozen at −20 °C for further analysis.

Determination of Total Phenol Content. For total phenol compounds determination, 1:10 dilutions of the juices were used. Total

Table 2. Correlation Matrix between Antioxidant Activities Methods, Total Polyphenols, Total Anthocyanins, and the Levels of the Four Hydrolyzable Tannins in Juice Prepared from the Arils of the 29 Pomegranate Accessions According To the Pearson Test in the 2006 Season[a]

	antioxidant activity FRAP	antioxidant activity DPPH	total polyphenols	total anthocyanins	punicalagin	punicalin	gallagic acid	% arils
antioxidant activity FRAP	1	0.83**	0.86**	0.68**	0.16	−0.02	0.10	0.01
antioxidant activity DPPH		1	0.62**	0.265	0.48**	0.12	0.39*	0.02
total polyphenols			1	0.71**	−0.10	0.01	−0.06	0.02
total anthocyanin				1	−0.34	−0.14	−0.31	0.07
punicalagin					1	0.14	0.45*	−0.31
punicalin						1	0.79**	0.19
gallagic acid							1	0.14
% arils								1

[a] The *r* value of the correlation is given and its significance ($p < 0.05$) is identified by one asterisk, while ($p < 0.01$) is identified by two asterisks.

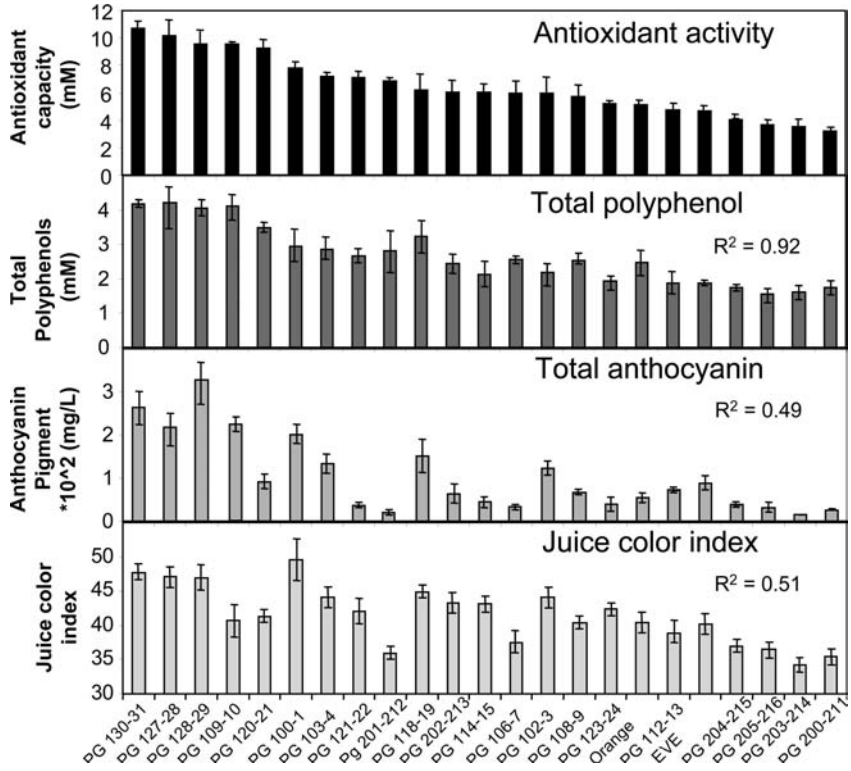

Figure 2. Antioxidant activity, total polyphenol content, total anthocyanin level, and juice color in aril juices prepared from 23 pomegranate accessions in the 2005 season. The data presented represent the mean ± SD of five replicates from each accession; each of the replicates is a pool of two fruits. The R^2 value was calculated against the antioxidant activity.

phenols were determined using the colorimetric method with a spectrophotometer, which modified the Singleton method for small volumes (*29*). The phenols were determined by the folin-Ciocalteu reagent (1:10 in water), 20% Na_2CO_3, using quercetin as a standard. The absorbance was measured at 760 nm.

Antioxidant Activity Evaluation. Two methods were used to analyze the antioxidant activity of PJ as previously recommended (*14*). The first method assayed used 2,2 diphenyl-1-picrylhydrazyl (DPPH), which is a radical generating substance widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants. Increased concentrations of juice (0–14 μmol of polyphenols/L) were mixed with 3 mL of 0.1 mmol DPPH/L in ethanol. The time course for the change in optical density at 517 nm was monitored kinetically.

The second method assayed was the FRAP method (*14*), which was developed to measure the ferric reduction ability of plasma at a low pH. An intense blue color is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form, which was recorded at 593 nm. Standard solution of 1 mM of L-ascorbic acid in deionized water and 1 mM 6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol were prepared. The pomegranate juices were diluted 1:50 (v:v) in water. Fifty microliters of diluted standards of samples were mixed with 950 μL Fe^{3+} solutions. These solutions were left to react for a period of time (15 min for the DPPH method and 4 min for the FRAP method) under continuous stirring. The changes in absorbance were then measured at 25 °C. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) (*14*).

Determination of Total Anthocyanin Content. Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra (*30*, *31*). The colored oxonium form predominates at pH 1.0 (25 mM potassium chloride buffer), and the colorless form predominates at pH 4.5 (0.4 M sodium acetate buffer). The samples were diluted by a potassium chloride buffer until the absorbance of the sample at a 510 nm wavelength was within the linear range of the spectrophotometer. This dilution factor was used later to dilute the sample with the sodium acetate buffer. The wavelength reading was performed after 15 min of incubation, four times for each sample diluted in the two different buffers and at two wavelengths of 510 and 700 nm. The absorbance was then calculated according to the following equation: $A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$. Results were expressed as mg of cyanidin-3-glucoside per 1 L of juice, using a molar absorptive coefficient (ε) of 26900 and a molecular weight of 449.2.

