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Absence of Pomegranate Ellagitannins in the Majority of Commercial Pomegranate Extracts: Implications for Standardization and Quality Control

YANJUN ZHANG, DAVID WANG, RU-PO LEE, SUSANNE M. HENNING, AND DAVID HEBER*

Center for Human Nutrition, David Geffen School of Medicine, University of California, Los Angeles, California 90095

The health benefits associated with pomegranate juice have led to the development of pomegranate extracts as botanical dietary supplements. Pomegranates contain hydrolyzable tannins in the form of punicalagins and punicalin as well as tannin-based complex oligomers that account for much of the antioxidant activity in juice. The content of ellagic acid has been used to standardize most pomegranate extract dietary supplements marketed. However, supplements can be adulterated with ellagic acid from less expensive plant sources and undercut this method of standardization. To compare the phytochemical contents and antioxidant activities of commercially available pomegranate extract dietary supplements beyond their content of ellagic acid, a total of 27 different supplements in the form of capsules, tablets, and soft gels were studied. Total phenolics were measured using both gallic acid equivalent (GAE) and ellagic acid equivalent (EAE) assays. Punicalagins, punicalin, and ellagic acid contents were determined by HPLC, whereas antioxidant capacity was measured using the Trolox equivalent antioxidant capacity (TEAC) assay. Of the 27 supplements tested, only 5 had the typical pomegranate tannin profile by HPLC, 17 had ellagic acid as the predominant chemical with minor or no detectable pomegranate tannins, and 5 had no detectable tannins or ellagic acid. Therefore, standardization of pomegranate extract supplements based on their ellagic acid content does not guarantee pomegranate supplement authenticity. Future research is needed to assess the health impact of substituting ellagic acid for the complex mix of phytochemicals in a pomegranate extract dietary supplement.

KEYWORDS: Pomegranate; polyphenols; punicalagins; ellagic acid; GAE; EAE; TEAC

INTRODUCTION

Pomegranate fruit (*Punica granatum*, Punicaceae) has a long history of use for health benefits in Iran, Turkey, India, China, Afghanistan, and Russia. The Wonderful variety of pomegranate, grown extensively in California, has become popular as a commercial juice with potential health benefits (*1*−*3*). This has led to the development of pomegranate extract dietary supplements claiming to contain extracts of pomegranate fruit. These are advertised to consumers to provide the key ingredient of hydrolyzable tannins (punicalin and punicalagins A and B, **Figure 1**), which account for 89% of the antioxidant activity of pomegranate juice (*4*, *5*). Although these large molecules are not absorbed directly into the body (*6*, *7*), they are hydrolyzed in the intestinal tract over several hours prior to absorption and lead to sustained blood levels of ellagic acid over 6 h (*8*). The ellagic acid is then metabolized to urolithins about 12 h after absorption, and urolithins can be detected in the blood and urine for up to 48 h after a single dose (*8*, *9*). These urolithins may account for some of the biological activities associated with pomegranate juice consumption. Pomegranate extract and juice have similar bioavailabilities and metabolism (*8*, *9*). The content of ellagic acid in dietary supplements has been selected as the method for assuring that supplements contain genuine pomegranate fruit extract.

However, research demonstrating health benefits from pomegranate fruit products does not specify that these benefits are provided by ellagic acid alone. The fact that ellagic acid has been selected as the intrinsic chemical marker for the commercial standardization of pomegranate supplements led to this investigation to determine the phytochemical profiles of available pomegranate extract supplements beyond their content of ellagic acid. Commercially available pomegranate dietary supplements (PDS) are produced as three main different types of formulations: capsules, tablets, and soft gels. The capsules and tablets primarily contain the hydrolyzable tannins, whereas the soft gels contain pomegranate seed oil as well as some ellagitannins. Among 27 commonly available commercial PDSs, we measured punicalagins A and B, punicalin, and ellagic acid contents using high-performance liquid

*Address correspondence to this author at the Center for Human Nutrition, David Geffen School of Medicine at UCLA, Warren Hall 12-217, Los Angeles, CA 90095 [telephone (310) 206-1987; fax (310) 206-5264; e-mail dheber@mednet.ucla.edu].

