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INFLUENCE OF ENVIRONMENTAL FACTORS ON THE CONTENTS OF ACTIVE INGREDIENTS AND RADICAL SCAVENGING PROPERTY OF *POTENTILLA FRUTICOSA* IN THE MAIN PRODUCTION AREAS OF CHINA

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Abstract

Extracts from *Potentilla fruticosa* have been applied in traditional medicine and exhibited antioxidant property, but little has been known about the diversity of phytochemicals and properties on this species from different growing environment. This study investigated the influence of environmental factors on the active ingredient contents and radical scavenging property of *P. fruticosa* from different production areas of China in order to discover a location could produce high-quality resources for pharmaceutical products. The contents of tannin, total flavonoids, and rutin were determined and varied within the range of 7.64±0.43~10.68±0.67%, 2.29±0.34~5.37±0.36%, and 0.19±0.053~0.79±0.125%, respectively. Radical scavenging property was quantified, with the IC_{50} of 7.24±0.423 to 17.23±0.551 μg mL^{-1}. Principal component analysis, multiple linear stepwise regression analysis, and path analysis were conducted to further analysis the relationship between the variations of active ingredients and radical scavenging capacity and growth environment. The results showed dominant environmental factors for these variations were rapidly available nitrogen, rapidly available phosphorus, pH, July average temperature, and annual sunshine duration. Furthermore, a significant positive correlation was observed between pH, annual sunshine duration and active ingredients and radical scavenging property (p<0.05). Considering the high active ingredient contents and strong radical scavenging property, leaf extracts from *P. fruticosa* could become useful supplements for pharmaceutical products as a new antioxidant agent, and Huzhu Northern Mountain in Qinghai Province and E-mei Mountain in Sichuan Province were selected as favorable production locations.

Key words: *Potentilla fruticosa*, Environmental factors, Active ingredients, Radical scavenging property, Influence.

Introduction

Potentilla fruticosa is a species of hardy deciduous flowering shrub in the *Potentilla* genus of the family *Rosaceae*, native to the cool temperate and subarctic regions of the northern hemisphere, often growing at high altitudes in mountains (Li *et al.*, 2003; Miliauskas *et al.*, 2004). In China, *P. fruticosa* commonly called "Jinlaomei drug" and "Gesanghua", its altitude ranges from 400 to 5000 m (Shimono *et al.*, 2009). Apart from its common application as a garden plant, it also has numerous medicinal virtues (Mitich, 1995). *P. fruticosa* have been widely used as folk medicinal herbs and functional tea for a long time in China to treat diarrhoea, hepatitis, rheuma and scabies (Miliauskas *et al.*, 2004; Wang *et al.*, 2013). Moreover the leaves of *P. fruticosa* which taste slightly sweet and cool have applications as food additives and an ingredient in cosmetic products (Nkiliza, 1999).

Modern scientific researches have confirmed that the medical foundations of *P. fruticosa* are tannins and flavonoids and powerful radical scavenging capacity that are contained in its leaf extracts (Aryayeva *et al.*, 1999; Miliauskas *et al.*, 2004; Bai *et al.*, 2007; Miliauskas *et al.*, 2007; Tomczyk *et al.*, 2010). The activity of some extracts was higher than that of the synthetic antioxidant BHT and of extracts isolated from sage (*Salvia officinalis*), which contains powerful antioxidants (Miliauskas *et al.*, 2004). The phytochemicals (secondary metabolites) are the result of the interaction between plants and the environment in the long evolution process, and its production and changes have a strong correlation with the local environment (Gershenzon, 1984). Previous studies have demonstrated that medicinal plants that grow in various environments produce different chemical constituents (Khan & Siddiqi 2014; Khan *et al.*, 2014), resulting in variations in their internal qualities as functional foods, nutritional supplements and medicines (Dong *et al.*, 2011; Wang *et al.*, 2014). In this process, the concept of so called geo-authentic herbal drugs was established. It is assumed that most geo-authentic traditional herbs produced in their native geographical area contain adequate effective chemical constituents. For example, only *Picrorhiza scrophulariiflora* Pennell., one of the well-known herbal drugs in traditional Chinese herbal medicine (TCHM), produced in Tibet, China is officially recognized for use in medicinal practice (China Pharmacopoeia Committee, 2010). By contrast, only *Panax ginseng* C. A. Mey., produced in northeastern China is officially recognized as medicinal drug (China Pharmacopoeia Committee, 2010).

Studies have reported on the influences of growth environment on chemical constituents of other medicinal plants. For example, altitude and annual average temperature were significantly and positively correlated to the contents of chlorogenic acid and flavonoids (P<0.05); annual sunshine duration was significantly and positively correlated to the content of geniposidic acid (P<0.05), while annual average temperature was significantly and negatively correlated to the content of geniposidic acid (P<0.05) in *Eucommia ulmoides* (Dong *et al.*, 2011). In *Betula pendula* Roth., altitude is also positively correlated to the contents of flavonoids (Wulff *et al.*, 1999). Among the *Sinopodophyllotoxin hexandrum* populations, the existing variations in podophyllotoxin content were

proved to be coupled with geographical altitude and local ecological conditions (temperature, rainfall, humidity, soil pH, etc.) but not with genetic basis (Alam *et al.*, 2008; Alam *et al.*, 2009). However in this aspect, studies on *P. fruticosa* are limited despite the fact that environmental factors strongly affect the secondary metabolism. Hence, the authors investigated the main phytochemicals (tannin, total flavonoids, and rutin) and radical scavenging properties of *P. fruticosa* leaves from representative growing regions throughout China combined with environmental factors including soil and climate factors. The present study aims at clarifying the environmental factors affecting the production of the phytochemicals and radical scavenging properties of *P. fruticosa* in order to suggest the best production areas for this wild species, promote its reasonable exploitation for the production of raw materials of pharmaceutical products rather than random harvesting this wild resources.

