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Stoichiometric and Free Radical-Scavenging Kinetic Studies of Extractable Polyphenols from Pomegranate Husk and Pistachio Hull

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In this work, the antiradical activity of fresh and aged skins of two Iranian varieties of pomegranate husk and pistachio hull was measured in order to assess their concentration in antioxidant potential usable in various fields. The radical scavenging capacity (RSC) of pomegranate husks and pistachio hulls samples were studied using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH˙) assay. To determine the RSC and stoichiometric factor of the samplers, the second-order rate constants *(k_2)* and total H-atom-donating capacities *(n)* for the oxidation of polyphenol extracts by DPPH˙ were evaluated. The resulting k_2 values were also compared with those of the natural and synthetic antioxidants. The order of relative second-order rate constants in methanol at 25 °C found to be pomegranate husk > gallic acid > tannic acid > pistachio hull. Furthermore, the RSCs based on the calculation of area under kinetic curve (AUC), total stoichiometric factor of natural phenolics and commercial antioxidants were also compared.

Keywords: Natural antioxidant, Radical scavenging, Pistachio, Pomegranate, Kinetic studies

INTRODUCTION

Generation of active oxygen and free radicals is important both in food and in biological systems. In foods, the process of autoxidation and development of rancidity is caused by free radicals [1]. Lipid peroxidation leads to the development of off-flavors and undesirable chemical compounds [2]. In living systems, free radicals may attack life important molecules such as DNA and membrane lipids and play a key role in the pathology of numerous chronic diseases [3].

However, few researchers have studied the rate of antiradical reaction to indicate how fast the antioxidants react with the free radicals [4,5a]. Meanwhile, since free radicals in the organism are short-lived species, the knowledge of the kinetics of atom transfer is important, as it implies that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals. Several kinetic models based on the structural complexity of the polyphones have been proposed to allow the determination of rate constants, especially for those corresponding to the first hydrogen atom transfer possessing rather fast kinetics. The most widely used one is the antiradical activity (EC50), defined as the amount of antiradical necessary to decrease the initial DPPH˙ concentration by 50%. However, the EC50parameter does not give any information about the rapidity of the kinetics. Thus, in order to define a parameter quantifying not only the antiradical activity but also giving information about the rapidity of the kinetics, Sanchez-Moreno *et al*. [5b] introduced the antiradical efficiency AE = 1/(EC50 × TEC50), where TEC50 is the time needed to complete the reaction when the

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initial concentration of antiradical is a value corresponding to EC50.

The antioxidant efficacy (AE) is a parameter that combines both factors. It is well-accepted that DPPH˙ scavenging capacity is strongly dependent on the time of reaction and the EC50 value is highly dependent on how the "steady state" is arbitrarily selected and also on DPPH˙ concentrations at which time domain of the antioxidant-radical reaction are used. Consequently, the AE method may not have adequate reproducibility and can not be used to compare the DPPH˙ scavenging capacity data between different laboratories and, thus, suggesting a need for a new method in this respect. Recently, a few high-throughput assays have been developed to rapidly examine the free radical scavenging capacities of natural antioxidants. These include but are not limited to the oxygen radical absorbance capacity (ORAC) [6], hydroxyl radical scavenging capacity (HRSC) [7] and peroxyl radical scavenging capacity (PRSC) [8] assays. The DPPH˙-based method has also applied to the estimation of RSC [9]. All of these high-throughput assays use an area under the kinetic curve (AUC) for RSC estimation, expressed as trolox equivalents (TEs) in μmol on a per sample weight basis. These approaches take into account both the kinetic and the thermodynamic measurements of the radical-antioxidant reactions and make it possible to compare data between different laboratories.

To the best of our knowledge, there is no previous report on the kinetic study of phenolic compounds from pistachio hull and pomegranate husk extract in the DPPH˙ system. The aim of the present work was to characterize kinetically the free radical scavenging capacity of these natural antioxidants sources, which can find applications in various fields including agro-industrial, cosmetic and pharmaceutical industries.

EXPERIMENTAL

Plant Materials

Dried byproducts of two different varieties of pistachio hull (Kaleghouchi and Aghaie) were a gift from Dr. Asadi from Rafsanjan Medical Science University. Pomegranate husks samples were taken from two Iranian cities Saveh and Kashmar. All plant materials were collected in year 2008.

Fresh plant samples were cleaned, freeze-dried and grounded into a fine powder by laboratory mill. An amount of 5 g of pistachio hull and pomegranate husk powders were respectively extracted with 40 and 120 ml water by applying sonication for 45 min at ambient temperature and then the extracts were filtered for further purification and analysis. These extracts then called as raw extracted materials. Some parts of the raw extracted materials were lyophilized to get the total dry mass. Afterwards, the raw extracted materials were mixed with two parts of methanol for removing some insoluble materials in water extract. The resulting mixture was centrifuged at 5000 rpm for 15 min and the supernatant, called as methanolic treated extracts, was stored in dark at 4 °C for further use. Some parts of methanolic treated extracts were lyophilized to get the total dry mass.

Additionally, for further purification of the raw extracted materials, a procedure using amberlite XAD nonionic polymeric resin was used to obtain the purified samples. Aliquots of 100 and 50 ml of the extracts of pistachio hull and pomegranate husk were applied into a column packed with 250 g of XAD resin (100 cm length × 2.5 cm ID). Pectins, salts, and sugars were eluted with 350 ml of water and then the phenolics were eluted with 310 ml of methanol. The later fraction was concentrated and dried under reduced pressure at 37 °C.