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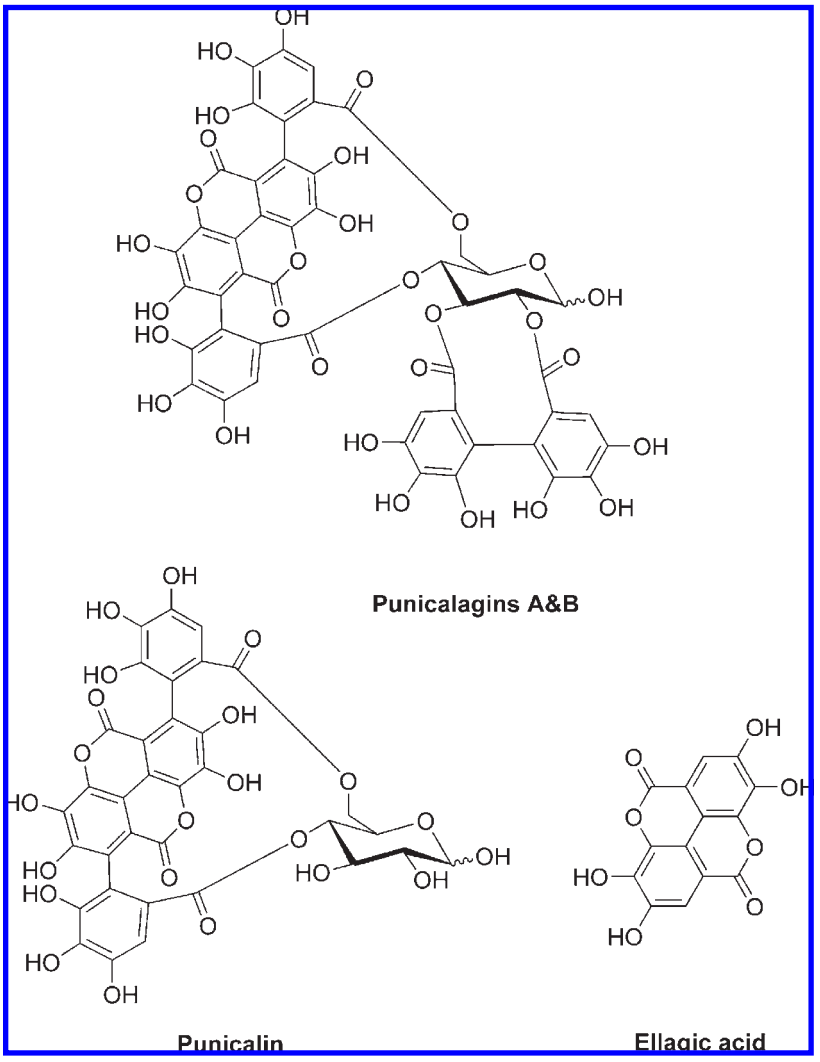

Figure 1. Structures of punicalagins A and B, punicalin, and ellagic acid.

chromatography−ultraviolet (HPLC-UV) and total polyphenol content and antioxidant capacity by several methods.

MATERIALS AND METHODS

Materials. All solvents were of HPLC grade and purchased from Fisher Scientific Co. (Tustin, CA). Gallic acid (>98%) standard was purchased from Sigma Aldrich Co. (St. Louis, MO). Ellagic acid (primary standard, lot 05071-241, adjusted purity of 91.0%) and punicalagins A and B (primary standard, lot 16983-1218, adjusted purity of 93.4%) standards were purchased from Chromadex (Irvine, CA). Punicalin was isolated and characterized at the UCLA Center for Human Nutrition (*10*).

Samples. Twenty-seven PDSs including capsules (20 samples, C1−C20), soft gels (3 samples, S1−S3), and tablets (4 samples, T1−T4) were obtained on the basis of accessibility to the average consumer (purchased from retail stores, chain pharmacies, Internet, or mail order). All pomegranate dietary supplements were analyzed prior to the expiration dates as stated on their packages. The main labeling information of all testing PDSs is listed in **Table 1**.