Materials and methods

Instrumentation and reagents: The amounts of rutin was quantified using RP-HPLC at ambient temperature, which was carried out with an Agilent Series 1260 liquid chromatograph equipped with a quaternary gradient pump system and a variable-wavelength detector system, connected to a reverse-phase SB-C 18 column (5 µm, 4.6×250 mm, Agilent, USA). Data collection was performed using ChemStation software (Agilent, USA).

Folin-Ciocalteus's phenol reagent was purchased from Solarbio Co., Ltd (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Co., St. Louis, USA. Standards including tannic acid and rutin were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol, acetic acid, sodium nitrite, sodium hydroxide and sodium carbonate were purchased from Tianjin Bodi Chemical Holding Co. Ltd (Tianjin, China). Other chemicals were of analytical grade and were purchased from Tianjin Bodi Chemical Holding Co. Ltd (Tianjin, China). Deionized water (18 MΩ cm) was used to prepare aqueous solutions. Stock solutions of all chemicals to be used were prepared in methanol and were diluted to the desired concentration.

Study area: This study was conducted at the Taibai Mt. National Nature Reserve (33°49′ to 34°10′N, 107°19′ to 107°58′E, Shaanxi Province), Zibai Mt. National Forest Park (33°34.9′ to 34°18.3′N, 106°24.9′ to 107°7.5′3E, Shaanxi Province), E-mei Mt. Scenic Regions (29°16.5′ to 29°43.7′N, 103°10.5′ to 103°37.1′E, Sichuan Province), Yungding Mt. Scenic Regions (37°51′ to 38°13′N, 111°31′ to 112°02′E, Shanxi Province), and Huzhu Northern Mt. National Forest Park (36°30′ to 37°9′N, 101°46′ to 102°45′E, Qinghai Province), located in Midwest China (Fig. 1). These areas were considered to be a research hotspot of biology in China, which span an altitudinal gradient of 530 to 3767 m. Mean annual rainfall ranges from 430 to 1600 mm, primarily falling in June through August, which are also the warmest months with mean monthly temperature of 13.7 and 12.2 , and December and January are the coldest months with monthly temperature of −5.3 and −4.1 . Annual mean temperature is 6 to 12 (Zhu & He, 1992). As the distribution areas of *P. fruticosa*, these areas have unique environmental features and geographical conditions which have high impact on the growth of the plant species.

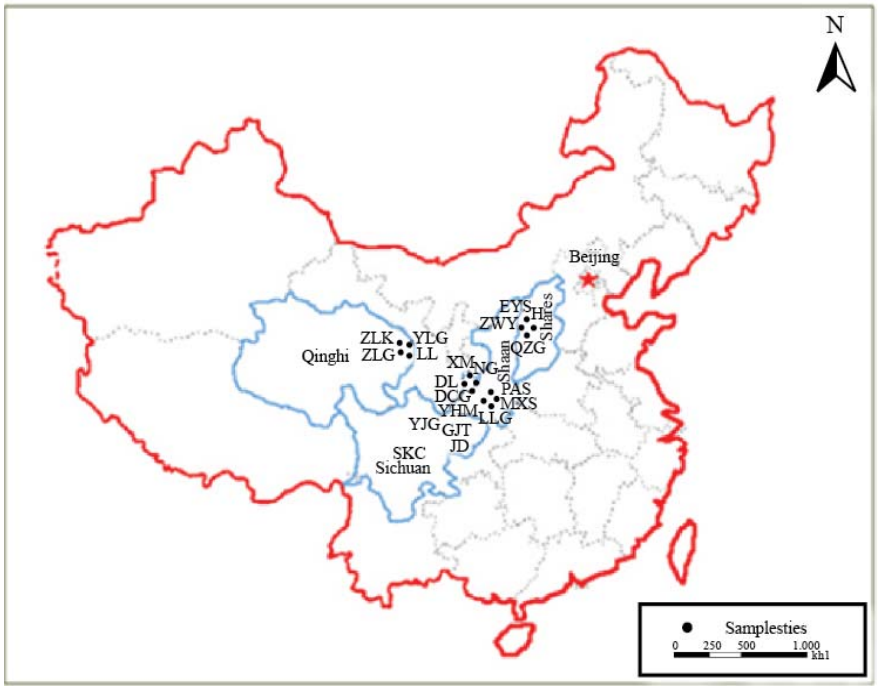

Fig. 1. Locations of *P. fruticosa* samples in different regions sampled for this study.

