SUPPLEMENTARY MATERIAL

Phenolic profiles and antioxidant capacities of crude extracts and subsequent fractions from *Potentilla fruticosa* L. leaves

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This work aimed to further investigate the phenolic profiles and antioxidant capacities of the crude extracts and the subsequent fractions of *P. fruticosa* leaves. Result showed that *P. fruticosa* leaves contained high amounts for hyperoside, ellagic acid and (+)-catechin contents, and the highest amount being registered for hyperoside (17.67 mg g⁻¹). Nine sub-fractions were obtained after column chromatographic separation. EF-3, EF-4, EF-5 and BF-2 presented higher values for their total phenolic or flavonoid, (+)-catechin, ellagic acid and hyperoside content. Besides, EF-3, EF-4, BF-2 and BF-3 showed significant *in vitro* antioxidant capacities and protective effects on *E. coli* under peroxide stress. The correlation between chromatograms and antioxidant activity showed that (+)-catechin, ellagic acid and hyperoside may play crucial roles in the antioxidant capacities of *P. fruticosa* and could be used as chemical markers for its quality assessment. Moreover, this is the first time *P. fruticosa* leaves have been systematically studied.

Keywords: *P. fruticosa* leaves; phenolic profiles; antioxidant capacities; HPLC-UV

Experimental

Plant material and extraction procedure

P. fruticosa leaves were collected from Huzhu Northern Mountain, Qinghai, and authenticated by the Herbarium of the Northwest A&F University, Yangling, China. The air-dried leaves (5000 g) were extracted with 80% chilled acetone for three days to obtain the crude acetone extract (CE, 1062.59 g; yield, 21.25%). An aliquot of the CE (800 g) was dissolved in distilled water (8 L) and further partitioned with petroleum ether, ethyl acetate, and n-butanol (1:1, v/v) in sequence to obtain four fractions: a petroleum ether fraction (PF, 29.72 g; yield, 3.72%), an ethyl acetate fraction (EF, 282.08 g; yield, 35.26%), an n-butanol fraction (BF, 324.12 g; yield, 40.52%) and a water fraction (WF, 106.40 g; yield, 13.30%). Then, 100 g of the EF was mixed with 100-200 mesh of silica gel, loaded into the chromatography column with 200-300 mesh of silica gel and then sequentially eluted with a mixed solvent (petroleum ether/acetone at 4:1, 1:1, 1:5 and 0:1 and methanol) to obtain ethyl acetate fraction 1 (EF-1, 5.04 g; yield, 5.04%), ethyl acetate fraction 2 (EF-2, 23.79 g; yield, 23.79%), ethyl acetate fraction 3 (EF-3, 35.22 g; yield, 35.22%), ethyl acetate fraction 4 (EF-4, 17.80 g; yield, 17.80%) and ethyl acetate fraction 5 (EF-5, 16.31 g; yield, 16.31%). Similarly, 100 g of the BF was also filled and sequentially eluted with mixed solvent (chloroform/methanol at 4:1, 1:1, 1:5 and 0:1) to obtain n-butanol fraction 1 (BF-1, 6.02 g; yield, 6.02%), n-butanol fraction 2 (BF-2, 43.53 g; yield, 43.53%), n-butanol fraction 3 (BF-3, 27.39 g; yield, 27.39%) and n-butanol fraction 4 (BF-4, 20.66 g; yield, 20.66%) (Zhang et al., 2014). The whole separation process is shown in Figure S1. Each sample was dissolved in methanol except for the water fraction (dissolved in distilled water) and stored at -20°C in the dark for further use.

Total phenolic and total flavonoid determination

The total phenolic content was determined using the Folin-Ciocalteu colorimetric method (Mocan et al., 2014a; Mocan et al., 2014b). The crude extracts and fractions were thawed, dissolved in 80% ethanol and prepared at a concentration of 0.2 mg/ml. All values were expressed as millimoles gallic acid equivalent per one hundred grams dry weight (GAE) (mM GAE 100 g⁻¹). The total flavonoid content was determined using the sodium borohydride/chloranil-based (SBC) assay (He et al., 2008). Each sample was thawed, dissolved in a mixed solvent of THF/EtOH (1:1, v/v) and prepared at concentration of 0.2 mg/ml. The total flavonoid content was expressed as millimoles quercetin equivalents per one hundred grams dry weight (QE) (mM QE 100 g⁻¹). All determinations were performed in triplicate. Data were reported as means ± SD for three replicates.