Quantification of Punicalagins A and B, Punicalin, and Ellagic Acid in Pomegranate Dietary Supplements. *Standards*. Punicalagins A and B, punicalin, and ellagic acid, all 3 mg, were individually dissolved in 1 mL of DMSO as stock solutions. Five hundred microliters of each standard stock solution was mixed and further diluted to afford 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL concentrations. Standard calibration curves were constructed for each reference standard. Sample punicalagins A and B, punicalin, and ellagic acid concentrations were determined from the peak areas by using the linear regression equations obtained from the calibration curves.

Samples. Once the label information was recorded, tablets or capsules were sampled from each bottle/packet in duplicate, weighed, analyzed by HPLC, and reported as an average value ± standard deviation (SD). Tablets were crushed or the contents of capsules and soft gels were collected, and 100 mg sample aliquots were quantitatively dissolved in DMSO in 100 mL volumetric flasks and sonicated for 20 min.

HPLC Conditions. The HPLC system consisted of a Waters Alliance 2695 module with a 996 photodiode array detector, controlled by Waters Empower 2 software (Waters, Milford, MA). The mobile phase, solvent A (acetonitrile) and solvent B (0.4% aqueous phosphoric acid), was used under binary linear gradient conditions as follows: 0−10 min, 5−15% solvent A in solvent B; 10−30 min, 15−25% solvent A in solvent B; with a flow rate of 1.0 mL/min. All samples were filtered (0.22 μm), loaded (25 μL injection volume), and analyzed on an Agilent Zorbax SB C_{18} 4.6 × 250 mm column with a guard column (C_{18} 5 μm, 3.9 × 20 mm). The monitored wavelength was 360 nm for the detection and quantification of punicalin, punicalagins A and B, and ellagic acid (**Figure 2**). The retention times for punicalin, punicalagins A and B, and ellagic acid at the above HPLC condition were 7.04, 10.97, 12.65, and 23.58 min, respectively.

Trolox Equivalent Antioxidant Capacity (TEAC). The assay was performed as reported (*11*). Briefly, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cations were prepared by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of $ABTS^+$ (20 mL using a 75 mM Na/K buffer of pH 7). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as an antioxidant standard. A standard calibration curve was constructed for Trolox at 0, 50, 100, 150, 200, 250, 300, and 350 μM concentrations. Capsule, tablet, and soft gel samples were extracted in propylene glycol (1 mg/mL). All samples were mixed for 30 min using a magnetic stirrer, diluted

Table 1. Labeling Information of All Testing PDSs

ID	main ingredients	per serving
C1	pomegranate extract	1000 mg
C2	pomegranate powder blend (fermented pomegranate juice, seed meal, and aqueous extracts of peel and leaves)	400 mg
C3	pomegranate extract (40% ellagic acid)	250 mg
C4	pomegranate herb powder	500 mg
C5	pomegranate extract (70% ellagic acid)	500 mg
C6	pomegranate extract (40% ellagic acid); pomegranate seed	250 mg
C7	pomegranate extract (30% punicalagins); pomegranate 5:1 extract	500 mg
C8	pomegranate extract (40% ellagic acid); pomegranate seed	350 mg
C9	pomegranate extract (40% ellagic acid), pomegranate seed	350 mg
C10	pomegranate extract (40% ellagic acid); pomegranate seed	250 mg
C11	pomegranate extract (40% ellagic acid)	250 mg
C12	pomegranate powder blend (seeds, fermented pomegranate juice, and aqueous extracts of peel and leaves)	440 mg
C13	pomegranate extract (10% ellagic acid), fruit and flower	800 mg
C14	pomegranate fruit extract (40% ellagic acid); pomegranate seed extract	250 mg
C15	pomegranate extract (70% ellagic acid)	250 mg
C16	pomegranate extract (5:1 concentrate); ellagic acid; (40% punicosides)	400 mg
C17	pomegranate extract (40% ellagic acid)	40 mg
C18	pomegranate seed extract (40% ellagic acid)	1000 mg
C19	pomegranate extract, (40% ellagic acid), pomegranate seed	250 mg
C20	pomegranate pure powder	500 mg
S1	pomegranate extract (40% ellagic acid), (30% punicalagin)	500 mg
S2	pomegranate peel, pulp, juice and seeds	1−2 soft gels
S3	pomegranate extract (60% ellagic acid) and seed oil extraction	1050 mg
T1	pomegranate extract (40% punicosides)	200 mg
T2	pomegranate juice (6000 mg of pomegranates)	240 mg
T3	pomegranate extract	15 mg
T4	pomegranate seed extract (40% ellagitannins)	1 g