Determination of Aril Juice Color. The color of the juice prepared from the arils was determined using a colorimeter (Chroma Meter CR-

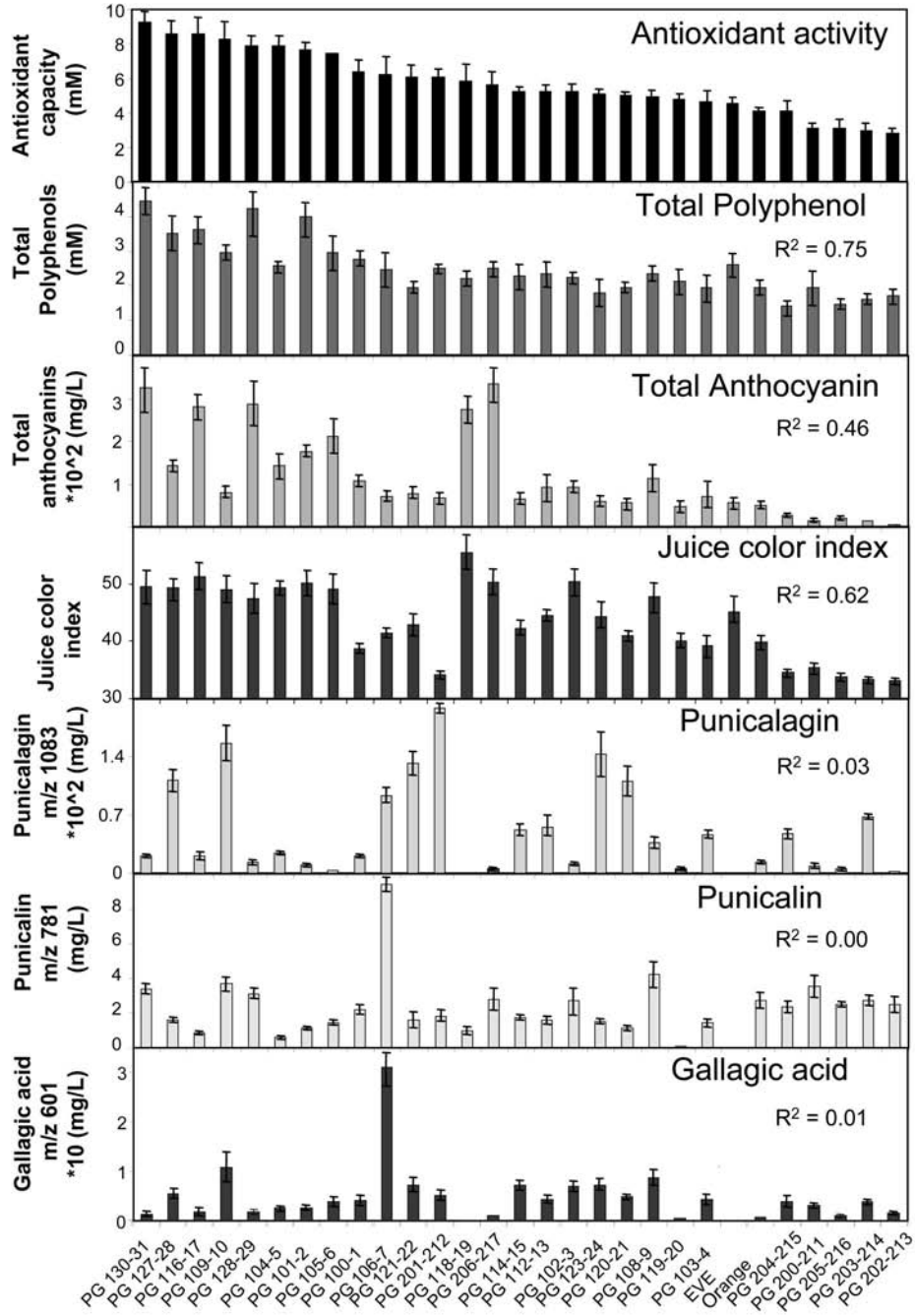

Figure 3. Antioxidant activity, total polyphenol content, total anthocyanin level, juice color, and the content of four members of the hydrolyzable tannins in aril juices prepared from 29 pomegranate accessions in the 2006 season. The data presented represent the mean ± SD of five replicates from each accession; each of the replicates is a pool of two fruits. The R^2 value was calculated against the antioxidant activity.

301, Minolta, Ramsey, NJ, USA) (*37*). The dimensions of *L*, *H*, and *C* were measured, and the hue angle (0° = red-purple; 90° = yellow) and chroma (departure from gray towards a pure chromatic color) parameters were calculated according to the following equation: $(180 - H)/(L + C)$. The values obtained are referred to as the juice color index.

Lipoprotein Isolation. LDL was isolated from fresh plasma samples taken from healthy volunteers by discontinuous density gradient ultracentrifugation, as previously described (*32*). The LDL was washed at d =1.063 g/mL, dialyzed against 150 mmol/L NaCl, 1 mmol Na_2EDTA (pH 7.4) at 4 °C. The LDL fractions were then sterilized by filtration (0.45 μm), kept under nitrogen in the dark at 4 °C, and used within 1 week. The lipoprotein concentration was determined by the Lowry assay. The LDL was dialyzed against EDTA-free, phosphate buffered saline (PBS) solution at pH 7.4 and 4 °C.

LDL Oxidation Studies. The inhibition of LDL oxidation by PJ was determined, as previously described (*10*). LDL (100 μg of protein/L) was incubated with 5 μmol/L of $CuSO_4$ for 2 h at 37 °C. The amounts of LDL-associated lipid peroxides and thiobarbituric acid reactive substances (TBARS) were then measured. The extent of lipid peroxidation was analyzed using 100 μL of pomegranate juice-treated LDL solutions as their capacity to convert iodide to iodine, measured colorimetrically at 365 nm (*33*). The extent of LDL oxidation was also measured by the TBARS assay at 532 nm using malondialdehyde (MDA) as a standard (*34*).