Chemicals and Reagents

2,2-Diphenyl-1-picryl-hydrazyl (DPPH˙), 6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid (trolox), and tannic acid were purchased from Sigma Chemicals. Gallic acid was obtained from Panreac.

Apparatus

Absorption spectra were obtained using a Sinco (model UVS-2100) UV-Vis spectrophotometer. Measurements were performed in 10-mm quartz cells and temperature was controlled to ±0.1 °C using a thermostatic cell holder and a thermostatic bath. A Bachrach/Coleman spectrophotometer (Model 35, USA) equipped with an advanced data acquisition and processing system (ADC-212 Picotech, UK) was used for monitoring the fast reaction kinetics. The decrease in absorbance at 515 nm was determined continuously with data capturing at 1 ms intervals until the reaction plateau step was

reached. Methanol was used to zero the spectrophotometer. Special care was taken to minimize the loss of free radical activity of the DPPH. The Datafit version 8.1 software was used for the data fitting.

Rate Constant and Stoichiometric Measurements

The second-order rate constant (k_2) was determined by having the antiradical compound [AH] in large excess as compared to the radical compound [DPPH˙], thus forcing the reaction to behave in a pseudo first-order:

$$-d[DPPH^{\cdot}]/dt = k_1[DPPH^{\cdot}] \quad (1)$$

where

$$k_1 = k_2[AH] \quad (2)$$

Therefore, DPPH˙ was depleted from the pseudo-first order conditions following the equation:

$$[DPPH^{\cdot}] = [DPPH^{\cdot}]_0 \, e^{-k1t} \quad (3)$$

Fitting of the experimental data to obtain k_1 values was carried out by plotting ln([DPPH˙]) *vs*. t by using the Microsoft Excel software.

The kinetic studies were conducted by measuring the disappearance of DPPH band at 515 nm under pseudo-first order conditions at a temperature of 25 °C to evaluate the H-transfer reactions from polyphenols to DPPH˙. The DPPH˙ solution in methanol was freshly prepared for each experiment (<1 day). Determinations of k_1 were conducted in duplicate using 12 different extract concentrations per sample. Briefly, 100 µl of testing antioxidant solution was mixed and reacted with 1900 µl of 130 µM DPPH˙.

Since each DPPH˙ molecule reacts with one active hydroxyl group, we can determine the quantity of active phenolic hydrogens in the reaction with DPPH˙ by the decrease in absorbance of DPPH˙ at 515 nm in the reaction solution under the condition of [DPPH˙] > [AH], which allows all of the AH to take part in the reaction with DPPH˙. The stoichiometric factor may be calculated from the decrease in absorbance of DPPH˙ band and the concentration of AH. A DPPH˙ radical-scavenging assay was employed as described by Brand-Williams *et al*. [10] and Espin *et al*. [11] to determine the hydrogen donating ability of the different crude and purified extracts. A volume of 1950 µl of 130 µM DPPH˙ methanol solutions was used. The reaction was started by the addition of 50 µl of diluted extracts. The bleaching of DPPH˙ was measured at 515 nm against the blank (130 µM DPPH˙ methanol solution) at 25 °C after 45 min. The difference in absorbance is proportional to the stoichiometric factor of the samples, expressed as milligrams or millimoles of antioxidant per millimole of DPPH˙.

Radical Scavenging Capacity (RSC) Assay Based on Area under Kinetic Curve

A volume of 1950 µl of 130 µM DPPH˙ methanol solution was used to determine the hydrogen-donating ability of the crude extract. The reaction was started by the addition of 50 µl of diluted extracts. The bleaching of DPPH˙ was monitored at 515 nm after each 3 s at 25 °C until 600 s. Menwhile, the normal decay of the blank solution (130 µM DPPH˙ in methanol) was also monitored. Four different concentrations were used for each antioxidant extract and antioxidant standard in the same experimental conditions. To estimate the total DPPH˙ scavenging capacity of a selected antioxidant sample, the %DPPH˙ quenched was determined according to the following equation:

$$\%DPPH^{\cdot} = (1-(A-A_b)/(A_0-A_b)) \times 100 \quad (4)$$

where A, A_b and A_0 represent the absorbance of the certain concentration of a selected antioxidant, blank, and the initial radical at 515 nm measured at the reaction time t, respectively. The values of %DPPH˙ quenched at different reaction times obtained from Eq. (4), were then used to evaluate the AUC values by using Eq. (5).

$$AUC = \sum_{i=0}^{i=t} (q_i + q_{i+1})/2 \times \Delta t \quad (5)$$

where q_0 is the initial DPPH˙ quenched reading at initial time, q_i is the total DPPH˙ quenched reading at time t, and Δt is the interval times between two subsequent points of absorbance readings. The data were processed with a Microsoft Excel program. The net AUC was calculated by subtracting the AUC of the blank from the AUC of the sample. Relative RSC values

(RRSC) expressed as millimoles of trolox equivalents (TE) per gram of material, were caculated from following equation:

$$RRSC = (\text{net } AUC_{sample})\ [Trolox]/[AH]\ (\text{net } AUC_{standard}) \quad (6)$$

A more precise RSC value was obtained by dividing the slope of the regression equation between net AUC and different antioxidant concentrations, for the sample, by the slope of the trolox curve for the same assay (regression method).

