JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Distribution of Phenolic Acids in Different Tissues of Jujube and Their Antioxidant Activity

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ABSTRACT: Free, esterified, glycosided, and insoluble-bound forms of eight phenolic acids in pulp, seed, and peel of jujube are separated and quantified by high performance liquid chromatography with electrochemical detection (HPLC-ECD). In the whole jujube, *p*-hydroxybenzoic and cinnamic acids are the most abundant phenolic acids. All quantified phenolic acids are mainly present in jujube peel. Phenolic acids in seed and peel are present in the insoluble-bound form, while, in pulp in the glycosided form, the glycosided and insoluble-bound phenolic acid fractions in jujube pulp represent the highest total phenolic content and the strongest antioxidant activity determined by DPPH and FRAP assays. Our results show that most phenolic compounds with antioxidant activity in different tissues of jujube are present as the glycosided and insoluble-bound forms.

KEYWORDS: Phenolic acids, antioxidant activity, jujube

INTRODUCTION

Natural phenolic compounds present in plants consist of groups such as phenolic acids, flavonoids, and tannins. Phenolic acids are hydroxy derivatives of aromatic carboxylic acids, which arise from either the benzoic acid group or the cinnamic acid group. Gallic, protocatechuic, and *p*-hydroxybenzoic acids are derivatives of benzoic acid, while caffeic, *p*-coumaric, and ferulic acids are derivatives of cinnamic acid. Phenolic acids constitute approximately 30% of the dietary phenolic present in plants in free and bound forms.[1] The latter is found more frequently and occurs in the form of esters, glycosides, and insoluble-bound complexes.[2]

Much more attention has been paid to the antioxidant activities exhibited by phenolic acids and their derivatives.[3,4] Phenolic acids have been found to have strong antioxidants against free radicals and other reactive oxygen species (ROS), to promote health benefits, and to prevent chronic human diseases such as cancer and cardiovascular diseases.[5−7] The antioxidant activity of phenolic acids and their derivatives depends on the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents.[8−10] The hydroxycinnamic acids have been found to have significantly higher antioxidant activity than the hydroxybenzoic acids, which may be attributed to the presence of the CH═CH—COOH group in the hydroxycinnamic acids and the COOH group in the hydroxybenzoic acids.[7] The presence of the CH═CH—COOH groups in hydroxycinnamic acids ensures greater H-donating ability and subsequent radical stabilization than the carboxylate group in hydroxybenzoic acids. Thus, caffeic, sinapic, ferulic, and *p*-coumaric acids are found to be more active than protocatechuic, syringic, vanillic, and *p*-hydroxybenzoate.[8]

Jujube is the fruit of *Ziziphus jujuba* Mill, a thorny rhamnaceous plant widely distributed in northern China. Jujube has a high nutritional value and numerous pharmacological effects, and it has been widely used as food, a functional food additive, and a traditional Chinese medicine for thousands of years. Most previous studies in the literature have revealed that jujube contains various components, including phenolic compounds with high antioxidant activity.[11−17]

As far as we know, little has been reported on the distribution of phenolic acids (in free, esterified, glycosided, and insoluble-bound forms) and their antioxidant activity in different tissues of jujube. Accordingly, it is necessary to make an in vitro study of the antioxidant activity of free, esterified, glycosided, and insoluble-bound phenolic acids present in jujube, thereby leading to a better understanding of their medicinal effects upon human health. For this reason, the objective of this study is to evaluate the distribution of phenolics of different forms in different tissues and their antioxidant activity.

MATERIALS AND METHODS

Jujube Materials. Jujube in this study was harvested successively at commercial maturity from Jia County, Shaanxi Province, China. They were picked up randomly from different parts of several jujube trees of the same species. The fruits were stored in airtight polyethylene bags at −18 °C until they were analyzed.

Chemicals. Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, chlorogenic acid,

Received: October 12, 2010
Accepted: December 15, 2010
Revised: December 11, 2010
Published: January 20, 2011

2,2-diphenyl-1-picryl-hydrazyl (DPPH), and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals used were of analytical grade and were obtained from Xi'an Chemical Co. (Xi'an, China). HPLC grade methanol was purchased from Merck (Darmstadt, Germany); analytical grade acetic acid was supplied by Beijing Reagent Co. Ltd. (Beijing, China); HPLC grade water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Preperation of Crude Jujube Extracts. The ten fruits (about 120 g) were carefully separated into three tissues; peel, pulp, and seeds. Subsequently, the fruit tissues were cut into small slices and lyophilized. The lyophilized fruit tissues were milled and sieved through a standard sieve (100 mesh). The powered samples (1 g) of different tissues of jujube were extracted with 15 mL of 80% (v/v) aqueous methanol at room temperature. The solution was sonicated for 30 min followed by centrifugation at 2000*g* for 10 min to collect supernatant. After four extractions, the combined supernatants were analyzed for free phenolic acids, soluble phenolic acid glycosides, and phenolic acid esters; and their residues were reserved for the determination of methanol−insoluble ester-bound phenolic acids.