LC-MS Analysis of Hydrolyzable Tannins. Sample preparation: the aril juice and homogenate samples from the different accessions were diluted with doubled distilled water (DDW) 1:10 or 1:1.000, respectively. The samples were further diluted at 1:1 with acetonitrile (Merck cat. no. 30) to achieve final concentrations of 1:20 and 1:2000, respectively. The samples were filtered with a 0.45 μm into testing vials. The samples were analyzed by an LC-MS instrument using a Waters 2790 HPLC system equipped with a Micromass triple quadrupole Quatro-Ultima mass

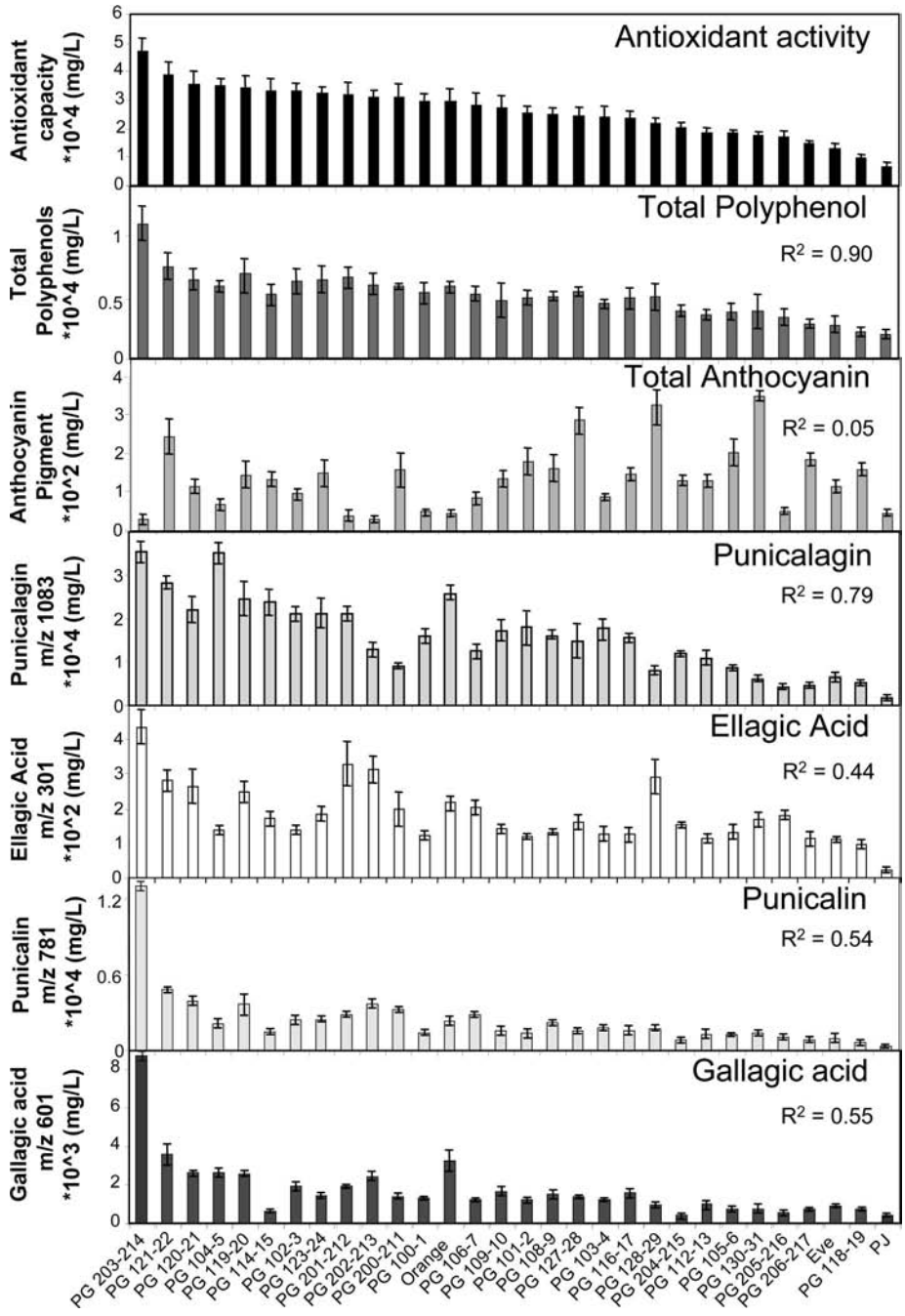

Figure 4. Antioxidant activity, total polyphenol content, total anthocyanin level, and the content of four members of the hydrolyzable tannins in homogenates prepared from the whole fruit, of 29 pomegranate accessions used in this study in the 2006 season. The data presented represent the mean ± SD of five replicates from each accession; each of the replicates is a pool of two fruits. The R^2 value was calculated against the antioxidant activity.

spectrometer in series, consisting of a HPLC quaternary pump, an autosampler, and a vacuum degasser. The system was controlled by Micromass–MassLynx ver. 4.0 software. Chromatographic separations were carried out on a reverse phase ODS Hypersill column (2.1 × 100 mm, particle size 5 μm, Thermo cat. no. 30105-102130) using acetonitrile (A) and DDW (B) as the mobile phases at a flow rate of 0.5 mL/min on a linear gradient mode. The solvent linear gradient for Punicalagin started with 100% (A), 3 min 70% (A), 5 min 100% (A) (gradient no. 1); the isocratic mode for Punicalin started with 50% (A) for 5 min (gradient no. 2); for gallagic acid with 70% (A), 2 min 100% (A), 4 min 70% (A), (gradient no. 3); and for ellagic acid with 100% (A), 3 min 50% (A), 5 min 100% (A) (gradient no. 4). The solute was inserted into the mass spectrometer using an electro spray ionization (ESI) probe in the negative mode. The high selectivity identity of the compound was obtained using the multiple reaction monitoring (MRM) method according to their mother and daughter ions. The mother ion (precursor ion) was fragmented by argon using different collision energies, as described in **Table 1**. The daughter ion areas of the standard solutions were compared to those received from the pomegranate samples. The specific LC-MS conditions for each of these phytochemicals were for punicalagin, ESI capillary voltage (kV) = 3.5 source temperature (°C) = 120, desolvation temperature (°C) = 300, cone gas flow (L/h) = off, desolvation gas flow (L/h) = 555, multiplier (V) = 650; for punicalin, ESI capillary voltage (kV) = 3.4 source temperature (°C) = 150, desolvation temperature (°C) = 400, cone gas flow (L/h) = off, desolvation gas flow (L/h) = 501, multiplier (V) = 650; for gallagic acid, ESI capillary voltage (kV) = 4.2 source temperature (°C) = 120, desolvation temperature (°C) = 350, cone gas flow (L/h) = off, desolvation gas flow (L/h) = 504, multiplier (V) = 650; and for ellagic acid, ESI capillary voltage (kV) = 3.0 source temperature (°C) = 120, desolvation temperature (°C) = 350, cone gas flow (L/h) = off, desolvation gas flow (L/h) = 529, multiplier (V) = 650.