RESULTS AND DISCUSSIONS

Reaction Kinetics of Natural Antioxidants-DPPH˙

The knowledge of the kinetics of atom transfer is important because free radicals in the organism are short-lived species, which implies that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals. The DPPH method permits to evaluate not only the antiradical capacity of antioxidants, but also the rate of their reaction towards the free radicals. The rate constant of the reaction of antioxidants with free radicals are indicative of the order of reactivity and shows how much an antioxidant reduces the rate of oxidation [5]. The kinetic information can be used in food systems to design strategies to inhibit lipid, flavor, and color oxidation and preserve the quality of foods. The more rapidly the absorbance decreases, the more potent is the antioxidant compound in terms of hydrogen donating ability.

Thus, in the presence of antioxidants, the decrease in the absorbance at 515 nm *vs*. time was measured until a steady state was observed (Fig. 1). From the slope of linear plot of $\ln([DPPH^{\cdot}]/[DPPH^{\cdot}_0])$ *vs*. t, the second-order rate constants (k_2) of the reactions of DPPH˙ radical with appropriate antioxidants were estimated and the results are reported in Table 1. For phenolics purified from natural sources by XAD resin, the k_2 found to be 2.03×10^{-4} l mg^{-1} s^{-1} for pomegranate of Saveh, 2.16×10^{-4} l mg^{-1} s^{-1} for pomegranate of Kashmar, 1.10×10^{-4} l mg^{-1} s^{-1} for pistachio of Aghaie, 0.84×10^{-4} l mg^{-1} s^{-1} for pistachio of Kaleghouchi, 1.84×10^{-4} l mg^{-1} s^{-1} for gallic acid and 1.80×10^{-4} l mg^{-1} s^{-1} for tannic acid. The order of deceasing rate constant k_2 is: pomegranate husk > gallic acid > tannic acid > pistachio hull. These results indicate that phenolics from pomegranate husk have faster reaction kinetics than phenolics from pistachio hull. Phenolics from pomegranate husk also react faster to stabilize DPPH˙ radicals as compared to gallic acid, which is considered to be a powerful antioxidant (gallic acid is 6.7 and 9.5 fold antiradical activity than trolox and vitamin E respectively) [11,12].

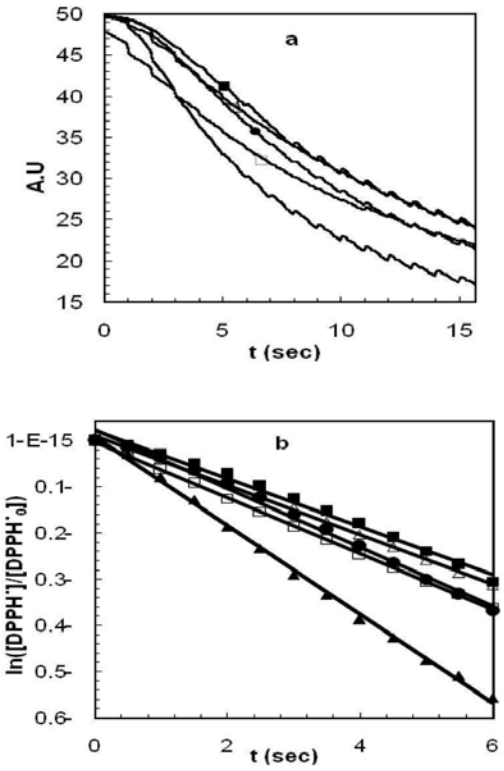

Fig. 1. (a) Absorbance decrease and (b) $\ln([DPPH^{\cdot}]/[DPPH^{\cdot}_0])$ *vs*. t for the pseudo-first-order reaction of phenolics with DPPH˙. ▲: pistachio of Aghaie, □: pistachio of Kaleghouchi, ●: pomegranate of Kashmar, Δ: gallic acid, and ■: pomegranate of Saveh.

Stoichiometric Factor (n_{tot})

Antioxidant can be characterized by their stoichiometry, which indicates the amount of oxidant molecules reduced by one molecule of antioxidant [13]. To determine the number of free radicals stabilized per unit of phenolic compounds present in each sample, the analyses stoichiometric factor were conducted, as follows. The reaction of DPPH˙ with a hydrogen-donating antioxidant can be represented by

Table 1. Phenolics.and Standards in l mg^{-1} s^{-1}

Samples	Variety	k_2 (l mg^{-1} s^{-1})	
		Fresh	Aged
Raw extracted	Pomegranate of saveh	5.11×10^{-5}	
	Pomegranate of kashmar	4.95×10^{-5}	
	Pistachio of aghaie	2.21×10^{-5}	
	Pistachio of kaleghouchi	2.05×10^{-5}	
Methanolic treatment	Pomegranate of saveh	7.32×10^{-5}	7.31×10^{-5}
	Pomegranate of kashmar	5.98×10^{-5}	6.35×10^{-5}
	Pistachio of aghaie	2.82×10^{-5}	2.94×10^{-5}
	Pistachio of kaleghouchi	3.49×10^{-5}	4.33×10^{-5}
Purified phenolics	Pomegranate of saveh	2.03×10^{-4}	1.87×10^{-4}
	Pomegranate of kashmar	2.16×10^{-4}	1.68×10^{-4}
	Pistachio of aghaie	1.10×10^{-4}	1.11×10^{-4}
	Pistachio of kaleghouchi	8.41×10^{-5}	1.15×10^{-4}
Standards	Tannic acid	1.80×10^{-4}	
	Gallic acid	1.89×10^{-4}	

$$DPPH^{\cdot} + AH \rightarrow DPPH^{\cdot} - H + A^{\cdot} \quad (7)$$

On the basis that each $DPPH^{\cdot}$ molecule reacts with active hydrogen, we can determine the number of active phenolic hydroxyl groups in 1 g of total phenolics, for each sample. Experiments extending over 45 min were used for the determination of the total stoichiometry (n_{tot}) of antioxidant at different concentrations in methanol using Eq. (8),

$$n_{tot} = (A_0 - A_f)/(\varepsilon bc) \quad (8)$$

where A_f is the final absorbance by taking into account the intrinsic decay of $DPPH^{\cdot}$, A_0 is the initial absorbance, ε is the molar absorptivity of $DPPH^{\cdot}$ (11260 M^{-1} cm^{-1}) and b and c are the cell path length and initial antioxidant concentration, respectively. Of course, the initial $DPPH^{\cdot}$-antioxidant molar ratio $A_0/\varepsilon bc$ must be higher than n_{tot} for Eq. (8) to be applied.