Plant materials and related data collecting: *P. fruticosa* distribution pattern and extent of in China were investigated from 2011 to 2012. The *P. fruticosa* populations are primarily distributed in Qinghai Province, Shaanxi Province, Sichuan Province and Shanxi Province. Simultaneously, there are small distribution in Inner Mongolia, Tibet, Xinjiang and Heilongjiang Province. According to the field survey information, leaves of *P. fruticosa* and the soil rhizosphere were sampled from five representative growing locations (Taibai Mt., Mei County in Shaanxi; Zibai Mt., Feng county in Shaanxi; E-mei Mt., E-mei in Sichuan; Yungding Mt., Loufan in Shanxi; Huzhu Northern Mt., Huzhu in Qinghai) in July, 2013 (Fig. 1). In details, four natural populations that were similar in growth statues (each population were separated geographically by at least 30 km) were selected at each test location. Five healthy individuals of each natural population were collected. The distance between the adjacent individuals was at least 5 m to increase the likelihood of sampling inter-individual variations within each population of the same location (Xiao *et al.*, 2006). A total of twenty individual samples were collected at each test location, as shown Table 1. Matured leaves were picked up respectively from four directions (north, south, east, and west) of three positions (up, middle and low part) of the plant and then mixed as one test sample in each test location, thereby obtaining 5 samples. All samples were dried under the vacuum at 40°C and ground into powders, and were stored at −20 and protected from light until further analysis. Soil samples (at least 1 kg each) were used to determine the key soil parameters including rapidly available nitrogen (X_1), rapidly available phosphorus (X_2), rapidly available potassium (X_3), total nitrogen (X_4), total phosphorus (X_5), total potassium (X_6), organic matter (X_7), and pH (X_8) according to "Soil Agrochemical Analysis" (Bao, 2000). Related data of climate factors including annual average temperature (X_9), January average temperature (X_{10}), July average temperature (X_{11}), annual accumulated temperature (≥10) (X_{12}), annual highest temperature (X_{13}), annual lowest temperature (X_{14}), annual average precipitation (X_{15}), annual sunshine duration (X_{16}), frost free period (X_{17}), and relative humidity (X_{18}) in the recent thirty years (1984~2013) was also collected from local meteorological bureaus (stations). The key soil indicators and main climate factors of the five study sites were summarized in Table 2.

Table 1. *P. fruticosa* samples collected from different regions in China.

No.	Locations	Population	Code	Coordinates	Number of samples	Altitude (m)	Soil type	Climate zone
S1	Taibai Mt., Mei county, Shaanxi	Pingansi	PAS	E107°43′N34°1′	5	2815	Dark brown earth, Dark brown soil	Semi-humid warm temperate continental monsoon climate zone
		Mingxingsi	MXS	E107°44′N34°0′	5	2637		
		Yuhuangmiao	YHM	E107°22′N34°5′	5	1780		
S2	Zibai Mt., Feng county, Shaanxi	Liulingou	LLG	E108°10′N33°52′	5	1013	Yellow brown earth, Blub soil	Humid warm temperate monsoon climate zone
		Xiaomo	XM	E106°21′N33°45′	5	2728		
		Dalong	DL	E106°14′N34°2′	5	2620		
		Dacaogou	DCG	E106°22′N33°52′	5	2677		
		Nagou	NG	E106°14′N33°51′	5	2963		
S3	E-mei Mt., E-mei, Sichuan	Yiajiageng	YJG	E103°27′N29°17′	5	2946	Frigid brown earth, Bleached podzolic soil	Subfrigid climate zone
		Guanjingtai	GJT	E103°29′N29°15′	5	3788		
		Shengkangcun	SKC	E103°25′N29°14′	5	3207		
		Jinding	JD	E103°26′N29°16′	5	3554		
S4	Yunding Mt., Loufan, Shanxi	Baiyunshan	BYS	E111°15′N37°37′	5	2232	Yellow earth, Meadow soil	Temperate continental monsoon climate zone
		Hougu	HG	E111°13′N37°31′	5	2370		
		Zhiwuyuan	ZWY	E111°18′N37°22′	5	2080		
		Qiaozigou	QZG	E111°22′N37°15′	5	2564		
S5	Huzhu Northern Mt., Huzhu, Qinghai	Zhalongkou	ZLK	E102°34′N36°53′	5	3064	Yellow brown earth, Alpine shrub meadow soil	Semi-arid continental plateau monsoon climate zone
		Zhalonggou	ZLG	E102°37′N36°47′	5	3098		
		Yuanlongogu	YLG	E102°27′N36°54′	5	3069		
		Lalagou	LL	E102°42′N36°44′	5	3169		

Table 2. Main environmental factors of five study sites throughout China.