Fractionation of Free and Bound Phenolic Acids. Phenolic acids present in crude extracts were fractionated into free and bound forms according to the methods of Zadernowski et al. [18] and Xu et al.[19] The combined supernatant was evaporated under vacuum at 35 °C to about 25 mL. The aqueous suspension was acidified to pH 2 using 6 M HCl, and it was extracted five times with diethyl ether−ethyl acetate (1:l) (v/v) at a solvent to water phase ratio of 1:l. The diethyl ether−ethyl acetate extracts were referred to as the free phenolic acids. The aqueous phase was treated by alkaline hydrolysis (4 M NaOH containing 10 mM EDTA and 1% ascorbic acid) [20] under nitrogen for 4 h at room temperature. After acidification to pH 2 with 6 M HCl, phenolic acids released from soluble ester were extracted from the hydrolysate five times as described above. Following this, the aqueous phase was hydrolyzed with 5 mL of 6 M HCl for 30 min at 85 °C under nitrogen. Phenolic acids released from soluble glycosides were separated from the hydrolysate five times as described above. The residues from the 80% methanol extractions were hydrolyzed directly with 8 mL of 4 M NaOH (containing 10 mM EDTA and 1% ascorbic acid) under the same conditions as those for the ester. After acidification to pH 2 using 6 M HCl, phenolic acids released from methanol−insoluble ester-bound phenolic acids were extracted from the hydrolysate 5 times as described above. Each sample was treated in triplicate. Each of the phenolic acid fractions, obtained as described above, was dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 35 °C. The dry residues were dissolved into 5 mL of 80% (v/v) aqueous methanol.

Determination of Total Phenolic Contents. Total phenolic contents were determined using a modified version of the Folin−Ciocalteu method.[21] Folin−Ciocalteu reagent (0.5 mL) was added to appropriately 0.5 mL of diluted sample to form the mixture. After the mixture was kept at room temperature for 5 min, 1.5 mL of sodium carbonate (20%) was added to the mixture and mixed thoroughly. Then the total volume of mixture was adjusted to 10 mL with distilled water. The absorbance was read using a spectrophotometer at 760 nm wavelength after incubation at 75 °C for 10 min. The standard calibration (0.02−0.12 mg/mL) curve was made using gallic acid. The total phenolic contents were expressed as the gallic acid equivalents per gram dry weight (mg GAE/g DW).

HPLC-ECD Analysis. To determine the phenolic acid distribution of jujube, the free and bound phenolic acids were separated and quantified using a HPLC fitted with an electrochemical detector (ECD) described by Wang et al.[17] HPLC analysis of phenolic acids was carried out on an Agilent 1100 HPLC System equipped with a vacuum degasser, a quaternary solvent delivery pump, a manual chromatographic valve, a thermostated column compartment, and a HP1049A programmable ECD (HP, USA). The column was a Zorbax SB-C18 column (150 mm × 4.6 mm, 5.0 μm) connected to a Zorbax SB-C18 guard column (20 mm × 4.0 mm, 5 μm). The mobile phase adopted was methanol (A) and 2% aqueous acetic acid (B) (v/v) using a linear gradient elution of 5−20% A at 0−10 min, 20−40% A at 10−15 min, 40−60% A at 15−25 min, and 60−70% A at 25−30 min. The flow-rate was kept at 1.0 mL min^{-1} at all times. The column was operated at 30 °C. The injection volume was 10.0 μL. The electrochemical detector was set at 0.8 V in the oxidative mode. The re-equilibration duration was 6 min by using the starting condition before injection of the next individual sample. Quantification of phenolic acids was carried out by an external standard method using calibration curves. The amount of each phenolic acid was expressed as microgram per gram dry weight (μg/g DW).