Because of the unknown molecular weights of natural phenolics, in Table 2 and Fig. 2, we have reported $1/n_{tot}$ with dimension of mg AH/mmol $DPPH^{\cdot}$ instead of n_{tot} with dimension of mmol $DPPH^{\cdot}$/mmol AH. The results show that the n_{tot} of antioxidants depends on the concentration used for analysis. Due to this concentration dependency, it is not possible to give a single value for n_{tot} of a given antioxidant and compare it with the stoichiometric factor of other products, as expressed by other researchers [5,12,14]. Table 2 shows that the relative standard deviations for stoichiometric factor of different samples used in this study are in the range of 6.9-52.0%, which obviously indicate huge deviations of n_{tot} from the mean values, in the concentration range studied. Thus, it is suggested that, for the comparison of n_{tot} values of different antioxidants, they should be evaluated over a wide concentration range graphically.

It is worth mentioning that, for some of antioxidants including gallic acid, tannic acid, and different samples of pistachio hull, the graph of $1/n_{tot}$ *vs*. concentration possess a negative slope before reaching a plateau, while it is not the case for pomegranate husk samples and trolox (Fig. 2). Since this plateau has been reached at high antiradical compound [AH] possessing higher reaction rates so that more $DPPH^{\cdot}$ radicals have been quenched over defined time intervals, more precise lower valued stoichiometric factors can be obtained.

Figure 2d shows the linear graphs of $1/n_{tot}$ *vs*. inverse of antioxidant concentration. By extrapolating the graphs to zero, where the stoichiometry is just satisfied with no further antioxidant to affect reaction with $DPPH^{\cdot}$, one obtains the

Table 2. Inverse of Total Stoichiometry ($1/n_{tot}$) of the Natural Phenolics and Standards at Different Concentrations and Extrapolation. The Data in the Parenthesis are the RSD%

Samples		Fresh		Aged	
		Concentrarion (mg l^{-1})	$1/n_{ot}$ (mg antioxidant/ mmol $DPPH^{\cdot}$)	Concentrarion (mg l^{-1})	$1/n_{tot}$ (mg antioxidant/ mmol $DPPH^{\cdot}$)
Raw extracted	Pomegranate of saveh	2.5	173.4		
		3.6	171.7		
		4.8	169.8		
		6.0	107.3		
		8.7	177.1		
			(18.5)		
	Pomegranate of kashmar	3.6	3.6		
		4.7	4.7		
		8.5	8.5		
			(46.5)		
	Pistachio of aghaie	7.3	452.9		
		10.8	330.8		
		14.3	307		
		17.7	309.2		
			(19.8)		
			$1/n_{extra} = 309$		
	Pistachio of kaleghouchi	6.7	549.3		
		10	398.3		
		13.1	287.8		
		16.3	286.2		
			(32.7)		
			$1/n_{extra} = 286$		
Methanolic treatment	Pomegranate of saveh	1.4	150.0	1.4	164.5
		2.7	178.8	2.7	116.0
		4.0	149.8	4.0	142.6
		5.2	143.6	5.2	111.1
			(10.1)		(18.6)
					$1/n_{extra} = 92$
	Pomegranate of kashmar	1.4	137.7	1.4	175.6
		2.8	156.9	2.8	158.1
		4.1	123.7	5.4	144.5
		5.3	114.2		
			(14.0)		(9.8)
					$1/n_{extra} = 135$
	Pistachio of aghaie	4.3	420.5	4.3	434.7
		8.4	310.6	8.4	350.8
		12.5	268.3	12.5	288.3
		16.6	249.9	16.6	273.6
		20.5	253.8	20.5	265.5
			(23.7)		(22.0)
			$1/n_{extra} = 253$		$1/n_{extra} = 265$

Table 2. Continued

	Pistachio of kaleghouchi	11.2	344.3	11.2	361.3
		14.7	318.2	14.7	328.7
		18.2	288.8	18.2	299.4
		21.7	289	21.7	295.8
			(18.4)		(9.5)
			$1/n_{extra}$ = 289		$1/n_{extra}$ = 296
Purified phenolics	Pomegranate of saveh	1.8	129.6	2.3	100.5
		3.5	94.4	3.4	89.4
		4.3	96.7	4.6	75.8
		5.2	87.5	5.7	78.9
			(18.4)		(13.0)
			$1/n_{extra}$ = 95		$1/n_{extra}$ = 73
	Pomegranate of kashmar	1.8	111.4	2.4	94.9
		2.7	82.2	3.5	84.0
		3.5	85.3	4.7	81.4
		4.4	90.3	5.8	89.8
			(14.3)		(6.9)
			$1/n_{extra}$ = 85		$1/n_{extra}$ = 85
	Pistachio of aghaie	2.8	192.6	2.5	287.2
		5.6	122.1	5	180.1
		8.4	111.8	7.5	158.7
		11.1	113.4	9.8	145
		13.7	110.8	12.2	144.5
			(27.0)		(32.7)
			$1/n_{extra}$ = 111		$1/n_{extra}$ = 144
	Pistachio of kaleghouchi	1.7	272.3	3	174.1
		3.3	131.5	6	146.6
		4.9	104	8.9	137
		6.5	102.1	11.7	135.1
		8	100.5	14.5	138.3
			(52.0)		(11.1)
			$1/n_{extra}$ = 100		$1/n_{extra}$ = 135
Standards	Trolox	1.2	141.6		
		2.73	116.4		
		3.7	138.2		
		4.93	101.4		
			(15.3)		
	Tannic acid	0.8	60.5		
		1.7	38.8		
		2.5	34		
		3.3	30.2		
			(33.2)		
			$1/n_{extra}$ = 24		
	Gallic acid	0.5	54.3		
		1	34.9		
		1.5	30.4		
		1.9	28.2		
			(32.2)		
			$1/n_{extra}$ = 22		

