Antioxidant activities and phenolic compounds of date plum persimmon (*Diospyros lotus* L.) fruits

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Abstract In the present study, phenolic compounds are extracted from the date plum persimmon fruits using water, methanol and acetone as solvents. Antioxidant activities of the phenolic extracts are measured using four different tests, namely, DPPH, hydroxyl radical scavenging activities, chelating and reducing power assays. All the extracts show dose dependent DPPH radical scavenging activity, reducing and chelating powers and moreover, they are well correlated with the total phenolic and total flavonoid substances, suggesting direct contribution of phenolic compounds to these activities. In further, the extracts are identified and quantified by HPLC-ECD. Results show that gallic acid is the most abundant phenolic compound, with amounts ranging between 45.49 and 287.47 $\mu\text{g/g}$ dry sample. Myricetin is the dominant flavonoid in all extracts. Its level varied from 2.75 $\mu\text{g/g}$ dry sample in acetone extract to 5.28 $\mu\text{g/g}$ dry sample in water extract. On the basis of the results obtained, the date plum persimmon fruits phenolic extract is a potential source of natural antioxidants owing to its significant antioxidant activities.

Keywords Date plum persimmon · Antioxidant activities · Phenolic compounds

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Introduction

The human body living in an oxygen-rich environment is inundated with various endogenous and exogenous sources of reactive oxygen species (ROS). During normal activities, various processes inside the body produce ROS such as superoxide anion (O_2^-), hydroxyl radical ($\text{OH}\cdot$) and hydrogen peroxide (H_2O_2). As a natural defense system, our body is protected against these free radicals by antioxidant molecules and antioxidant enzymes. However, when level of ROS exceeds the capacity for defense or antioxidant systems, the result is the induction of aging, abnormal physiological functions or various human diseases (Valko et al. 2007; Dreher and Junod 1996).

Dietary antioxidants may afford protection against oxidative damage. Among dietary antioxidants, phenolic compounds are by far the most abundant in common human diets. In recent years, phenolics have received considerable interest based on positive reports of their presumed role in the prevention of various human diseases (Hertog et al. 1993; Hoper and Cassidy 2006). This beneficial effect is considered to be mainly due to their antioxidant, radical scavenging properties and chelating activities (Rice-Evans et al. 1997). Numerous plant species have been analyzed for their phenolics and antioxidant activities, and the date plum persimmon being among the best sources (Ayaz et al. 1997; Loizzo et al. 2009).

Date plum persimmon (*Diospyros lotus* L.) (DPP) which belongs to the Ebenaceae family is native in China and Asia. It has been cultivated in several countries for its edible fruits. In traditional Chinese medicine, the fruits are febrifuge and commonly used to promote secretions and seed as a supplement for being sedative. According to prior studies, nutritional constituents of the DPP fruit have been measured (Glew et al. 2005; Ayaz and Kadioğlu 1999).

Changes in phenolic acid contents during fruit development of this plant have been published (Ayaz et al. 1997). Phytochemical and some antioxidant activities of the aqueous methanolic extract of *Diospyros lotus* L. fruits have been evaluated (Ebrahimzadeh et al. 2008; Loizzo et al. 2009; Nabavi et al. 2009). However, to the best of our knowledge, there has been no detailed report on the comparative of individual phenolic compounds and the correlation between total phenolic, total flavonoid contents and antioxidant activities of water, methanol or acetone extracts.

The objectives of this study are to evaluate the antioxidant activity and phenolic compounds of the DPP fruits extracts obtained from three solvents. In the present study, 10 individual phenolic compounds are investigated by high performance liquid chromatography with electrochemical detection (HPLC-ECD). Antioxidant activities of the extracts are measured using DPPH, hydroxyl radical scavenging activities, chelating activity and reducing power assays. Moreover, correlations between the total phenolic, total flavonoid contents and antioxidant activities are also evaluated.