Statistical Analysis. The data obtained from this study were analyzed statistically using SPSS software adapted to Windows, ver. 14. In this

Table 3. Correlation Matrix (Pearson Test) Conducted on Data Obtained from Homogenates Prepared from the Whole Fruit of 29 Pomegranate Accessions in the 2006 Season[a]

	antioxidant activity FRAP	antioxidant activity DPPH	total polyphenols	total anthocyanins	punicalagin	ellagic acid	punicalin	gallagic acid	% peels
antioxidant activity FRAP	1	0.77**	0.95**	−0.34	0.87**	0.665**	0.74**	0.74**	0.16
antioxidant activity DPPH		1	0.71**	−0.23	0.64**	0.38*	0.32	0.32	−0.00
total polyphenols			1	−0.33	0.87**	0.66**	0.78**	0.81**	0.08
total anthocyanin				1	−0.36	−0.18	−0.28	−0.33	−0.34
punicalagin					1	0.45*	0.61**	0.72**	0.36
ellagic acid						1	0.81**	0.73**	0.11
punicalin							1	0.95**	−0.04
gallagic acid								1	0.12
% peels									1

[a] The r value of the correlation is given and its significance [($p < 0.05$) is identified by one asterisk, while ($p < 0.01$) is identified by two asterisks].

software, Pearson or Spearman tests were used for the correlation studies and their significance.

RESULTS AND DISCUSSION

Twenty-three (in the 2005 season) and 29 (in the 2006 season) pomegranate accessions were selected from the Newe Ya'ar collection. This collection contains about 60 accessions that include many local and domestic types. The accessions chosen for the current study differed in their peel and aril colors (**Figure 1**), taste, and strength of seed shell.

Juices Prepared from Accessions Having a Darker Aril Color Exhibit Higher Antioxidant Activity in Comparison To Accessions Having Lighter Arils. The beneficial health effects attributed to pomegranate fruit consumption are related, at least in part, to their antioxidant activity (*23*, *35*). Since pomegranate arils are largely consumed in our region, eaten freshly or used for salad dressings and in desserts, we first examined the level of antioxidant activity of juices prepared from the arils of 23 and 29 pomegranate accessions harvested in the 2005 and 2006 seasons, respectively. The values calculated as equivalent ascorbic acid were very similar to those obtained for the Trolox equivalent; thus, the results are given as a Trolox equivalent. Both methods describing the antioxidant capacity of the juices correlated significantly to each other ($r = 0.83$) (**Table 2**). Since the FRAP method gave the highest correlation to most of the other parameters tested (**Table 2**), the results described in **Figures 2** and **3** show the value obtained using this method. The pomegranate accessions were graded from the accession having the highest antioxidant value (P.G. 130–31) in both seasons to the accession having the lowest value in line (P.G. 200–211 and line P.G. 202–213, **Figures 2** and **3**). Line 130–31 has an approximate 2.5- and 3-fold higher antioxidant activity level than that found in the lowest lines. Basically, the results of the two seasons were very similar; however, some differences were found that could be attributed to the different climate and growth conditions prevailing during these seasons.

In many fruits and vegetables, the level of antioxidant activity can be attributed to the level of total polyphenol content (*14*, *23*, *36–40*). Therefore, the total polyphenol levels were measured in these juices. It was found that the antioxidant level (according to FRAP methods) and polyphenol content were positively and significantly correlated ($r = 0.95$ for the season of 2005, $r = 0.86$ for the season of 2006) (**Table 2**; **Figures 2** and **3**), suggesting that polyphenols contributed significantly to the arils' antioxidant activity. Polyphenols are comprised of many different types of phytochemical compounds, in which one of the dominant classes in fruits are the anthocyanins belonging to the flavonoides group of polyphenols (*36*). Anthocyanins are water-soluble pigments primarily responsible for the attractive color of many fruits, including pomegranate juices, and they are well known for their antioxidant activity (*13*, *36*, *41–43*). Examination of the total level of anthocyanins in these jucies demonstrated that indeed the anthocyanins significantly correlated to the antioxidant activity ($r = 0.7$ and $r = 0.68$ for the 2005 and 2006 seasons, respectively) and to the polyphenol content ($r = 0.61$ and $r = 0.71$ for the 2005 and 2006 seasons, respectively) (**Table 2**).

The finding reported herein allows us to emphasize two points: (i) the antioxidant activity of pomegranate aril juice may be attributed, to a great extent, to total phenol content; and (ii) antioxidant efficiency appears to be related to the level of anthocyanins. In accordance with our data, total phenols and anthocyanins contents appeared to be the main components of the antioxidant activity of different berries (*38*, *44*, *45*), figs (*37*), apples and peaches (*39*), and wines (*46*). Similarly, a strong positive correlation was found between antioxidant and anthocyanin contents in seed coats of bean (*40*).