RP-HPLC analysis

According to the UV spectra and the retention times, the six peaks were identified as (+)-catechin, caffeic acid, hyperoside, rutin, ellagic acid and quercetin (Figure S2). Stock concentrated solutions of the crude extracts and the fractions were thawed, filtered through a pinhole and analyzed by RP-HPLC. Six phenolic compounds ((+)-catechin, caffeic acid, hyperoside, rutin, ellagic acid and quercetin) were detected and quantified at ambient temperature. The HPLC mobile phases consisted of water with 0.2% trifluoroacetic acid (solvent A) and methanol with 0.2% trifluoroacetic acid (solvent B). The flow rate was kept at 0.8 ml/min and the gradient elution program was set as follows: 5% B (0 min), 20% B (0–10 min), 25% B 10–15 min), 25% B (15–20 min),

30% B (20–25 min), 30% B(25–35 min), 35% B (35–40 min), 45% B (40–50 min), 100% B (50–60 min), 100% B (60–65 min). The injection volume was 20 μL and the detection wavelength was 254 nm. All analyses were performed in triplicate.

To ensure the accuracy of the measurements, validation of the method was also performed. The precision of this method was determined by assaying six replicates of these compounds. The repeatability of the method was measured by extracting one sample six times. A recovery experiment was performed by mixing the quantified samples with standard compounds in appropriate amounts. The relative standard deviations (RSD) of the peak areas were estimated to be less than 3%, demonstrating the analysis method to be repeatable and accurate (Table S1).

Evaluation of the antioxidant capacity in vitro

DPPH radical-scavenging activity

The capacities of the plant extracts to scavenge the DPPH free radical were assayed as described by Yen (Yen & Chen, 1995). The crude extracts and the fractions were diluted in ethanol (80%) at the following concentrations: 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.52 and 0.76 $\mu\text{g ml}^{-1}$. The absorbance was measured at 517 nm. The same concentration of Trolox was used as a positive control. The DPPH_{IC50} values were introduced to evaluate the DPPH radical-scavenging activity, which were the effective concentrations at which the DPPH radicals were 50% scavenged. So a lower IC₅₀ value indicated a higher DPPH radical-scavenging activity. All measurements were conducted in triplicate.

ABTS^{•+} radical cation scavenging assay

The scavenging effects of the samples for ABTS^{•+} were determined by a previously described procedure (Thaipong, 2006). The ABTS^{•+} solution was obtained by mixing an ABTS solution (7 mM in distilled water) with 2.45 mM potassium persulfate. Before test, the mixtures were kept in the dark for 12–16 hours at room temperature. For each analysis, the ABTS^{•+} solution was freshly prepared and diluted to an initial 734 nm absorbance of 0.70 $\pm$ 0.02 with a pH 7.4 phosphate buffered saline (PBS) solution. Then, 100 μL of sample (0.2 mg/ml) was added to 3.9 mL of ABTS^{•+} solution, and the 734 nm absorbance was determined. The absorbance was measured at 734 nm. Trolox standard was used for comparison. Results were expressed as (Trolox) ($\mu\text{Mequiv. Trolox g}^{-1}$). All determinations were carried out in triplicate.

Ferric reducing power (FRAP) assay

The FRAP assay was performed according to the method reported by Benzie and Strain with some modifications (Benzie & Strain, 2008). The method was based on the reduction of a colorless ferric complex 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+} -tripyridyltriazine) to a blue-colored ferrous form (Fe^{2+} -tripyridyltriazine) through electron-donating antioxidants. The FRAP reagent was prepared daily by mixing 300 mM acetate buffer (3.1 g $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$ and 1.6 mL $\text{C}_2\text{H}_4\text{O}_2$) and 10 mM TPTZ solution in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution at 37°C. The crude extracts and the fractions were diluted to 0.2 mg mL^{-1} with ethanol (80%). For each analysis, 3 mL fresh FRAP reagent was added to 400 μL sample solutions. After a 30 min dark incubation in a 37°C water bath, the absorbance of the reaction mixture was measured at 593 nm using the

spectrophotometer. Trolox standard was used for comparison. Trolox standard was used for comparison. Results were expressed as (Trolox) ($\mu\text{M equiv. Trolox g}^{-1}$). All determinations were performed in triplicate.