Materials and Methods

Chemicals and Reagents Rutin, gallic acid (GA), myricetin, kaempferol, galangin and quercetin standards were purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Caffeic acid, *p*-coumaric acid, ferulic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid monosodium salt (Ferrozine), 2-deoxyribose, protocatechuic acid, and Folin-Ciocalteu agent were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol was purchased from Merck (Darmstadt, Germany). HPLC grade water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium ferricyanide, ethylenediamine tetraacetic acid (EDTA), and ascorbic acid were purchased from Beijing Chemical Co. (Beijing, China). All other chemicals were of analytical grade and were purchased from Xi'an Chemical Co. (Xi'an, China).

Preparation of Extraction Plant material commercially available grade DPP fruits were purchased from Xingtang, China. The dried pulp of the DPP fruits was ground in a pulveriser. Water extract was obtained as follows. In brief, 10 g powder were suspended and extracted with 150 mL distilled water with refluxing for 1 h. The extract was filtered over Whatman No.1 paper and the supernatants were pooled. The residue was re-extracted under the same conditions. Pooled supernatants were condensed with vacuum-filtration and then condensed supernatants were dissolved and diluted to 30 mL with water. Methanol and

acetone extracts were produced by procedures analogous to the one used to prepare the water extract. Sample solutions were stored at 4 °C in amber bottles and served as the stock solution for subsequent analyses.

Total Phenolic Content (TPC) TPC was determined using a modified version of the Folin-Ciocalteu method (Singleton and Rossi 1965). One mL of sample was added to 1.0 mL of Folin-Ciocalteu reagent and the mixture was kept at room temperature for 5 min. Five mL of sodium carbonate (1 M) was added to the mixture and the whole mixed gently. The total volume of the mixture was adjusted to 10 mL with distilled water. After the mixture had been kept at room temperature (25 ± 1 °C) for 90 min, the absorbance was read at 760 nm. The standard calibration (0.02–0.12 mg/mL) curve was plotted using GA. The total phenolic content was expressed as the GA equivalents.

Total Flavonoid Content (TFC) TFC was measured by a modified colorimetric assay (Dowd 1959). Extracts (0.25 mL) were added to a test tube containing 0.75 mL of distilled water. Sodium nitrite solution (5%, 0.15 mL) was added to the mixture and reacted for 5 min followed by the addition of 0.3 mL of 10% aluminum chloride. After 5 min, 1 mL of 1 M sodium hydroxide was added. The absorbance of the mixture was measured at 510 nm. The total flavonoid content is expressed as mg of rutin / g dry sample, on the basis of a calibration curve performed with rutin.

Determination of Individual Phenolic Compounds by HPLC Individual phenolic compounds were analyzed according to the modified method of Gao et al. (2010). A HPLC with ECD detection was used to identify and quantify the rutin, gallic acid, myricetin, kaempferol, galangin, quercetin, caffeic acid, *p*-coumaric acid, ferulic acid and protocatechuic acid in the extracts. The HPLC system used was an Agilent 1100 liquid chromatography system (Agilent Technologies Deutschland, Waldbronn, Germany) with a ZorbaxSB-C18 column (150×4.6 mm, 5 µm) at room temperature. Two mg of water, methanol and acetone extracts were dissolved in 1.0 mL of methanol, and filtered through a 0.45 µm membrane filter prior to the injection of 10 µL into the HPLC system. The mobile phase consisted of 2% aqueous phosphoric acid (A) and methanol (B) (v/v) using a linear gradient elution of 5–20% B at 0–10 min, 20–40% B at 10–15 min, 40–60% B at 15–25 min, 60–70% B at 25–30 min, 70% B at 30–35 min. The flow-rate was 1.0 mL/min. Re-equilibration duration was 8 min between individual runs. The electrochemical detector was set at 0.8 V in the oxidative mode.