Anthocyanins contribute to the fruit is color; however, some of these compounds do not have a strong red color. To determine the relationships between anthocyanins levels and aril juice color, the juice colors were measured using a colorimeter, and the correlations to anthocyanins content were measured. A strong positive and significant correlation was found between the juice's color values and anthocyanin content ($r = 0.70$, $r = 0.76$ $p < 0.01$ for the 2005 and 2006 seasons, respectively). These color values are also positively correlated to the antioxidant level of the juice ($r = 0.70$, $r = 0.79$ $p < 0.01$ for the 2005 and 2006 seasons, respectively). The correlation values obtained suggest that in addition to the anthocyanins other antioxidant compounds, such as phenolic compounds that are colorless in comparison to anthocyanins but can influence fruit color (e.g., some hydroxycinnamic acids) (*47*), exist in the arils juices and also contribute to the color.

In general, the finding described here suggests that when the arils are used alone for juice preparation or in salads, the best accessions having the highest antioxidant activity are those that have red or darker colored arils.

Antioxidant Levels Are Significantly Enhanced in Homogenates Prepared from the Whole Fruit in Comparison To Juices Prepared from the Arils Alone. Pomegranates are also popularly consumed as PJ, which is usually prepared by the commercial juice industry. To prepare this kind of juice, the whole fruit is pressed hydrostatically, which also extracts a large amount of bioactive polyphenol compounds found in the fruit peels (*25*). However, the amount of these polyphenol compounds and their properties was only partially studied. Previously, Gil et al. (*14*) found that the antioxidant activity of CPJ has an

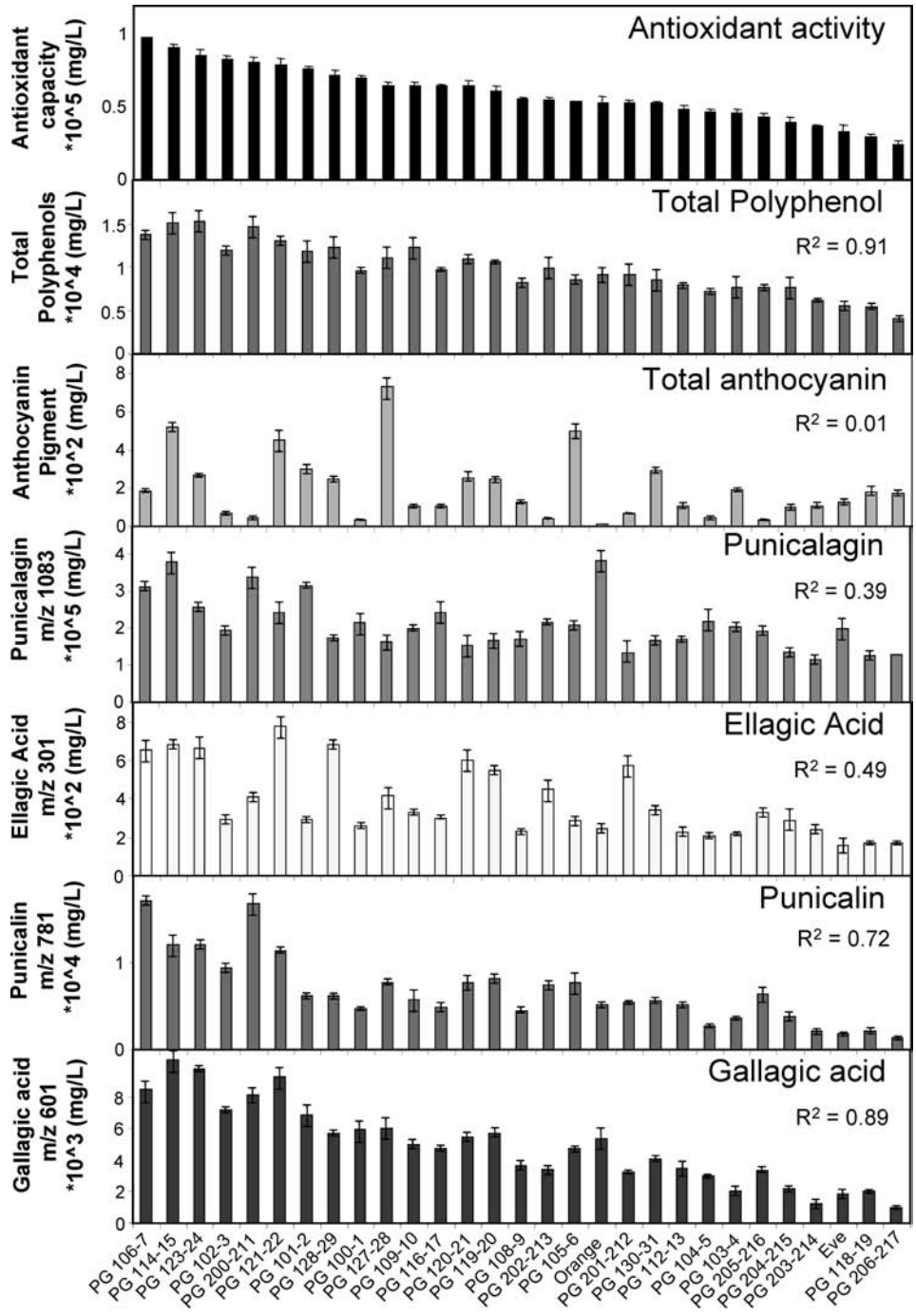

Figure 5. Antioxidant activity, total polyphenol content, total anthocyanin level, and the content of four members of the hydrolyzable tannins in homogenates prepared from the peels, of 29 pomegranate accessions used in this study in the 2006 season. The data presented represent the mean ± SD of five replicates from each accession; each of the replicates is a pool of two fruits. The R^2 value was calculated against the antioxidant activity.

approximately 2-fold higher antioxidant activity than that of fresh juice prepared from the arils alone. To study the contribution of phytochemicals extracted from the peels on antioxidant activity, we next measured the level of antioxidant activity in homogenates prepared from the whole fruit of 29 accessions. The antioxidant activity was dramatically and significantly increased in these juices, about 20-fold compared to the level found in juices prepared from the arils alone (**Figure 4**). Again, the 29 accessions were ordered from the best accession P.G. 203-214, having a 3.5-fold higher antioxidant activity, to the one having the lowest activity (accession P.G. 118-19).