Items	S1	S2	S3	S4	S5
X_1	7.31 ± 0.03[a]	28.12 ± 0.02[b]	32.65 ± 0.04[b]	9.09 ± 0.07[c]	6.34 ± 0.04[ac]
X_2	7.42 ± 0.06[a]	7.58 ± 0.05[a]	8.56 ± 0.02[a]	10.37 ± 0.01b	7.52 ± 0.04a
X_3	118.6 9 ± 0.02[a]	357.53 ± 0.03[b]	96.71 ± 0.01[c]	154.04 ± 0.03[d]	150.22 ± 0.01[d]
X_4	0.18 ± 0.002[a]	0.25 ± 0.001[b]	0.20 ± 0.005[a]	0.38 ± 0.006[c]	0.23 ± 0.004[ab]
X_5	0.04 ± 0.001[a]	0.11 ± 0.002[b]	0.14 ± 0.003[b]	0.16 ± 0.005[bc]	0.03 ± 0.001[a]
X_6	1.60 ± 0.01[a]	1.49 ± 0.02[b]	1.62 ± 0.04[a]	1.58 ± 0.03[a]	1.76 ± 0.05[c]
X_7	5.09 ± 0.01[a]	6.52 ± 0.01[b]	5.40 ± 0.01[a]	9.53 ± 0.04[c]	6.58 ± 0.03[b]
X_8	7.49 ± 0.05[a]	7.29 ± 0.03[a]	5.29 ± 0.05[b]	6.69 ± 0.07[c]	8.19 ± 0.06[d]
X_9	11.30 ± 0.03[a]	11.84 ± 0.08[a]	17.29 ± 0.04[c]	7.5 ± 0.02[d]	5.92 ± 0.05e
X_{10}	-2.06 ± 0.02[a]	-2.18 ± 0.04[a]	7.2 ± 0.02[b]	-7.6 ± 0.05[c]	-14.24 ± 0.02[d]
X_{11}	27.08 ± 0.02[a]	31.10 ± 0.07[b]	25.6 ± 0.05[c]	21.7 ± 0.01[d]	18.66 ± 0.03[d]
X_{12}	3803.80 ± 0.02[a]	3595.80 ± 0.03[b]	5274.22 ± 0.01[c]	3798.87 ± 0.04[a]	2129.10 ± 0.02[d]
X_{13}	44.94 ± 0.08[a]	42.44 ± 0.04[a]	41.5 ± 0.01ab	37.2 ± 0.03[c]	18.70 ± 0.06[d]
X_{14}	-22.54 ± 0.04[a]	-25.70 ± 0.06[b]	-4.3 ± 0.03[c]	-24.6 ± 0.01[b]	-12.38 ± 0.04[d]
X_{15}	626.40 ± 0.03[a]	557.44 ± 0.04[b]	1555.3 ± 0.02[c]	430.27 ± 0.06[d]	491.30 ± 0.02[d]
X_{16}	2194.12 ± 0.01[a]	2132.84 ± 0.04[a]	3130.60 ± 0.03[b]	2572.6 ± 0.06[c]	2295.98 ± 0.01[a]
X_{17}	202.60 ± 0.08[a]	190.00 ± 0.06[a]	310.64 ± 0.05[b]	135.45 ± 0.02[c]	128.00 ± 0.03[c]
X_{18}	75 ± 0.01[a]%	71 ± 0.05[a] %	82 ± 0.07[c]%	71 ± 0.06[a]%	64 ± 0.04[b]%

Each values represented in table are means±SD (n = 30). Values with different letters within same line were significantly different ($p<0.05$). X1(mg kg−1), rapidly available nitrogen; X2(mg kg−1), rapidly available phosphorus; X3(mg kg−1), rapidly available potassium; X4(%), total nitrogen; X5(%), total phosphorus; X6(%), total potassium; X7(%), organic matter; X8, pH; X9(°C), annual average temperature; X10(°C), January average temperature; X11(°C), July average temperature; X12(°C), annual accumulated temperature(≥10°C); X13(°C), annual highest temperature; X14(°C), annual lowest temperature; X15(mm), annual average precipitation; X16(h), annual sunshine duration; X17(d), frost free period; X18(%), relative humidity.

Optimization of extraction process and preparation of the extracts: Considering the impact of various factors on tannin, total flavonoids, and rutin yields, a single factor test was carried out using *P. fruticosa* leaves from Taibai Mt. as representative material, from which we ultimately screened the four main factors (Lu *et al.*, 2008). Next, using the response surface method, the extraction process of tannin, total flavonoids, and rutin was optimized (Kalil *et al.*, 2000). The optimized extraction process was analyzed using Design-Expert V7.1.6 software as follows: ethanol concentration, 70%; extraction temperature, 40 °C; extraction time, 2 h; extraction times, 3 times; and liquid-solid ratio, 20:1. Each powdered sample was treated as described in the optimized extraction process. The obtained filtrates were evaporated at 40°C under vacuum using a rotary evaporator and were stored at 4°C for further use. Extracts were diluted if necessary. All extractions were performed in triplicate.

Measurement of tannin: Tannin content was determined using the Folin-Denis method (Helrich, 1965). First, 1.0 mL of the diluted sample solution (2 mg mL^{-1}) was transferred into a 25 mL volumetric flask, and then 1 mL of F-D chromogenic reagent and 5 mL sodium carbonate solution (1 mol L^{-1}) were added and mixed. The solution was diluted to volume with methanol. After 30 min of incubation at room temperature, the absorbance at 720 nm was measured against a blank. Tannin acid (1 to 10 mg L^{-1}) was used for the standard curve calibration. All measurements were performed in triplicate.

Measurement of total flavonoids: Total flavonoids content was determined by sodium nitrite-aluminum nitrate colorimetric method (Jia *et al.*, 1999). Approximately 1.0 mL of the diluted sample solution (2 mg mL^{-1}) was transferred into 25 mL volumetric flasks, then 0.3 mL $NaNO_2$ (5%) was added and held for 6 min. Next, 0.3 mL $Al(NO_3)_3$ (10%) was added and held for another 6 min. Finally, 4 mL NaOH (1 mol L^{-1}) was added and the solution was diluted to volume with 70% ethanol solution. After 30 min of incubation at room temperature, the absorbance at 510 nm was measured against a blank. Rutin (4 to 40 mg L^{-1}) was used for the standard curve calibration. All measurements were performed in triplicate.

Quantification of rutin by reverse phase-high performance liquid chromatography (RP-HPLC): The diluted sample solution (1 mg mL^{-1}) was filtered through a 0.22 μm microporous filtering film. The resulting filtrate was then analyzed using RP-HPLC at ambient temperature (Sagdic *et al.*, 2011). The mobile phase consisted of acetic acid aqueous solution (0.5%, solvent A) and acetic acid in methanol solution (0.5%, solvent B) with a flow rate of 0.8 mL min^{-1}. The elution conditions were as follows: 0 min to 10 min, gradient elution, eluent

B was increased from 30% to 35%; 10 min to 20 min, isocratic elution with B at 35%. The injection amount was 20 μL, the detection wavelength was 360 nm, and the sampling time was 20 min. Rutin standard solutions (0.0025 to 0.2 mg L^{-1}) were used for the standard curve calibration using the external standard method. Analyses were performed in triplicate.

