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Opinions and Perspectives

Pomegranate (*Punica granatum*) supplements: Authenticity, antioxidant and polyphenol composition

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ABSTRACT

Pomegranates contain a complex mixture of gallotannins, ellagitannins, ellagic acid and anthocyanins. However, label claims on pomegranate supplements (PS) may not correlate with actual content of antioxidants, polyphenols or tannins. Nineteen PS were evaluated for their authenticity by determining ellagitannin composition by RP-HPLC and studying the relationship between total polyphenols as measured by the Folin–Ciocalteau assay and antioxidant capacity by oxygen radical absorbing capacity (ORAC), free radical scavenging properties by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and ferric reducing antioxidant power (FRAP). Only a limited number of pomegranate supplements were authentic. Product labels were inconsistent with polyphenol composition and antioxidant content. A majority of the samples ($n = 13$) contained disproportionately high amounts of ellagic acid and low or no detectable pomegranate tannins. Only six products had tannin composition that resembled pomegranates (punicalagin, punicalin, ellagitannins and gallotannins). PS-01 (natural pomegranate extract) was the most representative of pomegranate fruit polyphenols with 99% total pomegranate polyphenol and the highest antioxidant capacity across all measures. Correlations between total polyphenols and antioxidant content were high ($R^2 > 0.87$) in products that had polyphenol composition resembling pomegranates. Products that contained high amounts of ellagic acid and low or no detectable pomegranate tannins had poor correlations between total polyphenols and antioxidant content. The results indicate that reliable labeling information, better standardization, improved manufacturing practices and regulation of the market is required to assure consumers of the quality of pomegranate supplements.

1. Introduction

Improved methods for determining authenticity, standardization and efficacy of nutritional supplements are required for growth and regulation of the market. There are increasing numbers of pomegranate (*Punica granatum*) supplements in the retail markets in the United States. Most of these products are promoted based on their possible health benefits and antioxidant content, but their authenticity has not been evaluated and their manufacturing is not highly regulated.

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Hydrolyzable tannins are the most abundant polyphenols and antioxidant compounds in pomegranates and include gallotannins, ellagitannins and gallagyl esters such as punicalagin and punicalin (Fig. 1). Pomegranate also contains oligomeric ellagitannins with two to five glucose core molecules cross-linked by dehydrodigalloyl and/or valoneoyl esters (Tanaka et al., 1986a,b; El-Nemr et al., 1990; Clifford and Scalbert, 2000; Afaq et al., 2005; Reed et al., 2005). The objective of this study was to correlate polyphenol content of PS to antioxidant content. Authenticity and quality of PS were also studied using qualitative analysis of polyphenol composition as determined by reverse phase HPLC.

2. Materials and methods

2.1. *Pomegranate supplements (PS)*

The products used for the study (Table 1) are available in retail stores. All products were analyzed prior to their expiration dates as stated on their packages (see Table 1 for list and codes for pomegranate supplements tested).

2.2. *High performance liquid chromatography*

A 5-mg sample of each PS was solubilized in 5 mL of methanol (10%, v/v), sonicated for 15 min in a FS-14 ultrasonic bath (Fisher Scientific, Waltham, MA) and centrifuged for 10 min at 1000*g* in a 5415 C centrifuge (Eppendorf, Westbury, NY). One hundred microlitres of supernatant were injected onto a Spherisorb ODS2, C-18 column (10 μm, 25 cm × 0.45 cm, Waters Co., Milford, MA). The solvents for elution were trifluoroacetic acid (TFA)/water (0.1%; solvent A) and methanol (solvent B). The elution profile was a series of step gradients: 100–73% A over 30 min; 73–45% A over the next 15 min, 45–0% A over 5 min. The flow rate was maintained at 2 mL/min, and the elution was monitored using a 996 diode array detector (Waters Co., Milford, MA) and Millennium software (Waters Co., Milford, MA) for collecting and analyzing three-dimensional chromatograms. The amount of each phenolic compound or group was estimated by multiplying the percent composition by the amount of total polyphenols in the supplement.