Table 1. Total Phenolic Contents in Different Tissues of Jujube[a]

phenolic acid fraction	total phenolic contents (mg GAE/g of DW)		
	pulp	seed	peel
free	2.38 ± 0.17 [b]	2.31 ± 0.14 [b]	3.97 ± 0.26 [a]
esterified	5.59 ± 0.46 [b]	3.85 ± 0.30 [c]	9.83 ± 0.74 [a]
glycosided	15.07 ± 0.81 [a]	9.84 ± 0.77 [b]	13.81 ± 1.15 [a]
insoluble-bound	15.94 ± 0.84 [ab]	14.95 ± 0.93 [b]	18.62 ± 1.53 [a]
total phenol content	38.98 ± 2.10 [b]	30.95 ± 2.11 [c]	46.23 ± 3.23 [a]

[a] The data are presented as mean ± SD for three replications. Different lower case letters correspond to significant differences at $p < 0.05$.

Radical DPPH Scavenging Activity. Scavenging activity on DPPH free radicals by each phenolic acid fraction was assessed according to the method reported by Atoui et al. [22] with slight modifications. Briefly, 0.1 mL of properly diluted sample was placed in a cuvette with 4.0 mL of 0.1 mM ethanolic solution of DPPH radical added. The total volume of mixture was adjusted to 10 mL with distilled water. Then, the mixture was shaken evenly and allowed to stand at room temperature in the dark for 30 min. Thereafter, the absorbance of the assay mixture was measured at 517 nm against ethanol blank using a spectrophotometer. DPPH radical scavenging capacity is expressed as the percentage inhibition of DPPH radical. The percentage inhibition of DPPH radical by each fraction was calculated from the absorbance value according to the following equation: % inhibition of DPPH radical = $[(A_0 - A_1)A_0] \times 100\%$, where A_0 is the absorbance of control DPPH solution at 0 min and A_1 is the absorbance in the presence of test sample at 30 min.

Ferric Reducing Antioxidant Power (FRAP). FRAP assay of each sample was performed according to a modified protocol developed by Benzie and Strain.[23] Properly diluted sample (0.1 mL) was added to 4.0 mL of FRAP reagent to form a mixture. FRAP reagent should be preheated at 37 °C and should always be freshly prepared by mixing 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. Then the mixture was incubated at 37 °C for 4 min, and the absorbance was measured at 593 nm against a blank that was prepared using distilled water. A calibration curve was prepared, using an aqueous solution of ferrous sulfate (200−1000 μM). FRAP values were expressed on a dry weight basis as micromoles of ferrous equivalent Fe(II) per gram of jujube tissues (μmol $FeSO_4$/g DW).

Statistical Analysis. The results presented in the tables are expressed as the mean values ± SD (standard diviation) for three replications. Data analysis was carried out using SAS software, version 8.1. Statistically significant differences between the samples were evaluated by the Tukey's test. Differences at $p < 0.05$ were considered to be significant. The correlation analysis between phenolics and antioxidant activity was made using a standard Pearson correlation.

Table 2. Phenolic Acid Distribution in Different Tissues of Jujube (ug/g DW)

		hydroxybenzoics			hydroxycinnamics				
phenolic acid fraction	tissue	gallic	protocatechuic	*p*-hydroxybenzoic	caffeic	*p*-coumaric	ferulic	cinnamic	chlorogenic
free	pulp	1.72 ± 0.07	1.15 ± 0.05	nd[a]	3.54 ± 0.29	0.33 ± 0.01	1.15 ± 0.06	1.53 ± 0.09	2.71 ± 0.26
	seed	0.44 ± 0.04	0.65 ± 0.06	6.13 ± 0.41	2.96 ± 0.14	0.92 ± 0.11	2.05 ± 0.09	8.75 ± 0.66	12.98 ± 0.97
	peel	1.85 ± 0.13	2.25 ± 0.13	6.74 ± 0.38	11.27 ± 0.46	2.85 ± 0.22	6.53 ± 0.42	3.88 ± 0.24	3.94 ± 0.22
esterified	pulp	1.41 ± 0.06	4.38 ± 0.26	22.61 ± 1.70	3.84 ± 0.21	0.46 ± 0.03	1.55 ± 0.13	1.15 ± 0.06	4.08 ± 0.11
	seed	0.75 ± 0.07	1.73 ± 0.11	3.25 ± 0.22	3.57 ± 0.21	nd	nd	1.12 ± 0.08	nd
	peel	1.62 ± 0.15	3.45 ± 0.23	22.04 ± 1.36	3.66 ± 0.24	6.34 ± 0.29	4.05 ± 0.28	1.64 ± 0.05	4.55 ± 0.28
glycosided	pulp	2.54 ± 0.14	7.03 ± 0.65	42.12 ± 2.30	nd	nd	nd	12.53 ± 0.98	nd
	seed	2.58 ± 0.17	3.52 ± 0.23	nd	nd	nd	nd	13.51 ± 0.50	nd
	peel	7.53 ± 0.35	3.06 ± 0.20	nd	nd	nd	1.50 ± 0.09	155.12 ± 6.31	nd
insoluble-bound	pulp	3.25 ± 0.18	3.15 ± 0.18	9.56 ± 0.51	1.05 ± 0.05	2.07 ± 0.12	nd	5.53 ± 0.33	3.15 ± 0.13
	seed	2.85 ± 0.19	1.05 ± 0.09	71.08 ± 2.78	11.42 ± 0.81	1.74 ± 0.11	2.95 ± 0.13	1.35 ± 0.07	11.37 ± 0.55
	peel	19.54 ± 1.13	54.08 ± 2.69	82.51 ± 5.24	33.50 ± 2.60	145.28 ± 8.91	nd	40.19 ± 2.28	122.42 ± 6.71