Radical scavenging property assay: DPPH assay has been widely used for the determination of scavenging activity of pure antioxidant compounds as well as of different plant extracts (Hussain *et al.*, 2014; Mehmood *et al.*, 2013). IC_{50} values were the effective concentrations at which DPPH radicals were scavenged by 50% and were obtained from linear regression analysis, a lower IC_{50} representing stronger scavenging capacity (Brand-Williams *et al.*, 1995). In the present study, a modified DPPH method was used to determine the radical scavenging property of *P. fruticosa* leaves. Rutin standards and *P. fruticosa* leaves extracts from each production location were prepared in a solution of certain concentration gradient with methanol containing 13 to 17 concentration gradients ranging from 0.002 mg mL^{-1} to 0.07 mg mL^{-1}. Next, 2.0 mL of this solution was mixed with 2.0 mL of 0.1 mol L^{-1} DPPH in methanol. The reaction mixture was vortexed thoroughly and kept in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm against a blank. Rutin was used as control. All measurements were performed in triplicate. DPPH free radical scavenging activity (SA) can be expressed with the following formula:

$$SA(\%) = \left(\left[1 - \left(A_i - A_j\right)\right] / A_0 \times 100\right)$$

where A_i is the absorbance of 2 mL of the sample solutions mixed with 2 mL of DPPH, A_j is the absorbance of the blank sample (2 mL of the sample solutions mixed with 2 mL of methanol), and A_0 is the absorbance of 2 mL of methanol mixed with 2 mL of DPPH.

Statistical analysis: Four methodologies were performed step-by-step to analysis systematically the influence of environmental factors on the active ingredient contents and radical scavenging property of leaves of *P. fruticosa*. Principal component analysis (PCA) was carried out using SPSS software (SPSS for Windows 19.0, SPSS Inc., USA) (Lu, 2002). PCA has recently become the tool of choice for monitoring complex processes. PCA has the strong advantage of significantly reducing the dimension of the complex components while preserving most of the variance within by using dependencies among large numbers of variables without requiring knowledge of the data set in order to visualize high dimensional data and identify the most important variables (Wold *et al.*, 1987). PCA in the present study was used to screen principal components of environmental factors. Multiple linear stepwise regress analysis (MLSRA) was performed to exclude independent variables that were not significantly related to the dependent variables on the basis of PCA, obtaining dominant variables. Path analysis (PA) deals with the quantitative relationship between dependent and independent variables to explain the relative significance of each factor to the dependent variables. MLSRA and PA were conducted by DPS 2006 software to select the dominant environmental factors and evaluate correlations between the active ingredients, the radical scavenging property and these dominant environmental factors, respectively (Mo, 1983). Environmental factors were used as independent variables, and active ingredients and radical scavenging property were used as dependent variables in the each test. The data met the statistical assumptions in the each test (Yuan & Zhou, 2002).

The results were presented as the mean value ± SD (standard deviation). The data was analyzed by one-way ANOVA followed by Duncan multiple comparison based on the SPSS 19.0 software (P<0.05).

Results

Differences in active ingredient contents among various production locations: The contents of tannin, total flavonoids and rutin of *P. fruticosa* leaves differed significantly because of their various origins (P<0.05) (Fig. 2): the contents ranged from 7.64±0.43 to 10.68±0.67%, 2.29±0.34 to 5.37±0.36% and 0.19±0.053 to 0.79±0.125%, respectively. The contents of tannin and total flavonoids were abundant in all samples, while the content of rutin was found at lower concentration. The highest tannin (10.68%), flavonoids (5.37%), and rutin (0.79%) contents were found in leaves from the Huzhu Northern Mt. (S5), whereas the lowest tannin (7.64%) and rutin contents (0.19%) were observed in leaves from Yunding Mt. (S4). The lowest flavonoids content (2.29%) was found in leaves from E-mei Mt. (S3). Li *et al.* (2007) also found the contents of total flavonoids in *P. fruticosa* leaves from different environment displayed great differences, which quite agreed with the present results. These differences may be due to ecological factors, genetics, and the status of secondary metabolism of the leaves in different production locations (Zhao *et al.*, 2014).

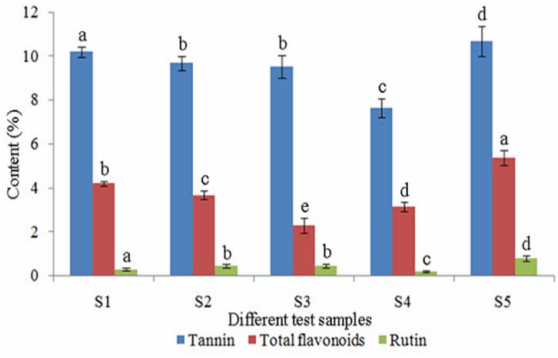

Fig. 2. Differences of active ingredient contents in *P. fruticosa* samples from different regions. For the same variable, bars with no letters in common are significantly different (p<0.05). S1, Mei county, Shaanxi; S2, Feng county, Shaanxi; S3, E-mei, Sichuan; S4, Loufan, Shanxi; S5, Huzhu, Qinghai.