As described above for the aril juice, the polyphenol content in homogenates prepared from the whole fruit correlated positively with antioxidant activity (FRAP method) ($r = 0.95$) (**Table 3**). Polyphenol content increased about 6.5-fold in comparison to the juices prepared from the arils alone (**Figure 4**). However, the differences between the increased level of antioxidant activity (about 20-fold) and the increase in polyphenol content (about 6.5-fold) suggest that in these kinds of homogenates, the polyphenols are not the only contributors to antioxidant activity, and other phytochemicals such the content of organic acids may also contribute. Taken together, these results reinforce the assumption proposed by Gil et al. (*14*) that the peels contain a high content of bioactive compounds, which contribute to the antioxidant activity of the homogenates.

In contradiction to the results described above for aril juice

Table 4. Correlation Matrix (Pearson Test) (Pearson test) Conducted on the Data Obtained from Homogenates Prepared from the Peels Alone of 29 Pomegranate Accessions in the 2006 Season[a]

	antioxidant activity FRAP	antioxidant activity DPPH	total polyphenols	total anthocyanins	punicalagin	ellagic acid	punicalin	gallagic acid	% peels
antioxidant activity FRAP	1	0.51**	0.95**	0.29	0.63**	0.70**	0.85**	0.94**	−0.19
antioxidant activity DPPH		1	0.55**	0.11	0.29	0.33	0.31	0.43*	0.11
total polyphenols			1	0.28	0.63**	0.77**	0.87**	0.93**	−0.18
anthocyanin				1	0.07	0.41*	0.27	0.41*	−0.27
punicalagin					1	0.27	0.6**	0.68**	0.01
ellagic acid						1	0.70**	0.72**	0.03
punicalin							1	0.85**	−0.19
gallagic acid								1	−0.13
% peels									1

[a] The r value of the correlation is given and its significance [($p < 0.05$) is identified by one asterisk, whereas ($p < 0.01$) is identified by two asterisks].

(**Figure 3**), no significant correlation was found between antioxidant activity and anthocyanin level (**Figure 4**; **Table 3**). Moreover, most of the best cultivars, which exhibit the highest antioxidant activity, were those having transparent and pink-colored arils, whereas some of the cultivars having red or darker colored arils exhibit an intermediate and low antioxidant activity (**Figure 1**). These data suggest that anthocyanins do not contribute significantly to the antioxidant activities of these homogenates. The results described here do not match those previously described by researchers who screened 20 pomegranate accessions in northern Greece. These researchers found a positive correlation between antioxidant activity and total anthocyanin content in the juice of these accessions, which was prepared in a food processor; therefore, they concluded that anthocyanin contributed to total antioxidant capacity (*48*). However, Gil et al. (*14*) estimated that anthocyanins contribute only 6% to the antioxidant activity of PJ.

Since the results described above showed that pomegranate peels contributed significantly to antioxidant activity, we next determined the correlation between the percentage of peels from the whole fruit weight and antioxidant activity. It was found (**Table 3**) that such a correlation does not exist. Therefore, we suggest that pomegranate accessions having the highest antioxidant activity have a higher content of certain compounds in their peels, and the thickness of the peels is not a major contributor to antioxidant activity.

Level of Antioxidant Activity Significantly Increased in the Peel's Homogenates. The results described above suggest that phytochemicals in the peels contributed significantly to antioxidant activity of the homogenates. Therefore, we next measured the levels of antioxidant activity, total phenol, and total anthocyanin contents in homogenates prepared from pomegranate peels. The results (**Figure 5**) showed that the antioxidant activity of the peels increased about 2-fold in comparison to homogenates prepared from the whole fruit. The polyphenol level correlated to the antioxidant level ($r = 0.95$) (**Table 4**), whereas no correlation was found between antioxidant activity and the anthocyanin level (**Figure 5**; **Table 4**). As expected, antioxidant activity in whole fruit homogenates correlated significantly to the antioxidant activity found in the homogenates of the peels ($r = 0.57$, $p < 0.01$). However, unexpectedly, this correlation was not high. This could be explained by the varying percentage of arils, which differs significantly between accessions (see also **Figure 1**), which dilutes the phytochemicals extracted from the peels. Alternatively, this could be explained by the presence of the fruit's white membranes that can contribute to the fruit's homogenates but not to the peel's homogenates. Indeed, it was recently shown that fruit membranes have the highest phenol and antioxidant contents in comparison to the other fruit components (arils, peel, seeds, and membranes) (*23, 49*).

The high antioxidant activity and total polyphenol content found in pomegranate peels can explain why extracts prepared from pomegranate peels were widely used in ancient times, as well as in the present day, for the treatment of respiratory diseases and the preparation of tinctures, cosmetics, and other therapeutic formulae (*18, 50*).

Levels of the Four Major Hydrolyzable Tannins Are Positively Correlated with the Antioxidant Activity of Homogenates Prepared from the Whole Fruit and the Peels. An analysis of pomegranate juice prepared by hydrostatic pressure applied to the whole fruit showed that the predominant type of polyphenolic compounds extracted from the peels during the process is the water-soluble compounds of the hydrolyzable tannins, which accounts for 92% of its antioxidant activity (*14*). This group of hydrolyzable tannins is found in the peel (husk, rind, or pericarp), membranes, and piths of the fruit (*49*). The hydrolyzable tannins group contains punicalagin isomers, which were suggested as being responsible for about half of the total antioxidant capacity of the juice, in addition to ellagic acid, gallagic acid, and punicalin (*14*).