[a] nd: not detected.

RESULTS AND DISCUSSION

Total Phenolic Contents. Total phenolic contents in different tissues of jujube estimated by the Folin-Ciocalteau assay are presented in Table 1. On the whole, the total phenol contents are significantly different in different tissues of jujube ($p < 0.05$). The total phenolic contents range from 30.95 mg of GAE/g of DW in jujube seed to 46.23 mg of GAE/g of DW in jujube peel. The total phenolic contents reported here are higher than those previously published for different tissues of jujube.[16] However, in other previous reports,[11,14] the total phenol contents of the edible tissues of jujube can only be determined, thereby it is difficult to directly compare those results with ours. As compared with the total phenolic contents in the edible tissues of jujube, the values found in our study are mostly higher than those previously published results;[12] this may be attributed to factors such as jujube varieties, material sources, regional differences, the degree of ripeness, and the analytical procedure used for extraction of phenolics. In the four phenolic acid fractions, insoluble-bound phenolic acids are the major fraction of phenolic acids constituting 40.9, 48.3, and 40.3% of the total phenols in peel, pulp, and seed, but free phenolic acids can only comprise 6.1, 7.5, and 8.6% of the total phenols in peel, pulp, and seed, respectively. In the previous studies, only soluble phenolic contents in extracts were evaluated, but insoluble-bound phenolic acids are generally ignored, thereby leading to the lower values.

Phenolic Acid Distribution in Jujube. Eight phenolic acids, including hydroxybenzoic acids (gallic, protocatechuic, and *p*-hydroxybenzoic), hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic, and cinnamic), and chlorogenic acids, are well separated and quantified by HPLC−ECD. The distribution of the phenolic acids in the four forms (free, esters, glycosides, and insoluble-bound) in pulp, seed, and peel of jujube is shown in Table 2. The results show that insoluble-bound phenolic acids are the most abundant form, followed by glycosided, esterified, and free form, in decreasing order.

Free phenolic acids constitute from 5.2% (peel) to 20.7% (seed) of the total phenolic acids present in corresponding tissues of jujube (Figure 1). In this fraction, the phenolic acid contents in jujube seed and peel are 34.88 and 39.31 μg/g DW, respectively. But their values are about 3-fold higher than those in pulp (12.13 μg/g DW). Caffeic acid and chlorogenic acid, an ester of caffeic acid, are the major phenolic acids in the pulp, with *p*-hydroxybenzoic acid not detected; *p*-hydroxybenzoic, cinnamic, and chlorogenic acid are the principal phenolic acids in the seed, while *p*-hydroxybenzoic, caffeic, and ferulic acid are mostly present in the peel. Gallic, protocatechuic, and *p*-coumaric acids are the minor phenolic acids in all tissues of jujube.

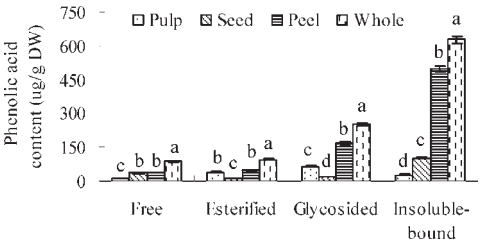

Figure 1. Phenolic acid contents in four forms in different tissues of jujube. Different lower case letters correspond to significant differences at $p < 0.05$.