2.3. *Folin–Ciocalteau assay for total phenolics (TPC)*

The Folin–Ciocalteau method was used to quantify total phenolic compounds in the pomegranate nutritional supplements (PNS), using a pomegranate polyphenol standard (PPS) developed in a previous work (Martin et al., in press). Stock solutions of PPS were serially diluted and used to generate a standard curve for the estimation of total polyphenols. Water (3 mL), undiluted Folin–Ciocalteau reagent (0.2 mL, Sigma, St. Louis, MO) and 0.1 mL of the PPS solution (1 mg/mL; 50% (v/v) methanol) were added to tubes and the solutions were mixed and incubated at room temperature for 10 min followed by addition of 0.6 mL 20% (w/v) Na_2CO_3. After mixing, tubes were incubated at 40 °C for 20 min then rapidly cooled to room temperature in an ice bath. All samples were analyzed at 755 nm by UV–Vis spectrophotometry. A 5-mg sample of each PS was solubilized in 5 mL of 50% (v/v) methanol, sonicated for 15 min in a FS-14 ultrasonic bath (Fisher Scientific, Waltham, MA) and centrifuged for 10 min at 1000*g* in a 5415 C centrifuge (Eppendorf, Westbury, NY). One millilitre of the supernatant was collected and diluted with 50% (v/v) methanol such that the absorbance fell within the range of the PPS calibration curve.

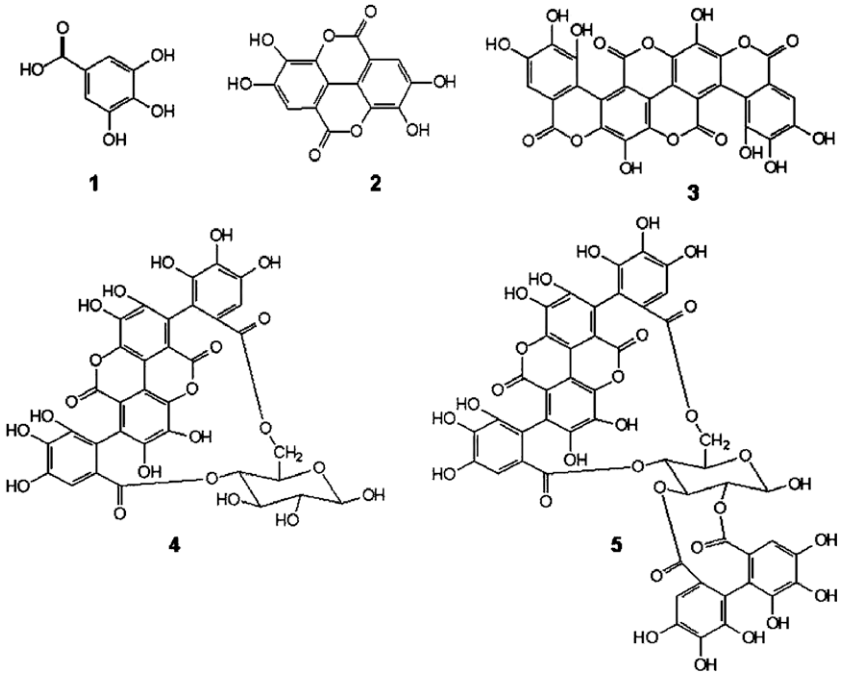

Fig. 1 – Structures of polyphenolic compounds found in pomegranate (*Punica granatum*). [Gallic acid (1), ellagic acid (2), gallagic acid (3), punicalin (4) and punicalagin (5).]

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Table 1 – Polyphenolic composition, total phenolics (TP) and total antioxidants (TA) of pomegranate supplements.