DPPH Radical Scavenging Activity DPPH radical scavenging activity was determined using the method outlined by

Singh and Rajini (2004). To evaluate the scavenging activity of the extract, sample solutions with concentrations 74, 148, 222 and 296 $\mu\text{g/mL}$ were added to 3 mL of a 1 mM DPPH solution in methanol. The mixture was shaken evenly and allowed to stand at room temperature for 30 min in darkness. Then, the absorbance of the assay mixture was measured at 517 nm against a blank solution using a spectrophotometer. Decreased absorbance of the reaction mixture correlates with greater reducing scavenging power. The percent inhibition of activity was calculated according to the following equation: $\text{DPPH scavenging activity}(\%) = [(A_0 - A_e)/A_0] \times 100$, where A_0 is the absorbance of control DPPH solution at 0 min and A_e is the absorbance in the presence of test sample at 30 min. The IC₅₀ value was calculated from the plots as the antioxidant concentration required for providing 50% free radical scavenging activity.

Hydroxyl Radical Scavenging Activity Scavenging activity of the extract on $\text{OH}\cdot$ was evaluated according to Chung et al (1997). 2-Deoxyribose is oxidized by the hydroxyl radical that is generated by the Fenton reaction in the system of FeSO_4 and H_2O_2 . The reaction mixture contained 0.2 mL of FeSO_4 -EDTA (10 mM), 0.2 mL of 2-deoxyribose solution (10 mM), 0.8 mL of sodium phosphate buffer (pH 7.4, 0.1 M), 75 μL of sample solution, and 1.525 mL of water to give a total volume of 2.8 mL. Finally, 0.2 mL of H_2O_2 (10 mM) was added to this reaction mixture and the whole incubated at 37 °C for 4 h. After this incubation, 1 mL each of trichloroacetic acid solution (2.8%) and thiobarbituric acid solution (1.0%) were added to the reaction mixture, and the whole boiled for 10 min, cooled in ice, and its absorbance measured at 520 nm. Hydroxyl radical scavenging activity was calculated as the inhibition rate of 2-deoxyribose scavenging ability. The IC₅₀ value was calculated from the plots as the antioxidant concentration required for providing 50% free radical scavenging activity.

Ferric to Ferrous Ion-reducing Power Reducing power of the extract was measured by the method of Jayaprakasha et al (2002). Different content ratios of the sample (22, 44, 66 and 88 $\mu\text{g/mL}$) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide in 10 mL test tubes. The mixture was incubated in a water bath for 20 min at 50 °C. At the end of the incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 5000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture correlates with greater reducing power.

Ferrous Ion-chelating Power Chelating power of the extract was investigated according to the method of Carter with slight modification (1971). Ferrous ion-chelating ability of the extract was evaluated by measuring the absorbance of ferrozine- Fe^{2+} complex at 562 nm. The reaction mixture, containing the extract, FeCl_2 , and ferrozine, was adjusted to a total volume of 1.0 mL with methanol, and the mixture shaken well and then incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against the blank. The ferrous ion-chelating power was calculated from the absorbance of the control and sample and expressed as Na_2EDTA equivalents (mg $\text{Na}_2\text{EDTA/g}$ dry sample).

Statistical Analysis Experiments were performed in triplicate and the data were processed by analysis of variance (ANOVA), followed by the Duncan's multiple range tests. Duncan's multiple range tests were used to determine significant differences at $p < 0.05$.

Results and Discussion

Total Phenolic Content (TPC) TPC of the water, methanol and acetone extracts from the DPP fruits are shown in Table 1. For all tested extracts, the TPC ranged from 1.59 to 14.48 mg GA/g dry sample. As it can be seen, there are significant effects of different solvents on the TPC ($p < 0.05$). The highest TPC is determined in the water extract, which is 4.35 and 9.11 times higher than TPC values of the methanol or acetone extracts, respectively. The variability in the TPC caused by different solvents may be due to the varying solubility of the phenolic compounds, which is directly related to the compatibility of the compounds with the solvent system. Based on the present TPC results, it is predicted that the DPP fruit contains diverse phenolic compounds with different polarity and water that is a polar solvent is a better extraction solvent for phenolics from the DPP fruits than less polar solvents such as acetone.