100-fold in Na/K buffer, and then centrifuged for 5 min at 9300*g*. Twenty microliters of the supernatants of the diluted samples was mixed with 200 μL of $ABTS^{\bullet+}$ radical cation solution in 96-well plates, and the absorbencies were recorded at 750 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA) after a 5 min incubation at 30 °C. Samples were assayed in six replicates. TEAC values were calculated from the Trolox standard curve and expressed as Trolox equivalents (in μmol/mg of sample). This assay was performed with a coefficient of variance (CV) under 5% for all absorbance readings.

Gallic Acid Equivalent (GAE) and Ellagic Acid Equivalent (EAE). The assays were performed as reported with some modification (*12*). Briefly, reaction mixture tubes were prepared by mixing 750 μL of methanol/water (1:1 v/v) with 50 μL of Folin−Ciocalteu reagent. Gallic acid and ellagic acid, dissolved in propylene glycol at 1 mg/mL, were used as total polyphenol content standards. Separate standard calibration curves for gallic acid and ellagic acid were constructed at 0, 40, 80, 120, 160, and 200 μg/mL concentrations. The samples were extracted in the previously described method for the TEAC assay. Extracted samples were diluted 5-fold in methanol/water (1:1, v/v), and then 50 μL of a sample was incubated at room temperature in a reaction mixture tube for 10 min. Each reaction tube was mixed with 150 μL of 20% sodium carbonate and incubated in a 40 °C water bath for 20 min. The tubes were then cooled to room temperature and centrifuged for 5 min at 9300*g*. The samples were assayed in six replicates by transferring 100 μL of the supernatant in a tube into 6 individual wells on a 96-well plate, and the absorbance of the plate was read at 755 nm in a ThermoMax microplate reader (Molecular Devices) at room temperature. The standard curves were used to convert the average absorbance of each sample into GAE or EAE, expressed in micrograms per milligram.

Statistical Analysis. For each solid sample, including tablets and capsules, 10 units (tablets or capsules) were taken from one bottle and mixed well and were sampled twice. For soft gel samples, 10 soft gel samples were cut into halves, and all of the contents were pooled in a vial and mixed well. HPLC analyses of each sample were done in duplicate and are reported as mean values ± SD. GAE, EAE, and TEAC values were all measured in six replicates, and the mean values ± SD are reported. All correlation analyses were performed using PRISM statistical analysis software package version 4 (GraphPad Software, San Diego, CA). Pearson correlation analysis was performed using a two-tailed *P* value, an $\alpha < 0.05$, and a 95% confidence interval. Once a significant correlation was determined, a best-fit linear regression curve was drawn to illustrate the data trend.

RESULTS AND DISCUSSION

The punicalagin A and B, punicalin, and ellagic acid contents of the various PDSs are expressed as percent weight to weight present using the known weight of each tablet, or the weight of the contents of a capsule or soft gel. The measured contents of punicalagins A and B, punicalin, and ellagic acid of all 27 commercial PDSs are listed in **Table 2**. According to their chemical profiles, the 27 PDSs can be divided into three categories. The first category contains 5 PDSs (C1, C7, C16, S1, and T1), which contain typical pomegranate hydrolyzable tannins with various amounts of punicalagins A and B ranging from 6 to 18%. These contain about 1% punicalin and various amounts of ellagic acid between 1 and 12%. The second group of PDSs includes 17 different PDSs (C3, C5, C6, C8, C9, C10, C11, C13, C14, C15, C17, C18, C19, C20, T2, S3, and T4) containing mainly ellagic acid at levels between 1.4 and 54.7%. Five PDSs (C2, C4, C12, S2, and T3) fall into the third category, containing very low contents of all three measured compounds between undetectable and 0.5%.