Sample ID	Description (supplier)	Polyphenolic composition (mg PPS/g dry matter)[a]					TA & TP values[b]				Antioxidant index (%)
		Punicalin	Punicalagin isomer A	Punicalagin isomer B	Ellagic acid	Olligomers	ORAC (uM/g)	DPPH (uM/g)	FRAP (uM/g)	PPS (mg/g)	
	Pomegranate skin composition	13	100	232	13	755	–	–	–	–	
	Pomegranate arils composition	5	6	20	4	712	–	–	–	–	
PS-01	Natural pomegranate polyphenol extract 1000 mg (A)	10	51	126	33	766	3210	4485	1680	986	100.0
PS-02	Pomegranate extract 500 mg (B)	13	1	5	39	382	2175	2100	781	440	53.0
PS-03	Pomegranate extract 285 mg (whole fruit and seed powder) (C)	0	0	0	608	126	516	2170	218	734	35.1
PS-04	Ellagic acid 50 mg, anthocyanins 80 mg (D)	11	52	104	67	119	81	223	92	354	7.2
PS-05	Pomegranate fruit extract 250 mg (E)	5	6	10	358	227	2410	1360	371	605	45.8
PS-06	Fermented pomegranate powder blend 400 mg (F)	0	0	0	16	3	59	74	3	19	1.5
PS-07	Whole pomegranate fruit extract 400 mg (G)	22	13	17	32	189	898	1435	589	272	30.8
PS-08	Pomegranate 200 mg (H)	45	17	34	42	187	824	1370	485	325	29.0
PS-09	Pomegranate extract 200 mg (whole fruit and seed) (I)	0	0	0	410	89	1815	985	252	499	34.3
PS-10	Pomegranate extract 500 mg (J)	0	1	3	604	31	542	2275	158	640	34.9
PS-11	Pomegranate extract 250 mg (whole fruit) (K)	0	0	1	734	43	532	2390	210	778	37.7
PS-12	Pomegranate 250 mg (fruit conc., fruit skin and whole fruit) (K)	0	95	201	172	245	1940	2910	1043	736	64.0
PS-13	Pomegranate 200 mg (seed and fruit extract) (L)	40	16	38	120	156	910	1190	445	369	28.1
PS-14	Pomegranate 400 mg (fruit extract) (M)	13	84	175	38	473	2475	3850	1590	783	83.9
PS-15	Pomegranate 200 mg (fruit extract and seed) (N)	20	6	11	346	142	785	1170	275	526	26.6
PS-16	Pomegranate blend 440 mg (seed powder, aqueous extracts of juice, peel, leaf and flower) (O)	1	0	0	37	13	148	74	42	52	3.0
PS-17	Pomegranate fruit extract 400 mg (P)	22	6	9	582	128	1034	927	239	746	28.4
PS-18	Pomegranate 350 mg (fruit extract and seed meal) (Q)	4	1	2	307	105	392	1027	59	420	18.3
PS-19	Pomegranate extract 250 mg (whole fruit) (R)	27	2	8	122	111	675	1130	457	270	24.4
	Mean	12	18	39	246	186	1127	1639	473	503	36.1
	SD	14	30	63	241	184	924	1182	486	259	25.2
	Variation	1.1	1.6	1.6	1.0	1.0	0.8	0.7	1.0	0.5	0.8

a Polyphenolic composition by RP-HPLC is expressed as (mg PPS/g dry matter) times the polyphenolic fraction obtained from the HPLC assigned peak area integration.

b ORAC, oxygen radical absorbing capacity; DPPH, free radical scavenging properties; FRAP, ferric reducing antioxidant capacity; PPS, total phenolics as pomegranate polyphenols standard equivalents.

2.4. Antioxidant assays

All antioxidant assays were performed at Covance Analytical Laboratories, Inc. Madison, WI.