Radical scavenging activity among various production locations: DPPH radical scavenging activity of *P. fruticosa* samples from different regions was compared and showed in Fig. 3A. The scavenging effects of different samples increased with concentrations between 1 and 100 μg mL^{-1}, while there was a significant decrease of absorbance with increase of the concentration of the extracts. To obtain 50% scavenging effect, the concentrations need for S1, S2, S3, S4, and S5 were 19, 15, 10, 45, and 24 μg mL^{-1}, respectively. For all the samples, the IC_{50} values were showed in Fig. 3B and ranged from 7.24±0.423 to 17.23±0.551%, which presented the obvious parabolic trend. The highest radical scavenging activity was obtained for *P. fruticosa* collected from S3 (E-mei Mt., Sichuan) with the lowest average IC_{50} value of 7.24±0.423 μg mL^{-1}, followed by S2 (Zibai Mt., Shaanxi) (IC_{50} value=11.12 ± 0.418 μg mL^{-1}). Compared with rutin standard, there was no significant difference (p<0.05) on the DPPH radical scavenging activity of S3 (Fig. 3B), implying that S3 could have same scavenging effect with rutin standard at adequate concentration. These data indicated that the phytochemicals of *P. fruticosa* leaves were free radical inhibitors, and the scavenging activity of the same *P. fruticosa* species varied immensely from region to region.

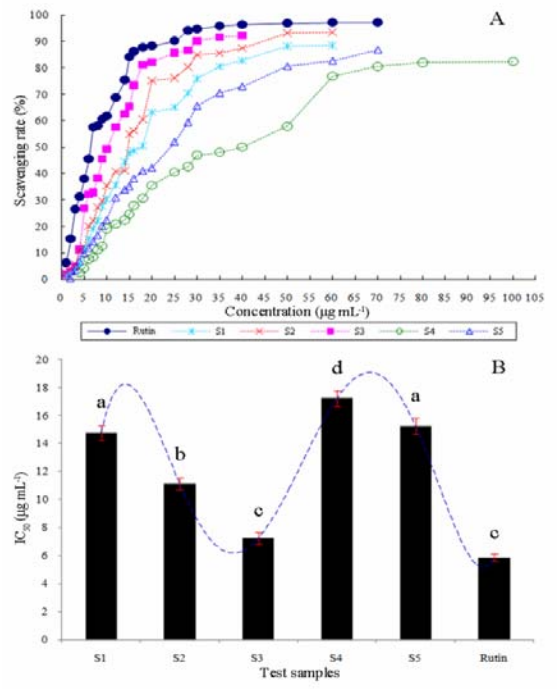

Fig. 3. The DPPH radical scavenging activity (A) and IC_{50} values (B) for *P. fruticosa* samples from different regions.

Analysis of environmental factors influencing the active ingredient contents and radical scavenging property of *P. fruticosa* leaves

Principal component analysis (PCA) of environmental factors: The synthesis and accumulation (secondary metabolism) of the active ingredients of medicinal plants is an extremely complex process affected by a series of ecological factors. The PCA was conducted to identify the principal components from a lot of independent variables (environmental factors). Contribution rate reflects the quantity of original information contained within each factor. The accumulated contribution rate of the first three eigenvalues reached 95.757% (Table 3), which indicates that the first three components nearly covered total original information of the eighteen environmental factors. Thus, these components can be extracted to obtain the loading level based on SPSS 19.0 software (Fig. 4). Fig. 4 indicated that the X_1 (0.504), X_2 (0.475), X_{11} (0.321), X_{15} (0.126), and X_6 (−0.138) were important environmental factors that influenced the first principal component (F_1) due to high loading values. For the second principal component (F_2), important environmental factors were the X_{16} (0.813), X_9 (0.523), X_8 (0.476), X_5 (0.137), X_{10} (−0.464), and X_{12} (−0.663). The third principal component (F_3) accounted for a larger proportion in the X_4 (0.481), X_3 (0.473), X_7 (0.114), X_{13} (−0.121), X_{14} (−0.253), X_{18} (−0.624) and X_{17} (−0.696) than that in other factors. However, its contribution rate was only 1.001 % and the F_3 was not be considered. Two principal components of environmental factors, F_1 (X_1, X_2, X_{11}, X_{15}, and X_6) and F_2 (X_5, X_8, X_9, X_{10}, X_{12}, and X_{16}) were thus screened for further analysis.

Multivariate linear stepwise regression analysis (MLSRA): MLSRA was conducted to establish a regression equation based on PCA and to reveal a more intuitive quantitative relationship between the two principal components (F_1 and F_2) and the active ingredients and radical scavenging property (Table 4). The variables fitted into the equation were the dominant factors. For example, dominant factors that affected tannin contents were X_1(rapidly available nitrogen), X_2 (rapidly available phosphorus), X_5(total phosphorus), X_8 (pH), X_9 (annual average temperature), X_{11}(July average temperature), X_{15}(annual average precipitation), and X_{16}(annual sunshine duration). The R^2 values of the regression equation were greater than 0.900, implying that the regression effect was significant and that the imitative effect was excellent (Table 4).

Although dominant factors could be clarified by MLSRA, the action degrees between active ingredients contents and radical scavenging property and dominant factors remain unclear.

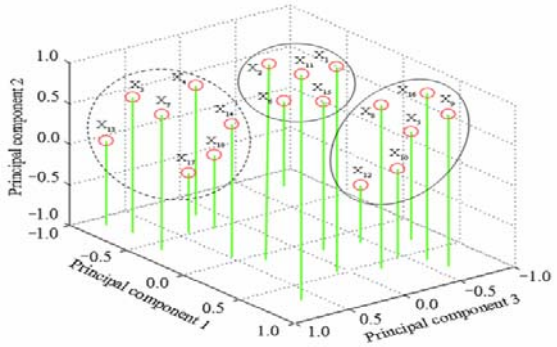

Fig. 4. The loading plot of principal component analysis (PCA) for various environmental factors.