The antioxidant capacity as TEAC and the polyphenol contents expressed in both EAE and GAE of these commercial PDSs are shown in **Table 3**. Total polyphenol content expressed as GAE, EAE, and ellagic acid content correlated well with the TEAC of each individual PDS as shown in **Figure 3** (**a**, GAE/TEAC; **b**, EAE/TEAC; **c**, EA/TEAC).

Pomegranate juice is a rich source of hydrolyzable tannins, and pomegranate extract dietary supplements are assumed by consumers to deliver the tannins found in the juice. In fact, punicalagins have been shown to be responsible for the majority of the free radical scavenging ability of pomegranate juice and may relate to the health benefits of juice and supplements (*4*). The dried pomegranate husk or pericarp/peel contains around 10% of

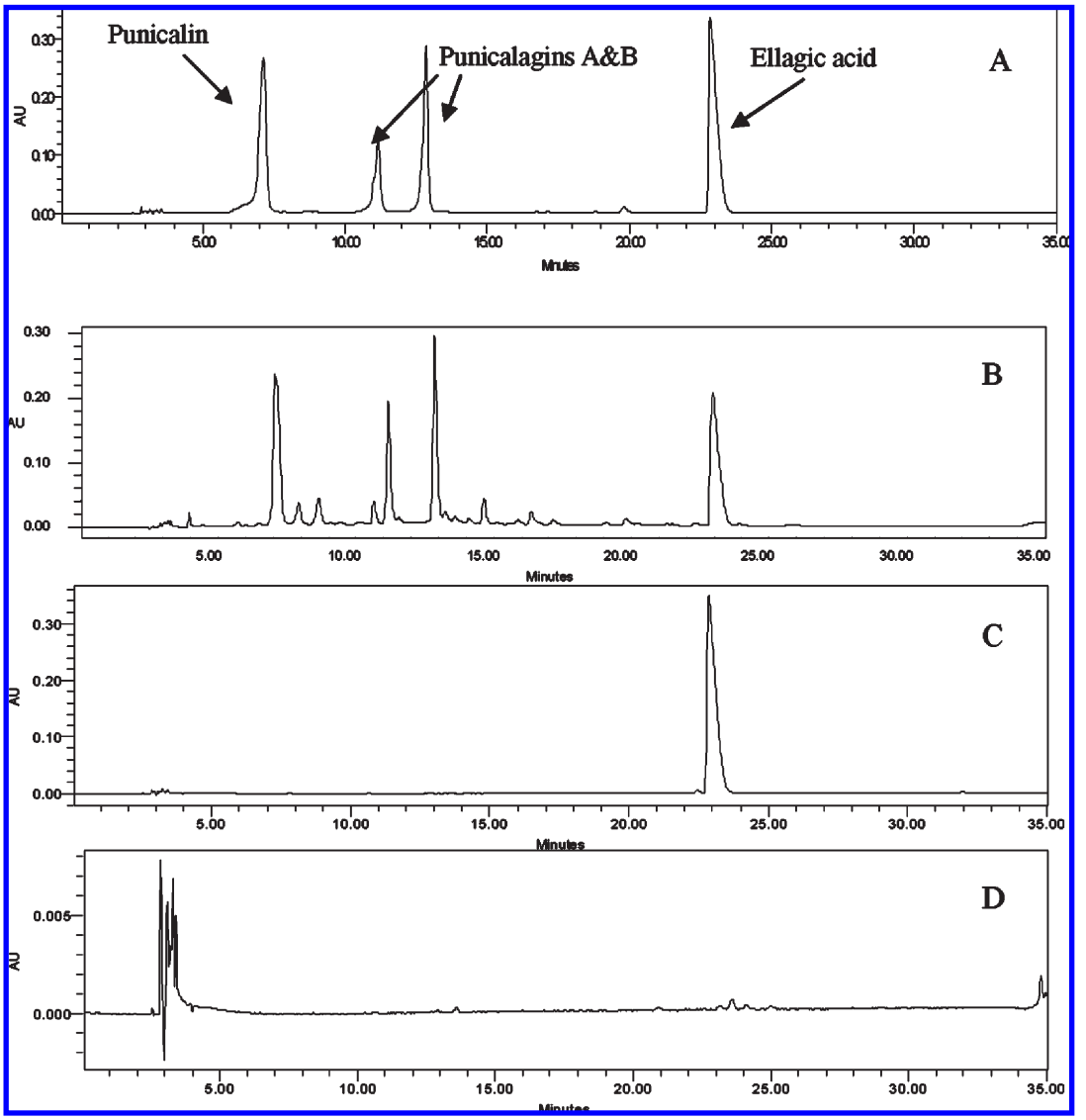

Figure 2. HPLC chromatograms: (**A**) reference mixture (punicalin, punicalagins A and B, and ellagic acid); (**B**) typical PDS of pomegranate extract; (**C**) typical PDS with only ellagic acid; (**D**) typical PDS with minor pomegranate chemical profiles.

punicalagins A and B (*13*). Gil et al. (*5*) found that laboratory-prepared pomegranate juice contained around 10 times less punicalagins than those in commercial pomegranate juice. This might be caused by differences in the processing conditions, such as temperature, pressure, and enzyme deactivation. The ellagitannins profiles of pomegranate supplements may vary among different products; however, the pomegranate dietary supplements should contain authentic pomegranate polyphenols. Ellagic acid was chosen as a common practical standard for pomegranate extract supplements, because vigorous hydrolysis can be used to produce ellagic acid from complex hydrolyzable tannins that would be difficult to measure on a routine basis. Several studies have demonstrated obtaining ellagic acid either by solid fermentation of pomegranate husk or creosote bush (*14*, *15*) or