Table 1 TPC and TFC in water, methanol and acetone extracts from DPP fruits

	TPC (mg GA/g dry powder)	TFC (mg rutin/g dry powder)
Water extract	14.5±0.20a	11.2±0.18a
Methanol extract	3.3±0.18b	2.8±0.10b
Acetone extract	1.6±0.12b	2.2±0.11b

Data are mean±SD of three determinations

Data with the same superscript letters in a column are not significantly different ($p > 0.05$)

However, a study conducted by Sun et al. (2007) proposed that a less polar solvent such as acetone could extract more phenolic compounds from buckwheat than more polar solvents, including methanol and ethanol. Some other authors suggested that aqueous mixtures of methanol and acetone solvent system are superior to pure water in the extraction of phenolic compounds from plant samples (Wang et al. 2007). These differences could be due to the different methods of extraction and standards used, but also to different varieties, genomics and harvest season of the samples (all these parameters affect the synthesis and accumulation of phenolic compounds in some parts of the plant) (Imeh and Khokhar 2002).

Total Flavonoid Content (TFC) Flavonoids are widely distributed group of plant phenolic compounds, which are very effective antioxidants. The present result shows that the total flavonoid of the three extracts is varied considerably from 2.21 to 11.19 mg rutin/g dry sample (Table 1). The highest flavonoid content of 11.19 mg rutin/g dry sample is observed in water extract and the lowest content of 2.21 mg rutin/g dry sample is observed in acetone extract. The total flavonoid content of the three extracts is well correspondence to the total phenolic content ($r^2=0.9952$), suggesting that the flavonoids are the major phenolic compounds present in the DPP fruits.

HPLC Analysis of Phenolic Compounds Individual phenolic compounds of the water, methanol and acetone extracts from the DPP fruits are determined by HPLC with ECD detection. The sample peaks are identified by matching retention times of phenolic standards and quantified by external standards. Six major phenolic compounds including gallic, protocatechuic, caffeic, *p*-coumaric, ferulic acids and myricetin are identified and quantified in all the extracts (Table 2). The experimental result in agreement

with previous investigation showed that gallic and protocatechuic acids are the most representative phenolic acids in the phenolic composition of persimmon fruits (Dreher and Junod 1996; Gorinstein et al. 1994). It should be noted that some individual phenolic compounds in the three extracts are not detected due to their low contents. Among the six compounds, gallic acid is the most abundant phenolic compound of the DPP extracts and the content of gallic acid in the water extract is 287.47 $\mu\text{g/g}$ dry sample, which is 4.58 and 6.32 times higher than the corresponding contents in the methanol or acetone extracts, respectively. The following contents of phenolic compounds are protocatechuic, caffeic, ferulic acids and myricetin, and the content of *p*-coumaric acid is relatively lower. In previous studies, gallic acid, *p*-coumaric acid and myricetin were detected in the DPP fruits (Dreher and Junod 1996; Loizzo et al. 2009). However, another three phenolic acids, namely protocatechuic, caffeic and ferulic acids are found in this investigation. Moreover, water extract reveals significantly higher ($p<0.05$) phenolic acid contents compared to methanol or acetone extracts when considering the sum of content of the phenolic compounds, a result agreeing with the total phenolic content determined by the Folin–Ciocalteu method. Phenolic compounds may contribute to the antioxidative action directly (Ramakrishnan et al. 2010). Phenolics have received considerable attention because of their physiological functions, including antioxidant, antimutagenic, and antitumour activities (Srivastava et al. 2007; Othman et al. 2007; Cao et al. 2008). Therefore, the DPP extract containing amounts of phenolic compounds could be used as a potent natural antioxidant.