Table 3. Cumulative contribution rates of principal components of environmental factors.

Ecological factors	Principal components	Eigenvalues	Contribution rates (%)	Cumulative contribution rates (%)
X_2	F_2	7.784	41.974	94.756
X_3	F_3	3.339	1.001	95.757
X_4	F_4	1.734	0.915	96.672
X_5	F_5	1.423	0.807	97.479
X_6	F_6	1.025	0.723	98.202
X_7	F_7	0.68	0.572	98.774
X_8	F_8	0.437	0.4105	99.184
X_9	F_9	0.342	0.377	99.561
X_{10}	F_{10}	0. 175	0.3135	99.875
X_{11}	F_{11}	0.072	0.1065	99.981
X_{12}	F_{12}	0.057	0.0065	99.988
X_{13}	F_{13}	0.041	0.0040	99.992
X_{14}	F_{14}	0.033	0.0024	99.994
X_{15}	F_{15}	0.024	0.0021	99.996
X_{16}	F_{16}	0.022	0.0015	99.998
X_{17}	F_{17}	0.018	0.0010	99.999
X_{18}	F_{18}	0.011	0.0009	100

Table 4. Multiple linear stepwise regression analysis (MLSRA) between principal components of environmental factors and the active ingredient contents and radical scavenging property.

Item	Regression equation	R^2	F value
Y_1	$Y_1=12.141□6.554X_{16}□2.825X_9□0.833X_2□0.334X_{15}□0.218X_5□0.553X_{11}□0.349X_8□0.013X_1$	0.956	29.83064**
Y_2	$Y_2=0.014□3.684X_{12}□1.8633X_2□1.661X_{15}□1.039X_{16}□0.885X_{11}□0.791X_6□0.494X_8□0.2071X_1□0.006X_{10}$	0.923	115.66372**
Y_3	$Y_3=2.783□1.833X_8□1.587X_2□0.603X_1□0.152X_{16}□0.136X_{11}□0.009X_{12}□0.003X_{15}$	0.941	271.23483**
Y_4	$Y_4=7.676□1.373X_9□0.604X_{16}□0.550X_2□0.371X_8□0.126X_1□0.084X_{11}$	0.912	72.29458**

** indicates significant difference at $p<0.01$. Y_1 (%), tannin content; Y_2 (%), total flavonoids content; Y_3 (%), rutin content; Y_4 (μg mL^{-1}), IC_{50}.

Path analysis (PA): The quantitative relationship among the dominant factors, active ingredients contents, and radical scavenging property were further explored through PA. The direct effect of dominant factors on active ingredients contents and radical scavenging property were uneven, as some had positive roles, while the others had negative roles (Table 5). The content of tannin was significantly and positively correlated to rapidly available phosphorus (X_2, 0.384), pH (X_8, 0.739) and annual sunshine duration (X_{16}, 0.147) ($p<0.05$), positively correlated to rapidly available nitrogen (X_1, 0.121), total phosphorus (X_5, 0.056) and annual average temperature (X_9, 0.041) (not significant at the level of $p<0.05$), whereas significantly and negatively correlated to annual average precipitation (X_{15}, −0.851), negatively correlated to July average temperature (X_{11}, −0.443) (not significant at the level of $p<0.05$) (Table 5). The content of flavonoids was significantly and positively correlated to annual sunshine duration (X_{16}, 0.501), pH (X_8, 0.462) and January average temperature (X_{10}, 0.179) ($p<0.05$), positively correlated to total potassium (X_6, 0.017) and rapidly available phosphorus (X_2, 0.006) (not significant at the level of $P<0.05$), whereas negatively correlated to rapidly available nitrogen (X_1, −0.897), July average temperature (X_{11}, −0.459), annual average precipitation (X_{15}, −0.333) and effective accumulated temperature (≥10°C) (X_{12}, −0.029) (also not significant at the level of $P<0.05$) (Table 5). The content of rutin was significantly and positively correlated to pH (X_8, 0.865), annual sunshine duration (X_{16}, 0.850), July average temperature (X_{11}, 0.711) and annual average precipitation (X_{15}, 0.217) ($p<0.05$), positively correlated to effective annual accumulated temperature (≥10°C) (X_{12}, 0.150) and rapidly available nitrogen (X_1, 0.136), whereas negatively correlated to rapidly available phosphorus (X_2, −0.368) (not significant at the level of $P<0.05$, Table 5). Rapidly available phosphorus (X_2, 0.406), annual sunshine duration (X_{16}, 0.308) and pH (X_8, 0.235,) exhibited significant and positive direct effect on IC_{50} values, whereas rapidly available nitrogen (X_1, −0.413), July average temperature (X_{11}, −0.302) and annual average temperature (X_9, −0.103) displayed negative direct effect (not significant at the level of $p<0.05$) (Table 5).

The PA results demonstrated that the higher the pH (X_8) or annual sunshine duration (X_{16}), the higher the contents of tannin, flavonoids and rutin, and the larger the IC_{50} values (the lower the radical scavenging property); the lower July average temperature (X_{11}), the higher the contents of tannin, flavonoids and the IC_{50} values, and the lower the content of rutin. All the analysis approaches showed a high degree of consistency among the results and demonstrated that the multiple statistical analyses were reasonable.