by the chemical processing of pomegranate husk or seeds (*16*, *17*). However, ellagic acid alone does not represent pomegranate polyphenols, and it is not as strong as apparently numerous synergistic chemical relationships that exist within the pomegranate fruit. Furthermore, this method of standardization has made possible the addition of ellagic acid from less expensive sources, such as chestnut bark (*18*), as an adulterant in pomegranate extract supplements. The problem has previously been noted by Lansky and co-workers (*19*). This is a significant issue because ellagic acid is poorly soluble in the aqueous phase. An alternative explanation of the findings here is that genuine pomegranate extract may have been the starting material but that the conditions of processing were such that the actual extract was inadvertently lost. However, the responsibility for ensuring the presence of the ellagitannins would still rest with manufacturers under these circumstances. Our research has clearly demonstrated that many pomegranate extracts contain ellagic acid alone with minimal or no detectable hydrolyzable tannins. For example, supplement C20 contained only two detectable peaks by HPLC at 360 nm. The dominant peak was ellagic acid, which accounted for 80% of total peak area, whereas the other 20% could not be identified. In supplement C4 there was nothing detected, suggesting that this PDS was made from something other than pomegranate fruit or that the concentrations of pomegranate tannins and ellagic acid were below the limit of detection of the HPLC-UV method utilized. Ellagic acid is poorly soluble in the water phase of the stirred layer in the intestine, where the hydrolysis of punicalagins occurs (*20*). It is thus unlikely that an ellagic acid supplement has the same benefits as a pomegranate supplement. Therefore, it is misleading the consumer to claim that a pure ellagic acid supplement is a pomegranate extract supplement.