DPPH Radical Scavenging Activity DPPH is a widely used stable free radical to evaluate antioxidant activities of bioactive compounds and food extracts. In this study, the DPPH radical scavenging activities of the three extracts increase in a concentration-dependent manner and also increased with the increment of the incubation time (data not

Table 2 Content of phenolic compounds in water, methanol and acetone extracts from DPP fruits ($\mu\text{g/g}$ dry powder)

Compounds	Water extract	Methanol extract	Acetone extract
Gallic acid	287.5 $\pm$ 5.31	62.8 $\pm$ 2.26	45.5 $\pm$ 2.12
Protocatechuic acid	3.3 $\pm$ 0.17	2.4 $\pm$ 0.20	2.9 $\pm$ 0.13
Caffeic acid	5.9 $\pm$ 0.81	2.5 $\pm$ 0.45	3.0 $\pm$ 0.31
<i>p</i> -coumaric acid	0.57 $\pm$ 0.09	0.96 $\pm$ 0.05	0.75 $\pm$ 0.05
Ferulic acid	3.7 $\pm$ 0.11	3.7 $\pm$ 0.09	4.3 $\pm$ 0.20
Rutin	ND	ND	ND
Myricetin	2.8 $\pm$ 1.01	3.3 $\pm$ 0.96	5.3 $\pm$ 1.54
Quercetin	1.5 $\pm$ 0.63	ND	ND
Kaempferol	ND	2.1 $\pm$ 0.34	ND
Galangin	ND	1.9 $\pm$ 0.06	2.2 $\pm$ 0.23
Total	305.1 $\pm$ 8.13	79.7 $\pm$ 4.41	64.0 $\pm$ 4.58

The peaks were identified on the basis of the relative standards. For some peaks, it was not possible to calculate the concentration. ND: not detectable
Data are mean $\pm$ SD of three replicates

shown). As it can be seen from Fig. 1a and the IC_{50} in Table 3, water extract shows the largest DPPH scavenging

Fig. 1 DPPH scavenging activities (a), OH $\cdot$ -radical scavenging activities (b) and reducing powers (c) of water, methanol and acetone extract from the DPP fruits. Data are mean $\pm$ SD of three determinations. Bars having different letters are significantly different ($p < 0.05$)

activity (IC_{50} =86.34 μ g/mL) than those of methanol (IC_{50} =201.03 μ g/mL) and acetone (IC_{50} =336.10 μ g/mL) extracts. This find is not in agreement with previous investigations which reported that the IC_{50} values for DPPH scavenging activity by methanol DPP fruit extract were 72.62 μ g/mL by Loizzo et al. 2009 and 1.45 mg/mL by Nabavi et al. 2009. To our knowledge, no studies have so far been evaluated on DPPH scavenging activity by the other two extraction solvents. The DPPH scavenging activities of the DPP extracts shows similar trend with the result of TPC (r^2 =0.9794) and TFC (r^2 =0.9553), indicating direct contribution of phenolic compounds to this activity through their hydrogen donating ability (Soares et al. 1997).

Hydroxyl Radical Scavenging Activity Among the oxygen radicals, OH $\cdot$ is an extremely reactive species that can induce severe damage to all classes of biological macromolecules and cause severe oxidative damage to the cells. Therefore, it is essential to remove the OH $\cdot$ to ensure cellular homeostasis. Figure 1b shows the OH $\cdot$ scavenging activities of water, methanol and acetone extract from the DPP fruits, however, even if the concentration of the extracts is increased, the scavenging activity is not largely shift at higher concentration (111–148 μ g/mL). The scavenging activities toward OH $\cdot$ for the extracts reported as IC_{50} values are also given in Table 3. Result indicates that methanol extract containing lower phenolics reflects an increase in its OH $\cdot$ scavenging activity (IC_{50} =273.49 μ g/mL). This suggests that the physicochemical nature of the individual phenolic compounds in the extracts may be more important in contributing to the OH $\cdot$ scavenging activities than the total phenolics content measured by the Folin–Ciocalteu assay.