To enhance the data about the nature of these compounds in different fruit components, and to study the relationships between antioxidant activity and the content of compounds belonging to the hydrolyzable tannins, we measured the levels of these tannins. The results showed that the levels of these four compounds are positively and significantly correlated to antioxidant activity and phenol level of homogenates prepared from the whole fruit and from the peels alone (**Figures 4** and **5**; **Tables 3** and **4**). The highest correlation value was obtained between antioxidant activity and punicalagin in the whole pomegranate homogenate ($r = 0.87$), and to gallagic acid in homogenates prepared from the peels ($r = 0.94$). The contents of these four compounds also usually positively correlated to one another (**Tables 3** and **4**). However, the contents of these compounds significantly differed in the homogenates. For example, the level of punicalagin, the major compound in the pomegranate whole fruit homogenate, was about 10-fold higher than the punicalin and gallagic acid contents, and 100-fold higher than the level of ellagic acid. The proportion changed in the peel's homogenates, where the content of punicalagin was further increased and its content was 30-, 50-, and 500-fold higher in comparison to punicalin, gallagic acid, and ellagic acid, respectively (**Figure 5**).

These results showed that hydrolyzable tannins are the major compounds contributing to the high antioxidant activity found in pomegranate homogenates, as previously suggested (*14*). The results also support the previous assumption (*14, 50*) that

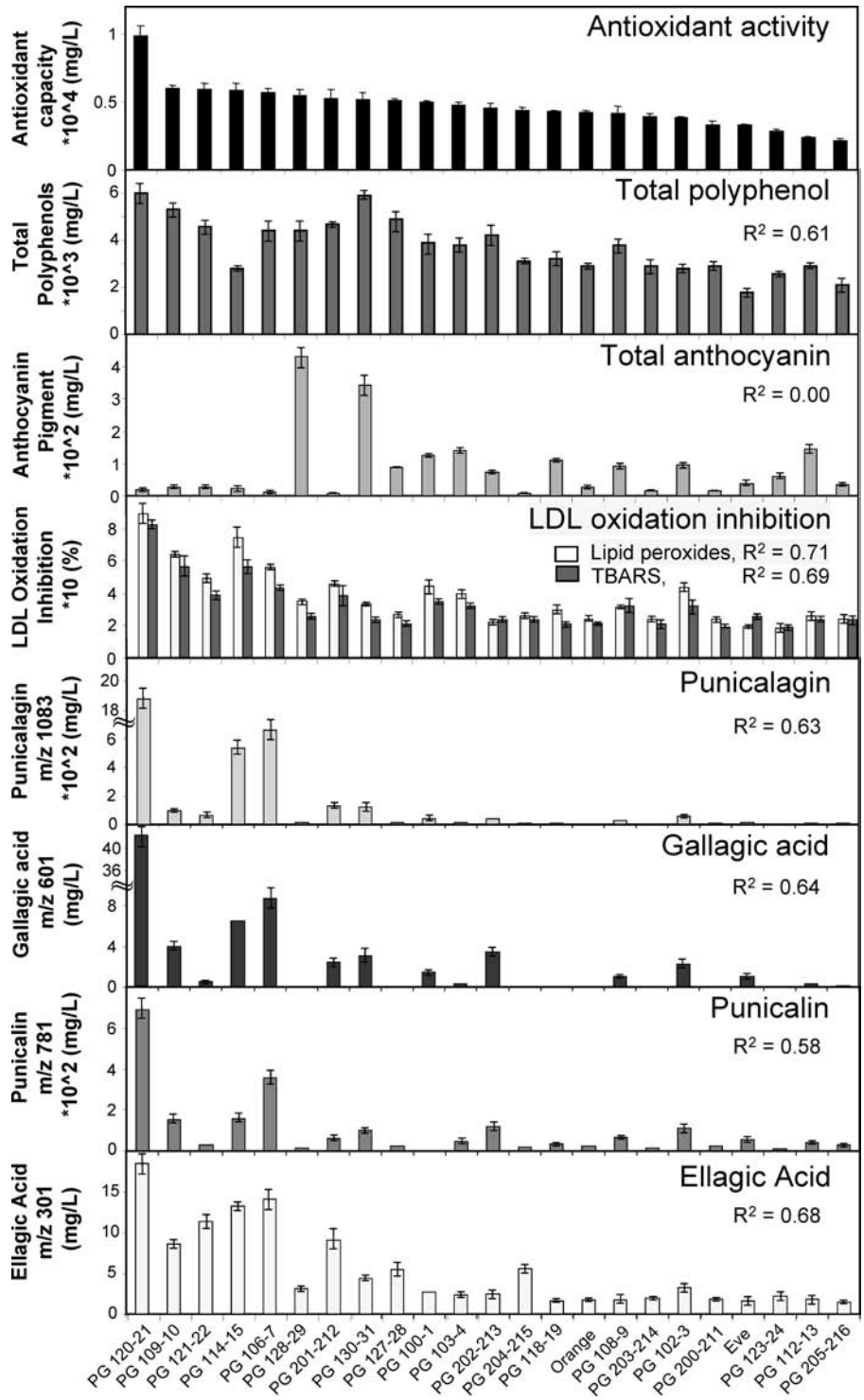

Figure 6. Antioxidant activity, total polyphenol content, total anthocyanin level, the inhibitory effect on LDL oxidation, and the content of four members of the hydrolyzable tannins in homogenates prepared from the peels of 23 pomegranate accessions in the 2005 season. The effects of pomegranate juice supplementation on LDL oxidation were detected by two ex vivo assays. The data presented represent the mean ± SD of five replicates from each accession; each of the replicates is a pool of two fruits. The R^2 value was calculated against the antioxidant activity.

punicalagin originating from the peels is one of the major phytochemicals contributing to the total antioxidant capacity of pomegranate juice, while ellagic acid plays only a minor role in this activity (*14*).