Table 2. Punicalin, Punicalagins A and B, and Ellagic Acid Contents in Testing PDSs

sample	punicalin (%)	punicalagins (%)	ellagic acid (%)
Group 1: Typical Pomegranate Tannin HPLC Profile			
C1	0.6 ± 0	10.7 ± 0.3	2.7 ± 0
C7	0.7 ± 0.1	18.0 ± 0.3	2.5 ± 0
C16	1.4 ± 0.1	8.8 ± 0.8	12.3 ± 0.6
S1	0.6 ± 0	6.6 ± 0.3	10.2 ± 0.2
T1	1.1 ± 0.1	18.0 ± 0.8	0.5 ± 0.1
Group 2: Ellagic Acid as Dominant Component			
C3	nd	nd	31.9 ± 0.3
C5	nd	nd	51.0 ± 6.1
C6	1.7 ± 0	1.1 ± 0.1	9.0 ± 0.4
C8	0.9 ± 0.1	0.6 ± 0	26.3 ± 2.2
C9	nd	nd	20.1 ± 1.3
C10	1.4 ± 0.1	1.4 ± 0.3	4.7 ± 0.4
C11	2.1 ± 0.3	0.3 ± 0	1.4 ± 0.1
C13	0.8 ± 0.2	1.2 ± 0	1.6 ± 0
C14	1.6 ± 0	1.0 ± 0	7.4 ± 0.4
C15	nd	nd	54.7 ± 0.4
C17	nd	1.0 ± 0	7.5 ± 0.6
C18	nd	nd	52.4 ± 1.0
C19	1.9 ± 0.1	1.1 ± 0.2	9.1 ± 0.9
C20	0.2 ± 0	0.6 ± 0	7.9 ± 0.2
S3	0.4 ± 0	0.9 ± 0.1	13.6 ± 0.4
T2	nd	nd	9.5 ± 0.2
T4	1.2 ± 0.1	nd	30.5 ± 0.3
Group 3: Punicalin + Punicalagins + Ellagic Acid ≤ 1%			
C2	nd	nd	nd
C4	nd	nd	nd
C12	0.3 ± 0.1	0.2 ± 0	0.5 ± 0.1
S2	nd	0.6 ± 0.1	0.1 ± 0
T3	nd	nd	0.4 ± 0.1

Table 3. TEAC, GAE, and EAE Values of Testing PDSs

sample	TEAC (μmol/mg)	EAE (μg/mg)	GAE (μg/mg)
Group 1: Typical Pomegranate Tannin HPLC Profile			
C1	6.83 ± 0.21	666.3 ± 9.0	671.9 ± 13.3
C7	6.29 ± 0.23	561.5 ± 18.0	471.8 ± 14.5
C16	5.53 ± 0.27	464.7 ± 6.9	393.5 ± 5.9
S1	3.69 ± 0.30	390.9 ± 54.1	307.7 ± 42.7
T1	5.67 ± 0.33	452.1 ± 2.1	398.0 ± 1.8
Group 2: Ellagic Acid as Dominant Component			
C3	5.85 ± 0.35	535.3 ± 5.8	421.7 ± 4.6
C5	7.15 ± 0.50	544.6 ± 7.6	478.6 ± 6.7
C6	3.28 ± 0.18	267.6 ± 4.6	223.0 ± 3.8
C8	5.05 ± 0.09	357.3 ± 15.0	315.3 ± 13.1
C9	3.90 ± 0.14	356.6 ± 9.3	304.1 ± 7.8
C10	2.58 ± 0.27	231.1 ± 2.2	205.3 ± 1.9
C11	2.27 ± 0.20	170.0 ± 2.8	154.9 ± 2.3
C13	2.83 ± 0.11	187.9 ± 6.6	169.4 ± 5.4
C14	2.83 ± 0.19	241.2 ± 7.4	212.5 ± 6.0
C15	8.04 ± 0.42	616.5 ± 24.6	485.8 ± 19.5
C17	2.31 ± 0.20	154.6 ± 1.6	138.7 ± 1.4
C18	7.23 ± 0.49	530.5 ± 26.4	417.9 ± 20.8
C19	3.18 ± 0.23	282.9 ± 3.3	246.3 ± 2.6
C20	2.71 ± 0.30	196.6 ± 6.7	175.3 ± 5.9
S3	1.55 ± 0.12	134.6 ± 2.8	121.2 ± 2.4
T2	1.64 ± 0.37	88.7 ± 1.2	81.2 ± 1.0
T4	5.63 ± 0.23	453.2 ± 2.9	399.0 ± 2.5
Group 3: Punicalin + Punicalagins + Ellagic Acid ≤ 1%			
C2	0.61 ± 0.09	0.4 ± 1.5	0.3 ± 1.3
C4	0.55 ± 0.12	0	0
C12	0.57 ± 0.19	13.3 ± 2.1	11.1 ± 1.7
S2	0.52 ± 0.16	0	0
T3	0.25 ± 0.20	59.9 ± 32.6	46.4 ± 25.7