Phenolic acids released from soluble esters can range from 6.2% (seed) to 27.5% (pulp) of the total phenolic acids present in jujube (Figure 1). The content of soluble esters of phenolic acids is much higher in jujube peel and in pulp (47.35 and 39.48 μg/g DW) than that in seed (10.42 μg/g DW). *p*-Hydroxybenzoic acid is the most predominant phenolic acid present as soluble esters in both pulp and peel of jujube, while *p*-hydroxybenzoic and caffeic acids are present with minor concentration in seed. In addition, soluble esters of *p*-coumaric, ferulic, and chlorogenic acids are not detected in jujube seed.

Glycosided phenolic acids constitute 44.7, 11.6, and 22.3% of the total phenolic acids present in jujube pulp, seed, and peel, respectively (Figure 1). In pulp, most detected phenolic acids are present in glycosided form. Though the percentage of glycosided phenolic acids in peel is lower, the content is the highest in three tissues of jujube (167.21 μg/g DW), accounting for 66.7% of the total glycosided phenolic acids in the whole jujube. Glycosided *p*-hydroxybenzoic acid dominates in jujube pulp but is not detected in seed and peel. Glycosided cinnamic acid is the most predominant phenolic acid in seed and peel, particularly in peel, and nearly all other phenolic acids in peel are negligible.

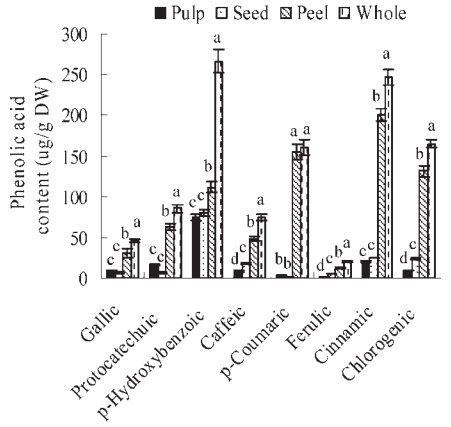

Figure 2. Total contents of individual phenolic acid in different tissues of jujube. Different lower case letters correspond to significant differences at $p < 0.05$.

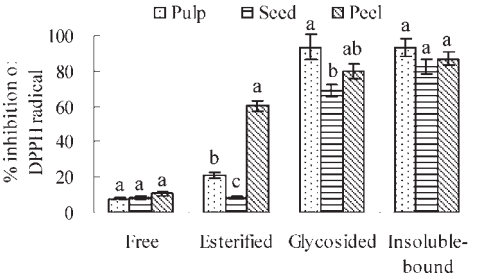

Figure 3. DPPH radical scavenging capacities of each phenolic acid fraction in different tissues of jujube. Different lower case letters correspond to significant differences at $p < 0.05$.

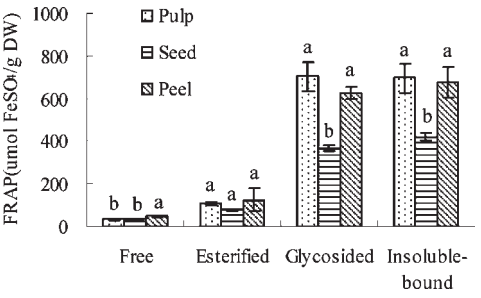

Figure 4. FRAP values of each phenolic acid fraction in different tissues of jujube.

Furthermore, ferulic acid in the glycosided form is unique in the peel, while glycosided caffeic, *p*-coumaric, and chlorogenic acids are not detected in all tissues of jujube.

The insoluble-bound phenolic acids represent 19.3, 61.5, and 66.2% of total phenolic acids in pulp, seed, and peel of jujube, respectively (Figure 1). The total contents of insouble-bound phenolic acid in seed and peel are 103.81 and 497.52 μg/g DW, respectively, but pulp only contains 27.76 μg/g DW. Moreover, insoluble-bound *p*-hydroxybenzoic acid is the major phenolic acid in pulp and seed, while insoluble-bound *p*-coumaric and chlorogenic acids dominate in the peel. Insoluble-bound ferulic acid can only be detected in seed, but the quantified phenolic acids in insoluble-bound form are mainly found in peel.