The high antioxidant activity value of punicalagin and its high level in the peels attracted researchers to learn more about its nature. As a result, knowledge about this compound has significantly increased in recent years. It has been shown that punicalagin possesses remarkable pharmacological attributes, including anti-inflammatory, antiproliferative, apopotic, and antigenotoxic properties (*8*, *51–53*). It was suggested that one of the factors responsible for these properties is its high antioxidant activity (*54*). A study of the toxicity of punicalagin revealed that repeated oral administration of high doses of punicalagin showed no evidence of toxicity (*54*, *55*). A similar study of the bioavailability of punicalagin in rats showed that this compound and its metabolites were observed in feces, urine, and plasma (*11*). Punicalagin's health beneficial properties,

Table 5. Correlation Matrix (Pearson test) (Spearman test) Conducted on Data Obtained from Juice Prepared from the Fruit Using a Juice Extractor of 23 Pomegranate Accessions in the 2005 Season[a]

	antioxidant activity FRAP	total polyphenols	total anthocyanins	TBARS 250	LP250	punicalagin	ellagic acid	punicalin	gallagic acid
antioxidant activity FRAP	1	0.80**	−0.24	0.71**	0.82**	0.75**	0.62**	0.42*	0.84**
total polyphenols		1	−0.04	0.44*	0.59**	0.58**	0.45	0.3	0.63**
total anthocyanins			1	−0.21	−0.13	−0.16	−0.41	−0.12	−0.35
TBARS 250				1	0.87**	0.85**	0.68**	0.64**	0.66**
LP250					1	0.78**	0.68**	0.52*	0.76**
punicalagin						1	0.91**	0.76**	0.79**
ellagic acid							1	0.88**	0.72**
punicalin								1	0.42*
gallagic acid									1

[a] The r value of the correlation is given and its significance [($p < 0.05$) is identified by one asterisk, while ($p < 0.01$) is identified by two asterisks].

coupled with its bioavailability and nontoxic nature, render it as a promising multifunctional molecule (*54*).

Punicalagin, punicalin, and gallagic acid were also found in juices prepared from the arils alone (**Figures 2** and **3**). However, the total contents of these compounds were very low in comparison to the level in the peels. For example, the levels of punicalagin and gallagic acid in these juices decreased to about 6×10^3 and 10^3, respectively, when compared to their content in the peel's homogenate. The level of ellagic acid was below the detection level under these conditions (i.e., below 0.5 mg/L). The level of these four compounds in juices prepared from the arils did not correlate to antioxidant activity or polyphenol content (**Table 2**; **Figures 2** and **3**). Taken together, these results strengthen the assumption that anthocyanins, which belong to the flavonoides group of polyphenols, and not the hydrolyzable tannins, are the major contributors to antioxidant activity in aril juice unlike in the juice prepared from the whole fruit.

Protection against LDL Oxidation by Pomegranate Juice Prepared Using a Juice Extractor Correlates to Antioxidant Activity of These Juices. Pomegranate juice is freshly prepared in many streets and open markets in Middle Eastern countries using a juice extractor. Therefore, in the 2005 season, we extracted the juice using this method from 23 accessions. The levels of antioxidant activity, total polyphenol content, and anthocyanin were measured in these juices. Since previously using in vitro and ex vivo assays it was shown that PJ inhibits lipid peroxidation in plasma and LDL oxidation (*10*), we next studied the ability of juices from different pomegranate accessions to inhibit LDL lipid peroxidation induced by copper ions. These measurements are important since oxidative modification of LDL is thought to play a key role during early atherogenesis (*10, 12, 19, 28, 56*). It was previously shown that pomegranate juice consumption by humans for a period of one year significantly reduced the oxidation of both LDL and HDL (*10*). Furthermore, it was observed that in patients with carotid artery stenosis who consumed PJ for three years, the oxidative stress of their blood and atherosclerosis lesion size were both significantly reduced (*20*). Therefore, the routine consumption of PJ including the peel extracts is important for maintaining good health.

The results obtained for antioxidant activity, total polyphenol, and total anthocyanin contents (**Figure 6**) showed that while the antioxidant activity significantly correlated, as expected, with the polyphenol level ($r = 0.8$, **Table 5**), it did not correlate with the level of anthocyanins, although the arils contribute the main color to the juice. LDL oxidation was reduced significantly in the presence of these juices, as shown by the two assays used (**Figure 5**). Both the percentage reduction in the oxidation of LDL measured by the thiobarbituric acid reactive substances (TBARS) assay and the percentage inhibition of LDL oxidation determined by the lipid peroxides assay (**Figure 6**, **Table 5**) positively and significantly correlated with the antioxidant capacity of the pomegranate juice having values of $r = 0.71$, and $r = 0.82$, respectively (**Table 5**). This finding suggests that antioxidant activity is an important factor contributing to the health beneficial properties of pomegranates. In general, the antioxidant levels of juices prepared in a juice extractor were about 3-fold higher compared to juices prepared from the arils alone, and about 10-fold less compared to the level observed in pomegranate homogenates (compare the values shown in **Figure 3** to those in **Figure 6**).

All in all, in the current study, it was shown that juices prepared from the arils alone exhibit relatively poor antioxidant activity and low polyphenol content, as well as low content of the four hydrolyzable tannins, relative to homogenates prepared from the whole fruit. Anthocyanins, which contribute to the antioxidant activities of different berries (*38, 44, 45*), also contribute significantly to the antioxidant activity of the arils. Therefore, if only the arils are used, the preferred pomegranates are those that have red or darker colored arils when considering the health beneficial properties of the fruit. However, if health benefits is a criterion for pomegranate consumption, then the PJ or the whole fruit homogenates should be the preferred choice since their antioxidant activity is about 20-fold higher than that found in the arils. This high level correlated with the level of phytochemicals derived from the hydrolyzable tannins found in pomegranate peels.

The data described here enhance our knowledge about the natural variation in the content of antioxidant activity, total polyphenol, and total anthocyanin contents among different pomegranate accessions. It also identified the location and concentration of some phytochemical compounds in the fruit. In thte future, such information will enable breeders to select and breed genotypes having higher levels of health beneficial compounds and also to provide useful information for addressing consumer choices for healthier products.

ABBREVIATIONS USED

PJ, pomegranate juice; CPJ, commercial pomegranate juice; LDL, low density lipoprotein; TEAC, Trolox equivalent antioxidant capacity; TBARS, thiobarbituric acid reactive substances.

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NOTE ADDED AFTER ASAP PUBLICATION

Several lines of text have been deleted from the original Web posting of October 4, 2007. The last paragraph under Plant Materials and Fruit Processing has been deleted as have been several lines from the second section under Results and Discussion. These deletions are reflected in the posting of October 16, 2007.

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