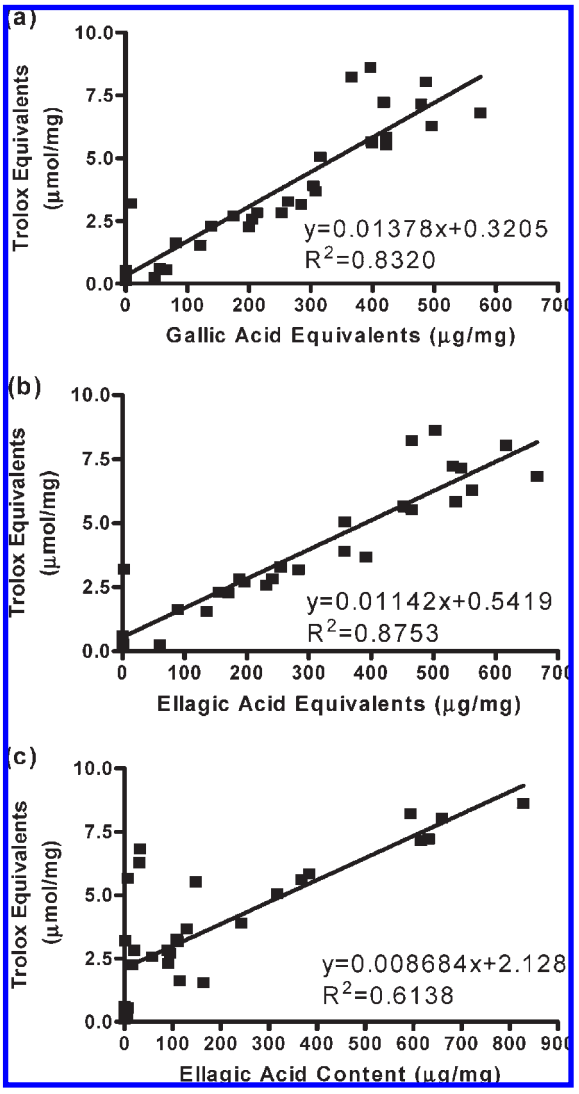

Figure 3. Correlation of antioxidant activity determined by the TEAC assay to (**a**) total pomegranate polyphenol concentration measured by the GAE assay, (**b**) total pomegranate polyphenol concentration measured by the EAE assay, and (**c**) ellagic acid content measured via HPLC-UV. Values are means ± SD; $n = 6$ for pomegranate polyphenol content assays, $n = 6$ for the TEAC assay, and $n = 2$ for HPLC-UV measurements.

Because our data demonstrate that antioxidant capacity in the laboratory can be reproduced with added ellagic acid rather than punicalagins from pomegranate, it is only by measuring the pomegranate polyphenols specifically that this form of adulteration can be prevented. Research on the health benefits of pomegranate dietary supplements should be conducted with only supplements demonstrated to contain authentic pomegranate polyphenols and standardized on this basis. Use of pomegranate supplements adulterated with ellagic acid and not providing pomegranate tannins may lead to false-negative research results and a failure of the public to derive health benefits from these supplements.

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