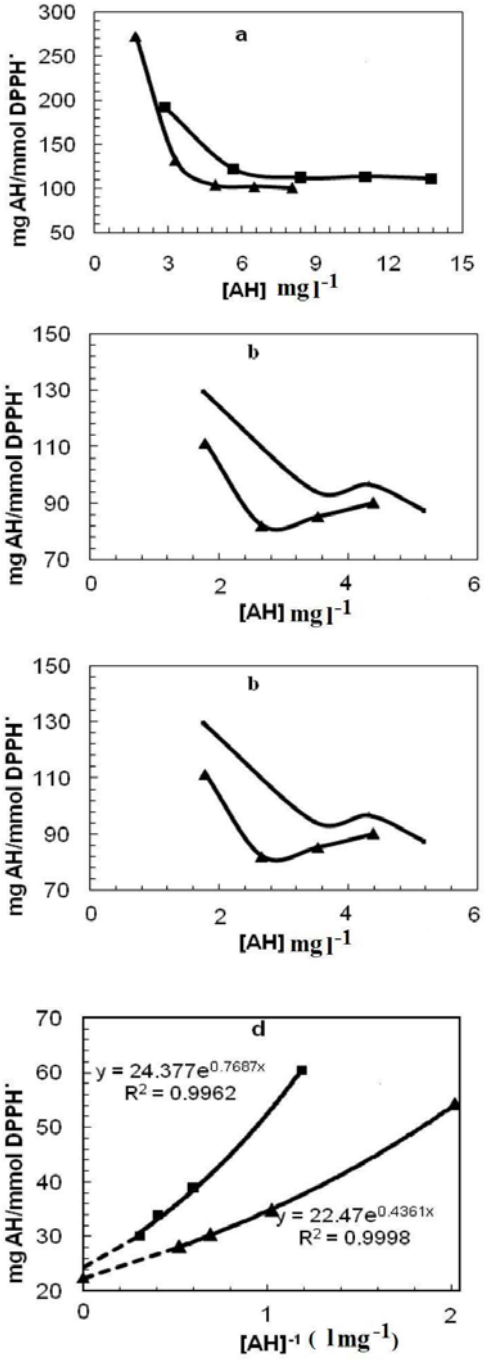

Fig. 2. Concentration dependency of $1/n_{tot}$ of different phenolics. (a) pistachio hull, ■: Aghaie and ▲: Kaleghouchi; (b) pomegranate husk, ■: Saveh and ▲: Kashmar; (c) standard samples, ▲: gallic acid and ■: tannic acid; and (d) extrapolation of $1/n_{tot}$ for ▲: gallic acid and ■: tannic acid.

ratio of antioxidant to DPPH· (stoichiometry factor). In this case we can compare the n_{tot} values of different antioxidant at the steady state. According to these results, the n_{tot} values for gallic acid and tannic acid indicate that they contain 7.7 and 70 mol of active hydroxyl groups per mol to reduce DPPH·, respectively. Meanwhile, the n_{tot} values for pistachio hull purified phenolics were determined to be 0.0100 and 0.0090 (mmol DPPH·/mg AH) for Kaleghouuchi and Aghaie varieties, respectively. The respective n_{tot} values for pomegranate husk purified phenolics for Saveh and Kashmar varieties were also determined as 0.0105 and 0.0117 (mmol DPPH·/mg AH). These results indicate that, for the quenching of 1 mmol of DPPH·, around 100.0-111.0 mg of phenolics from pistachio hull or around 85.0-95.0 mg of phenolics from pomegranate husk are necessary, the equivalent mass for the gallic acid and tannic acid being 22.0 and 24.0 mg, respectively.

Use of Area under Kinetic Curve (AUC) for the Estimation of RSC

The AUC values for different concentrations of each standard antioxidant compounds and purified natural phenolics were obtained from the %DPPH· quenched-reaction time plots. Figure 3 shows this plot for the tannic acid at four concentrations. The results obtained for three standard antioxidants including trolox, gallic acid, and tannic acid, as well as the natural phenolic extracts including two pomegranate husk varieties and two pistachio hull varieties are summarized in Table 3. The order of decreasing RSC values found to be: gallic acid > tannic acid > pomegranate husk > pistachio hull, materials with higher RSC values being associated with stronger DPPH· radical scavenging capacity. This order was in agreement with that of the total stoichiometric factor values. It should be noted that the RSC values of natural phenolic extracts of pomegranate husk and pistachio hull obtained in this work are larger than the values reported for botanical extracts by Zhihong *et al*. [9].