Ferric to Ferrous Ion-reducing Power The presence of reductants in samples would result in the reduction of the ferric ion/ferricyanide complex to its ferrous form. The amount of ferrous ion complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 1c shows the reducing power of the three extracts ranging in concentrations from 22 to 88 μ g/mL. The order of reducing power of the three extracts is water extract >

Table 3 IC_{50} values of DPP extracts on DPPH and OH $\cdot$ scavenging activities

	IC_{50} DPPH (μ g/mL)	IC_{50} OH $\cdot$ (μ g/mL)
Water extract	86.3 $\pm$ 0.99c	429.4 $\pm$ 0.87a
Methanol extract	201.0 $\pm$ 1.02b	273.5 $\pm$ 1.05b
Acetone extract	336.1 $\pm$ 0.125a	447.8 $\pm$ 0.92a

Data are mean $\pm$ SD of three determinations

Data with the same superscript letters in a column are not significantly different ($p > 0.05$)

methanol extract > acetone extract. Water extract exhibits significantly higher reducing power than the methanol or acetone extracts ($p < 0.05$), but there is no statistically significant difference ($p > 0.05$) between the methanol and acetone extracts, a result agreeing with the scavenging effect on DPPH radicals (Fig. 1a). Strong correlations are found between reducing power with TPC ($r^2 = 0.9987$) and TFC ($r^2 = 0.9989$), supporting the former statement on the contribution to antiradical of phenolic compounds (Yildirim et al. 2001). The r^2 value of 0.9678 for reducing power and DPPH radical scavenging activity indicates that reducing power may serve as a significant indicator of its potential antioxidant activity.

Ferrous Ion-chelating Power Ferrous ions chelation may render important antioxidative effects by retarding metal-catalyzed oxidation. Ferrous ion-chelating powers of all the three extracts from the DPP fruits are tested at a dosage of 1.5 mg. In accordance with DPPH and reducing power results, potent chelating power is again detected in water extract (Fig. 1). However, water and methanol extracts have the similar chelating power in their Na_2EDTA equivalent (34.59 ± 0.69 and 33.03 ± 0.29 mg Na_2EDTA /g dry sample), and their chelating powers are significant higher than that of acetone extract (25.47 ± 0.32 mg Na_2EDTA /g dry sample). In contrast, other authors have claimed that acetone extracts of all botanical samples showed higher chelating powers than did the corresponding 80% methanol extract (Su et al. 2007). This indicates solvent polarity might greatly alter the chelating power estimation of the extract due to different plant origin, growing season or the types of phenolic compounds in the plant materials. Phenolics might have also contributed to the extract chelating powers, but the correlation is relatively low ($r^2 = 0.5537$), whilst correlation tests show that chelating powers of the extracts are relatively low correlated with their DPPH scavenging activities and reducing power ($r^2 = 0.6649$, $r^2 = 0.4875$, respectively), suggesting that besides the phenolics, other factors such as polysaccharides, phytochelators and/or some peptides as well as proteins may display the chelating power as antioxidant mechanisms of the extracts. Since ferrous ions are the most effective pro-oxidants in food systems, the higher chelating power of the extracts would be beneficial.

Conclusion In conclusion, the type of extraction solvent has great impact both on total and individual phenolic contents and antioxidant activities of the extracts, water as the extraction solvent for phenolics is superior to methanol or acetone. Water extract is found to be the highest effective antioxidant, which may be attributed to the highest total phenolic content. The data suggest that the DPP fruits are a good source of natural antioxidants. We believe the DPP might have health benefits for consumers as a functional

food or value-added ingredient. Further studies should be carried out on the distribution of phenolic acids (in free, esterified, glycosided and insoluble-bound forms) and their antioxidant, antimicrobial activities of DPP fruits.

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