The total of quantified phenolic acid contents in the four forms achieves the highest value (751.39 μg/g DW) in jujube peel, while the lowest value (143.59 μg/g DW) in pulp. These values are lower than those reported in the literature.[16,17] In addition, phenolic acids are mainly found in the insoluble-bound form in both seed and peel, while in the glycosided form in the pulp.

The level of total phenolic acid contents in different tissues of jujube is calculated as a sum of individual phenolic acid present in all four forms. Apparently, all determined phenolic acids are mainly present in the jujube peel, ranging from 41.8 (*p*-hydroxybenzoic) to 96.5% (*p*-coumaric) of the total contents of individual phenolic acid present in the whole jujube (Figure 2). *p*-Hydroxybenzoic acid is the dominant phenolic acid in both pulp and seed of jujube, even in the whole jujube, with 51.7, 47.7, and 25.0% of the total contents in the pulp, seed, and whole jujube, respectively, while *p*-coumaric, cinnamic, and chlorogenic acids are present in large quantities in peel. Thus, the whole jujube is rich in *p*-hydroxybenzoic, cinnamic, chlorogenic, and *p*-coumaric acids.

Antioxidant Activity and Correlation Coefficient. The pattern observed for the antioxidant activity of free, esterfied, glycosided, and insoluble-bound phenolic acid fractions in different tissues of jujube closely resembles those recorded for the total phenolic contents (Figures 3 and 4). Both DPPH and FRAP assays appear to have similar trends, with very high antioxidant activity in glycosided and insoluble-bound phenolic acids present in all three tissues of jujube and low antioxidant activity in free phenolic acids. The glycosided and insoluble-bound phenolic acid fractions in jujube pulp possess the strongest antioxidant activities by two assays in spite of a low content of the total phenol content, and this may be due to other antioxidant phytochemicals. An unknown peak in the fractions (not shown) is present and considered one such compound, which is now being identified. It is also found that the phenolic acid fractions in peel have higher antioxidant activities than in seed because they have more phenolic acids detected and total phenols.

Table 3. Correlation Analysis of Phenolics and Antioxidant Activity

	DPPH	FRAP
total phenolic content	0.97[a,b]	0.94[b]
DPPH		0.93[b]

[a] Corrrelation coefficient R^2. [b] $p < 0.01$.

To further investigate the relationship of phenols in different forms in different tissues of jujube and their antioxidant activities, the correlation between the antioxidant activity measured by DPPH and FRAP assays and the total phenolic content of all fractions is established, and correlation coefficients (R^2) and *p* values are shown in Table 3. The antioxidant activity determined by DPPH assay is high positively correlated ($R^2 = 0.93$; $p < 0.01$) to that detected by FRAP assay. Moreover, both DPPH and FRAP assays are significantly in positive correlation ($R^2 = 0.97$ and 0.94, respectively; $p < 0.01$) with the total phenolic contents, suggesting that these phenolic compounds may be responsible for a large proportion of the antioxidant activity. The result is well in agreement with the data reported by some groups.[16,24] Therefore, total phenolic contents could be used as an important indicator of antioxidant activity evaluated by DPPH and FRAP assays.

The present data indicate that there are great differences in total phenolic content, phenolic acid distribution, and antioxidant activity of free, exterified, glycosided, and insoluble-bound phenolic acid fractions in different tissues of jujube. There are eight phenolic acids in whole jujube, with *p*-hydroxybenzoic and cinnamic acids being the most abundant phenolic acids in eight

detected ones, whereas ferulic acid is the least. All quantified phenolic acids in the four forms are mainly present in jujube peel. Phenolic acids in seed and peel are in the insoluble-bound form, while, in pulp, they are in the glycosided form. The antioxidant activities are significantly correlated with total phenolic contents, and the glycosided and insoluble-bound phenolic acid fractions in jujube pulp and peel exhibit the highest total phenolic content and the strongest antioxidant activity, determined by DPPH and FRAP assays. Similarly, jujube seed and peel, the byproducts of jujube juice, show plentiful phenolic compounds with antioxidant activity. Consequently, they can be used as a valuable source of antioxidant ingredients. But further studies are still needed to identify the unknown antioxidant substance and the major phenolic compounds present in jujube and to establish the effect of the regional differences, the degree of ripeness, postharvest storage conditions, and processing on the phenolic acid distribution of jujube.

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Funding Sources

This work is financially supported by the Science & Technology Research Program of Shaanxi Province (2009K01-09), the Industrialization Program of Shaanxi Provincial Department of Education (09JC03), and the Science Foundation of Northwest University (09NW07).

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