As we mentioned above, one of the problems associated with the conventional DPPH· scavenging capacity assay is that the percentage of DPPH· quenched is depend on the concentration of antioxidant used in the reaction. This makes it hard to compare the results from different laboratories. The effect of concentrations of antioxidants on RSC values was

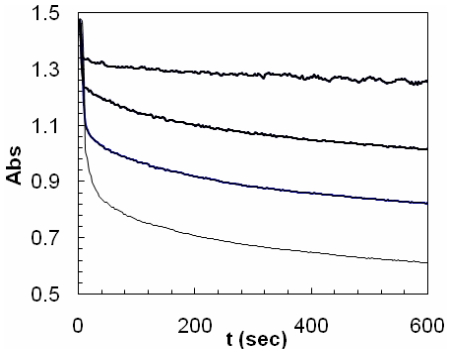

Fig. 3. %DPPH˙ quenched-reaction time plot for the estimation of AUC and RSC of gallic acid. Concentrations of gallic acid are 0.49, 0.98, 1.46, and 1.92 mg l^{-1} top to bottom, respectively.

evaluated and reported as relative standard deviation for standard antioxidants and natural phenolic extracts (Table 3). The RSDs were in the range of 2.0-20.0%, which shows that the RSC values reported by this method are more accurate than n_{tot} with RSDs values in the range of 6.9-52.0% (Table 2). These data demonstrated that the RSC assay using AUC is a more practical approach for radical scavenging capacity estimation and for comparison of different concentration of antioxidant samples at different laboratories [9].

Effect of Extraction and Purification Procedures on the Rate Constant, and RSC of Natural Phenolics

The extract yield is defined as the amount of freeze dried extract (grams) obtained from 1 kg of starting dried

Table 3. The Results of Area under the Kinetic Curve (AUC) and Relative Radical Scavenging Capacity (RSC) for Different Phenolic Extracts and Standards. The Data in the Parenthesis are the RSD%

Samples		Concentrarion (mg l^{-1})	AUC	Fresh RRSC for each concentration (mmol g^{-1})[a]	Slope	Intercept	r2	RRSC based regression (mmol trolox/g)[b]
Raw extracted	Pomegranate of saveh	2.5	102.4	2.9	33.9	26.4	0.9956	2.34
		3.6	157.1	3.0				
		4.8	192.1	2.7				
		8.7	320.2	2.5				
				(6.9)				
	Pomegranate of kashmar	3.6	152.9	3.0	34.1	29.4	0.9990	2.35
		4.7	186.3	2.7				
		8.5	320.2	2.6				
				(6.8)				
	Pistachio of aghaie	7.3	191.7	1.8	18.9	36.6	0.9421	1.30
		10.8	224.7	1.4				
		14.3	287.0	1.4				
		17.7	390.0	1.5				
				(12.5)				
	Pistachio of kaleghouchi	6.7	146.9	1.5	20.9	10.0	0.9340	1.44
		10.0	206.7	1.4				
		13.1	317.9	1.7				
		16.3	331.9	1.4				
				(7.8)				
	Pomegranate of saveh	1.4	65.5	3.2	58.0	-27.1	0.9732	3.99

Table 3. Continued

Methanolic treatment		2.7	109.4	2.7				
		4.0	217.0	3.7				
		5.2	276.8	3.6				
				(13.5)				
	Pomegranate of kashmar	1.4	68.8	3.3	64.0	-34.1	0.9851	4.41
		2.8	125.4	3.1				
		5.3	311.3	4.0				
				(14.0)				
	Pistachio of aghaie	4.3	99.6	1.6	24.1	-1.5	0.9964	1.66
		8.4	200.9	1.6				
		12.5	312.8	1.7				
		16.6	384.5	1.6				
		20.5	497.2	1.7				
				(2.9)				
	Pistachio of kaleghouchi	11.2	231.6	1.4	17.9	34.7	0.9952	1.23
		14.7	298.6	1.4				
		18.2	368.9	1.4				
		21.7	417.4	1.3				
				(3.1)				
	Pomegranate of saveh	1.8	93.2	3.7	94.4	-59.8	0.9726	6.49
		3.5	301.8	6.0				
Purified phenolics		4.3	331.1	5.3				
		5.2	423.0	5.6				
				(20.0)				
	Pomegranate of kashmar	1.8	111.7	4.3	97.8	-49.0	0.9830	6.73
		2.7	229.9	5.9				
		3.5	299.8	5.8				
		4.4	371.7	5.8				
				(14.2)				
	Pistachio of aghaie	2.8	172.7	4.2	50.4	43.6	0.9958	3.47
		5.6	341.9	4.2				
		8.4	466.6	3.8				
		11.1	613.8	3.8				
		13.7	719.1	3.6				
				(6.2)				
	Pistachio of kaleghouchi	1.7	111.6	4.6	52.0	21.6	0.9935	3.58
		3.3	179.1	3.7				
		4.9	291.2	4.1				
		6.5	360.3	3.8				
		8.0	434.1	3.7				
				(9.3)				