2.4.1. Total oxygen radical absorbance capacity (ORAC)

A mixture of 125 μL fluorescein (0.16 μM) and 250 μL, 2,2′-azo-bis-amidinopropane dihydrochloride (AAPH, 147 mM) at 37 °C was combined with 250 μL of each dietary supplement sample diluted in phosphate buffer (0.2 M; pH 7.0). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards ranged from 5 to 40 μM. The decrease in fluorescence of fluorescein was determined by collecting readings at excitation 535 nm and emission 595 nm every minute for 45 min in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The ORAC value was evaluated as area-under-curve (AUC) as previously reported by Cao et al. (1995).

2.4.2. Free radical scavenging capacity (DPPH)

The colorimetric DPPH scavenging capacity assay was performed according to a previously described laboratory protocol (Cheng et al., 2006). An aliquot of 500 μL of different concentrations of supplement sample dilutions in 50% (v/v) acetone was added to 500 μL of 0.208 mM DPPH solution. The initial concentration was 0.104 mM for DPPH in all reaction mixtures. Each mixture was vortexed for a few seconds and left to stand in the dark for 40 min at ambient temperature. The absorbance of each reaction mixture at 517 nm was measured against a blank of 50% (v/v) acetone using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

2.4.3. Ferric reducing antioxidant capacity (FRAP) assay

A 300-μL portion of the reaction solution (2,4,6-tri[2-pyridyl-S-triazine] 10 mM TPTZ, 20 mM ferric chloride and 300 mM (pH 3.6) sodium acetate buffer in a 1:1:10 volume ratio) was heated at 37 °C for 10 min and then 25 mg of the dietary supplements samples were added to the reaction mixture and its absorbance was read at 593 nm in a Molecular Devices SpectraMax M2 plate reader. Ferrous sulphate standards ranged from 100 to 1000 μM. Results were expressed as μM Fe (Fe^{3+} ions converted to Fe^{2+}).

2.5. Statistical analysis

Statistical analysis was performed using commercial software. The direction and the magnitude of the correlation between the variables were calculated using analysis of variance (ANOVA test). The criteria for statistical significance were $p \leqslant 0.05$ (*), $p \leqslant 0.01$ (**), $p \leqslant 0.001$ (***).

An overall Antioxidant Index was determined by assigning all assays an equal weight, assigning a index value of 100 to the best score for each test, and then calculating an index score for all other samples within the test as follows: Antioxidant Index Score = [(sample score/best score) × 100] and then averaging all the four tests for each PS for an antioxidant index. All assays were given equal weight and an overall mean index value was calculated on a normalized basis for each PS.

3. Results and discussion

Qualitative analysis of polyphenol composition of PS indicated that only seven products had tannin composition that resembled pomegranates. Only sample PS-01 (natural pomegranate extract) had an ellagitannin composition that was similar to pomegranate arils and skin with 99% purity and the highest antioxidant capacity across of measurements (Table 1). The polyphenol composition of pomegranate arils and skins is characterized by a high proportion of punicalagin, punicalin, ellagitannins and gallotannins in comparison to ellagic acid (Table 1). Pomegranate arils contain anthocyanins, in small amounts, but the rind does not (data not shown). None of the PS contained anthocyanins. However, the pomegranate anthocyanins have not been shown to correlate to antioxidant capacity (Tzulker et al., 2007). The majority of PS ($n = 13$) contained disproportionately high amounts of ellagic acid compared to pomegranate arils and rind, including PS ($n = 6$) in which low or no pomegranate tannins were detected. In PS that contained detectable amounts of punicalagin and other pomegranate tannins, the absence of anthocyanins indicates that these supplements were most likely derived from extractions of the press cake which is the by-product of pressing juice from the whole fruit and is primarily composed of rind and seeds. However, the absence of punicalagin and other pomegranate tannins in PS indicates either these products did not contain pomegranate and that ellagic acid was added or pomegranate tannins were degraded during manufacturing.