Protective effect on H_2O_2 -induced *E. coli*

Escherichia coli (ATCC No. 25922) was used to assess the *in vivo* antioxidant activity. The strain was procured from Microbial Culture Collection Center of Guangdong Institute of Microbiology, China. The protective effects were assayed as described by Smirnova with some modifications (Smirnova et al. 2010). Bacteria were cultivated on LB medium. Cells were aerobically grown overnight at 37°C in 250-mL flasks on an orbital shaker at 100 rpm, and the growth was monitored by measuring its optical density at 600 nm. Cultures in mid-log phase growth were selected, centrifuged for 5 min at 8000 rpm and re-suspended in fresh LB medium. Then, 1.0 ml of the cell suspensions (final $\text{OD}_{600} = 0.25$) were transferred to test tubes containing 8.9 mL of fresh LB medium and 0.1 mL of samples (50 mg mL^{-1}) dissolved in DMSO (less than 0.1%). The test tubes were incubated with shaking for 30 min at 37°C . Then, the cultures were treated with 6.0 mM hydrogen peroxide (H_2O_2) and incubated with shaking for another 30 min at 37°C . Finally, the optical density was measured at 600 nm. The specific growth rate for each sample was calculated according to the following equation:

$$\mu = \ln\left(\frac{N}{N_0}\right)/t$$

Where μ is the specific growth rate, N_0 and N is the optical density at time zero and t respectively. Quercetin (0.1mM) was used as the positive control. The protective activity of each sample was calculated as follows: the specific growth rate of the *E. coli*-containing samples and 6.0 mM H_2O_2 was divided by the specific growth rate of the samples containing only H_2O_2 .

Statistical analysis

All measurements were carried out in triplicate and each result was indicated as the mean value of at least three independent experiments $\pm$ the standard deviation of the mean. Statistical analyses were performed using a one-way analysis of variance followed by Duncan's test. Data were analyzed using the SPSS 18.0 (SPSS Inc., Chicago), and figures were made using the Origin 6.0. Differences were considered significant for $P < 0.05$.

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Table S1 Method validation for the quantitative determination of the six phenolic compounds using RP-HPLC.

Peak no.	Compounds	Retention time (min)	Regression equation	Precision experiment		Repeatability		Recovery experiment	
				Area of peak	RSD (%)	Area of peak	RSD (%)	Average recovery rate (%)	RSD (%)
1	(+)-catechin	17.74	$y = 2004.7x - 25.13$ ($R^2=0.9973$)	83.12±0.61	0.73	12.82±0.22	1.74	101.54±3.08	1.03
2	Caffeic acid	24.60	$y = 146577x + 12.70$ ($R^2=0.9998$)	323.05±1.83	1.32	285.04±2.94	2.94	102.66±2.96	2.88
3	Hyperoside	50.20	$y = 66172x + 37.96$ ($R^2=0.9986$)	960.32±5.22	2.11	5801.02±9.51	2.66	105.72±1.56	1.48
4	Rutin	50.58	$y = 43663x + 30.06$ ($R^2=0.9964$)	89.79±0.65	0.76	239.68±7.85	0.65	96.21±0.34	0.36
5	Ellagic acid	53.55	$y = 62654x - 82.44$ ($R^2=0.9999$)	101.25±1.43	0.93	181.42±2.40	3.28	102.60±1.20	1.17
6	Quercetin	58.68	$y = 89391x + 4.47$ ($R^2=0.9989$)	207.32±2.23	1.94	20.86±1.24	1.05	98.76±2.30	0.78

Note: Values were expressed in mean ± SD (N = 6).

Figure S1 The separation flow chart of the crude extracts and sub-sequent fractions of *P. fruticosa* leaves.

Figure S2. HPLC chromatogram of reference compounds, crude extracts and subsequent fractions of *P. fruticosa* leaves. Six compounds were identified: 1, (+)-catechin; 2, caffeic acid; 3, hyperoside; 4, rutin; 5, ellagic acid; 6, quercetin.

Figure S3. The growth curve of *E. coli* induced by different concentration of H₂O₂.

Figure S4. Protective effect of different samples on *E. coli* induced by 6 mM H₂O₂.

Figure S5 Correlations between DPPH, ABTS^{•+} radical-scavenging capacity, chelating capacity and protective effect on *E. coli* induced by H₂O₂.