Table 3. Continued

Tandards	Trolox	1.2	82.0	4.7	58.1	6.6	0.9946	4.00
		2.7	158.4	4.0				
		3.7	217.0	4.0				
		4.9	299.2	4.2				
				(7.7)				
	Tannic acid	0.8	124.0	10.1	165.4	-15.7	0.9993	11.38
		1.7	262.7	10.8				
		2.5	387.5	10.8				
		3.3	528.8	11.1				
				(3.9)				
	Gallic acid	0.5	110.3	15.3	235.5	11.0	0.9796	16.20
		1.0	258.1	18.1				
		1.5	373.6	17.7				
		1.9	444.9	15.9				
				(8.0)				
Methanolic treatment	Pomegranate of saveh	1.4	81.9	4.0	55.0	0.7	0.9624	3.79
		2.7	158.3	4.0				
		4.0	196.0	3.4				
		5.2	304.2	4.0				
				(8.3)				
	Pomegranate of kashmar	1.4	60.6	2.9	53.1	20.0	0.9976	3.65
		2.8	124.0	3.0				
		5.4	268.2	3.4				
				(9.0)				
	Pistachio of aghaie	4.3	62.1	1.0	20.9	-24.3	0.9955	1.23
		8.4	148.1	1.2				
		12.5	244.0	1.3				
		16.6	334.1	1.4				
		20.5	393.5	1.3				
				(12.3)				
	Pistachio of kaleghouchi	7.5	150.2	1.4	20.9	-16.1	0.9759	1.44
		11.2	196.7	1.2				
		14.7	305.5	1.4				
		18.2	363.4	1.4				
				(6.9)				
	Pomegranate of saveh	2.3	161.1	4.8	94.1	-46.2	0.9902	6.4800
		3.4	285.4	5.7				

Table 3. Continued

\Urified phenolics		4.6	399.5	6.0				
		5.7	476.2	5.8				
				(9.5)				
	Pomegranate of kashmar	2.4	180.0	5.2	75.0	11.4	0.9815	5.16
		3.5	279.3	5.4				
		4.7	382.8	5.6				
		5.8	433.8	5.1				
				(4.1)				
	Pistachio of aghaie	3.0	128.1	2.9	40.4	3.1	0.9998	2.78
		6.0	243.1	2.8				
		8.9	359.4	2.8				
		11.7	475.5	2.8				
		14.5	593.0	2.8				
				(2.0)				
	Pistachio of kaleghouchi	2.5	102.3	2.8	37.3	0.2	0.9912	2.57
		5.0	182.0	2.5				
		7.5	265.0	2.4				
		9.8	377.7	2.6				
		12.2	407.0	2.3				
				(7.3)				

[a]According to Eq. (6). [b]According to regression method.

byproducts [extract (g)/(kg dried weight)] in different extraction and purification procedures. Such mean extract yield for the ultrasound extraction step is 427.5 g freeze dried extract per 1 kg starting dried matter (g kg^{-1} dm) for pistachio hull and is 477.0 (g kg^{-1} dm) for pomegranate husk. While, the mean extract yield for the methanolic treatment step is 351.5 g kg^{-1} dm for pistachio hull is 427.5 (g kg^{-1} dm) for pomegranate husk and, after passing the first extracted materials through XAD resin, the mean extract yield for pistachio hull and pomegranate husk become 107 g kg^{-1} dm and 130 g kg^{-1} dm, respectively. As can be seen, for pistachio hull samples the residue mass ratio (RMR = extraction yield at each step/extraction yield of raw) obtained after methanolic treatment and amberlite purification are 1.2- and 4.0-folds lower than that of raw extracts respectively, which resulted in phenolic enriched extracts. Also, the corresponding RMRs for pomegranate husk were 1.1- and 3.7-folds lower than that of raw extracts.

The values for the rate constant and RSC for different steps of extraction and purification are presented in Tables 1 and 3. The mean value for each assays for the samples obtained after amberlite purification and methanolic treatment relative to mean value for raw extracts was defined as relative purification factor (RPF). The RPF of these assays shows a trend similar to the inverse of RMR (Fig. 4). The RMR mean values for RSC and k_2 for the samples obtained after amberlite purification was in the range of 2.6-4.5-folds (for pistachio hull) and 2.8-4.2-folds (for pomegranate husk), and the mean values for methanolic treatment was in the range of 1.1-1.5-folds (for pistachio hull) and 1.3-1.7-folds (for pomegranate husk) higher than that of corresponding raw extracts. As the RMR in methanolic treatment step is 1.2 fold (for pistachio hull) and 1.1 fold (for pomegranate husk) higher than that of raw extracts, then the RSC and k_2 will possess an increase of 1.1-1.5-folds (for pistachio hull) and 1.3-1.7-folds higher (for pomegranate husk) over the raw extracts. Also, the RMR after amberlite purification is 4.0 folds (for pistachio hull) and 3.7 folds (for pomegranate husk) higher than that of raw extracts and, consequently, the RSC and k_2 revealed 2.6-4.5 folds (for pistachio hull) and 2.8-4.2 folds (for pomegranate husk)