Pomegranate supplements that had similar tannin composition to rind readily dissolved in aqueous methanol to give a brownish colored solution. On the other hand, PS that contained high levels of ellagic acid did not dissolve in aqueous methanol because ellagic acid has low solubility in this solvent. These products were characterized by white suspen-

Table 2 – Correlations between total phenolics and total antioxidants of pomegranate supplements.

	Correlation coefficient values (R^s)		
	ORAC	DPPH	FRAP
Folin	0.3706***	0.6472***	0.2991***
ORAC	–	0.4983***	0.6897***
DPPH	–	–	0.7019***

*$p \leqslant 0.05$.
**$p \leqslant 0.01$.
*** $p \leqslant 0.001$.

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Table 3 – Correlations and significances between total phenolics and total antioxidants for pomegranate supplements resembling pomegranate ellagitannins composition and ellagic acid.

Sample group		Correlation coefficient (R^2)		
		ORAC	DPPH	FRAP
Pomegranate resembling samples				
	Folin	0.8737***	0.9209***	0.8989***
	ORAC		0.9541***	0.9355***
	DPPH			0.9925***
EA resembling samples				
	Folin	0.0404	0.4912**	0.3293*
	ORAC		0.0083	0.6368***
	DPPH			0.0779

* $p \leqslant 0.05$.
** $p \leqslant 0.01$.
*** $p \leqslant 0.001$.

sions when agitated but quickly precipitated. Free ellagic acid in pomegranate fruit is low and may increase in juice and supplements as a result of the hydrolysis of ellagitannins (Bala et al., 2006; Ignarro et al., 2006).

The variation among PS in content of total polyphenols (TP) and antioxidant assays was large with coefficients of variation exceeding 50%. The total polyphenol content ranged from approximately 1% to close to 100% of the sample weight (Table 1).

All of the assays for content of polyphenols and antioxidant are REDOX reactions. Polyphenolic molecules undergo REDOX reactions because phenolic hydroxyl groups readily donate hydrogen to reducing agents. The Folin–Ciocalteau reagent is a REDOX reagent used to estimate total polyphenolic compounds. Previous studies have reported that the content of total polyphenols, as determined by the Folin–Ciocalteau reagent, is highly correlated with other antioxidant assays (Wang et al., 1996; Benzie and Szeto, 1999; Apak et al., 2007), such as ORAC, DPPH and FRAP. However, correlations among TP, ORAC, DPPH and FRAP in PS were lower ($R^2 < 0.65$) than expected (Table 2). These low correlations may be a result of the low solubility of ellagic acid in the 10 supplements in which ellagic acid was essentially the only polyphenol present.

Correlations between TP and TA were high ($R^2 > 0.87$) in products that had a polyphenol composition resembling pomegranates (Table 3). Meanwhile, correlations among TP and TA was low or not significantly different from 0 in products that contained high amounts of ellagic acid and low or no detectable pomegranate tannins.

4. Conclusions

Product labels were inconsistent with polyphenol composition and antioxidant activity. Two samples contained no detectable polyphenols. The majority of the samples ($n = 13$) contained disproportionately high amounts of ellagic acid compared to pomegranate, including samples in which low or no pomegranate tannins were detected. Only six products contained pomegranate ellagitannins (punicalagin, punicalin, ellagitannins and gallotannins). PS-01 was most similar to the ellagitannin composition of pomegranate fruit and the highest purity and antioxidant capacity across all measures.

The correlations among total polyphenols and antioxidant capacity were high in PS that contained pomegranate ellagitannins. However, in PS that had high levels of ellagic acid and low or no detectable pomegranate ellagitannins, the correlations among total polyphenols and antioxidant capacity were low or not significantly different from 0, which may be explained in part by the low solubility of ellagic acid. High levels of ellagic may result from addition of ellagic acid to the product and/or extensive hydrolysis of the pomegranate ellagitannins during processing. Reliable labeling information, better standardization, improved manufacturing practices and regulation of the market is required to assure consumers of the quality of pomegranate supplements.

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