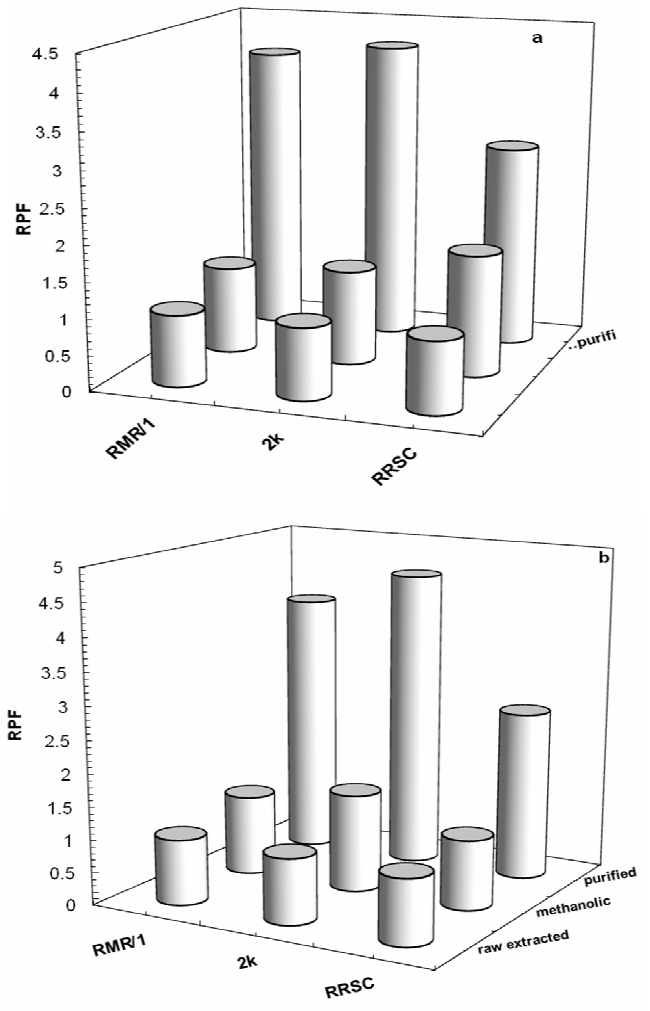

Fig. 4. Effect of extraction and purification procedures on the extract yield, rate constant, and RRSC of natural phenolics. (a) pomegranate husk samples and (b) pistachio hull samples (RPF = mean value for each assays/mean value for raw extracts).

increase over that of the raw extracts. These results show that, by the progression of extraction and purification procedures, the RSC and k_2 parameters will become larger, which proved that some impurities of extracted materials have been eliminated and a strong correlation among the values of RSC, and k_2 and amount of residual mass has been obtained at each purification step.

Effect of Aging on RSC, Rate Constants, and Stoichiometric Factors of Methanolic Treated and Purified Samples of Natural Phenolics

Different methanolic treated solution and aqueous purified samples of pomegranate husk and pistachio hull were stored at 4 °C for one year to evaluate the stability of their constituents by means of RSC, k_2 and n_{tot}. The data for the RSC, k_2 and n_{tot} are also included in Tables 1-3, for comparison with the fresh solutions. As is obvious, no significant difference between fresh and aged samples was observed for k_2 and n_{tot} values. However, for the AUC method, a difference of about 22% was observed for different samples in a one year period, which indicate that the AUC method can detect some differences, better than the other classical antioxidant methods.

CONCLUSIONS

The H-atom-donating capacity of polyphenols can be conveniently and quantitatively assessed from the stoichiometry and the kinetics of their reaction with DPPH˙. This study showed that the phenolics from pistachio hulls and pomegranate husks are fast radical scavengers relative to the standards like gallic acid and tannic acid. Due to the concentration dependency of n_{tot}, as a limiting factor, one may use the extrapolated value of n_{tot} at the highest concentrations as a more precise value for this parameter. Using the proposed method, the total stoichiometric factors are found to be 100-110 mg of phenolics from pistachio hull and around 87-90 mg of phenolics from pomegranate husk necessary for quenching of 1 mmol of DPPH˙, the corresponding mass for the gallic acid and tannic acid being 28.2 and 30.2 mg, respectively. The AUC assay used in this work does not have the problems of other classical antioxidant assays and also makes it possible to compare the DPPH radical scavenging capacity data between different research laboratories. The higher phenolics RSC for pistachio hulls and pomegranate husks, compared to other antioxidants, indicates that these byproducts have the potential to be considered as important natural antioxidant sources for the functional food and dietary supplement markets.

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REFERENCES

[1] F. Shahidi, P.K. Janitha, P.D. Wanasundara, Crit. Rev. Food Sci. Nutr. 32 (1992) 67.

[2] A.J. Angelo, Crit. Rev. Food Sci. Nutr. 36 (1996) 175.

[3] I.S. Young, J.V. Woodside, J. Clin. Pathol. 54 (2001) 176.

[4] J.C. Espin, C. Soler-Rivas, H.J. Wichers, J. Agric. Food Chem. 48 (2000) 648.

[5] a) H. Shi, E. Niki, Lipids 33 (1998) 365; b) C. Sanchez-Moreno, J.A. Larrauri, F. Saura-Calixto, J. Sci. Food Agric. 76 (1998) 270.

[6] D. Huang, B. Ou, M. Hampsch-Woodill, J.A. Flanagan, R.L. Prior, J. Agric. Food. Chem. 50 (2002) 4437.

[7] J. Moore, J.J. Yin, L.L. Yu, J. Agric. Food Chem. 54 (2006) 617.

[8] K.K. Adom, R.H. Liu, J. Agric. Food Chem. 53 (2005) 6572.

[9] C. Zhihong, M. Jeffrey, Y. Liangli (Lucy), J. Sci. Food Agric. 54 (2006) 7429.

[10] W. Brand-Williams, M.E. Cuvelier, C. Berset, Lebensm. Wiss. Technol. 28 (1995) 25.

[11] J.C. Espin, C. Soler-Rivas, H.J. Wichers, C. Garcia-Viguera, J. Agric. Food Chem. 48 (2000) 1588.

[12] D. Villano, M.S. Fernandez-Pachon, M.L. Moya, A.M. Troncoso, M.C. Garcia-Parrilla, Talanta 71 (2007) 230.

[13] O. Dangles, G. Fargeix, C. Dufour, J. Chem. Soc., Perkin Trans. 2 (2000) 1653.

[14] B.A. Cevallos-Casals, L. Cisneros-Zevallos, J. Agric. Food 51 (2003) 3313.