Table 5. Path analysis between dominant factors and the contents of active ingredients and radical scavenging property.

Active ingredients	Dominant factors	Direct action	Indirect action								
			Total	→X_1	→X_2	→X_5	→X_8	→X_9	→X_{11}	→X_{15}	→X_{16}
Tannin	X_1	0.121	0.454		−0.893	0.997	−0.003	0.096	−0.074	0.162	0.169
	X_2	0.384*	−0.958	−0.244		−0.793	−0.094	0.298	−0.226	0.013	0.091
	X_5	0.056	0.755	0.272	0.243		0.445	−0.327	−0.087	0.010	0.199
	X_8	0.739*	1.510	0.388	0.286	0.254		0.244	−0.075	0.231	0.182
	X_9	0.041	0.202	0.230	0.010	−0.045	0.141		0.029	−0.012	−0.151
	X_{11}	−0.443	0.556	−0.058	0.127	−0. 646	0.469	0.064		−0.005	−0.041
	X_{15}	−0.851*	1.020	0.081	0.324	−0. 413	0.265	−0.059	0.429		0.393
	X_{16}	0.147*	−0.062	0.001	0.031	0.673	−0.637	−0.025	−0.009	−0.096	
			Total	→X_1	→X_2	→X_6	→X_8	→X_{10}	→X_{11}	→X_{12}	→X_{15}	→X_{16}
Total flavonoids	X_1	−0.897	1.744		0. 710	−0. 164	0. 054	0. 171	−0. 030	−0. 425	0. 452	0. 976
	X_2	0.006	−0.887	0. 042		−0. 010	0. 038	0. 024	−0. 522	0. 135	0. 300	−0. 894
	X_6	0.017	0.597	0. 099	0. 104		0. 185	0. 012	0. 221	0. 038	−0. 438	0. 176
	X_8	0.462*	0.47	−0. 387	0. 142	0. 269		0. 274	0. 121	0. 039	−0. 022	0. 034
	X_{10}	0.179*	−0.357	−0. 476	0. 061	0. 974	0. 756		−0. 234	−0.861	0. 329	−0. 906
	X_{11}	−0.459	−0.148	0.743	0. 525	-0. 933	0. 170	0. 279		-0. 365	0. 047	−0. 614
	X_{12}	−0.029	0.534	0. 976	0. 671	−0. 830	0. 295	0. 236	−0. 852		0. 211	−0.173
	X_{15}	−0. 333	0.862	0. 305	−0. 042	0. 408	0.522	0.693	−0.756	0. 496		−0. 764
	X_{16}	0.501*	0.322	−0. 801	−0. 368	0. 022	0. 063	−0. 509	0. 416	−0. 532	0. 187	
			Total	→X_1	→X_2	→X_8	→X_{11}	→X_{12}	→X_{15}	→X_{16}		
Rutin	X_1	0.136	−0.725		−0. 015	0. 030	0. 066	0. 040	−0. 380	−0. 466		
	X_2	−0.368	−0.115	−0. 160		−0. 331	−0. 033	0. 182	0. 046	0. 181		
	X_8	0.865*	0.029	−0. 037	0. 173		0. 009	−0. 011	−0. 240	0. 135		
	X_{11}	0.711*	−1.436	0. 036	0. 193	−0. 543		0. 012	-0. 609	−0.543		
	X_{12}	0.150	0.471	0. 105	0. 132	−0. 019	0. 097		0. 154	0. 002		
	X_{15}	0.217*	−0.329	0. 124	0. 279	0. 141	0. 005	−0. 039		−0.839		
	X_{16}	0.850*	−0.143	−0. 062	−0. 775	0. 124	0. 434	−0. 006	0. 142			
			Total	→X_1	→X_2	→X_8	→X_9	→X_{11}	→X_{16}			
IC_{50}	X_1	−0.413	0.096		−0. 144	0. 430	0. 346	0. 050	−0.586			
	X_2	0.406*	−0.385		0. 010	−0. 525	−0. 004	0. 539	−0. 155			
	X_8	0.235*	0.141	0. 768	0. 204		−0. 433	−0. 054	−0. 044			
	X_9	−0.103	−0.75	0. 167	−0. 029	0. 015		−0. 008	0. 005			
	X_{11}	−0.302	−0.319	0. 023	0. 133	0. 272	−0. 076		0. 023			
	X_{16}	0.308*	−0.316	0.008	−0. 185	0. 030	0. 010	−0. 747				

* indicates significant difference at $P<0.05$.

Discussion

The internal quality of herbal medicine is a reflection of the integrated influences of multiple ecological factors during their developmental and growth periods (Dong *et al.*, 2011). Environmental variations in different production locations contribute to the differences in active ingredient contents and radical scavenging property of the plants, which result in internal quality and therapeutic effects of TCHM. In this study, significant differences were observed in the active ingredients contents and radical scavenging property of *P. fruticosa* leaves obtained from different production locations (Figs. 2 and 3). The altitude in Huzhu Northern Mt. is higher than other locations, the contents of active ingredient contents (tannin, flavonoids, and rutin) were the highest, and significantly different from other locations (Fig. 2). Altitude is an overall reflection of multiple ecological factors, such as temperature, humidity, and solar radiation. Many studies have confirmed that the contents of flavonoids are positively correlated to the altitude of the growing location (Cuadra *et al.*, 1997; Wilson *et al.*, 1998; Wulff *et al.*, 1999; Zidorn & Stuppner, 2001), which was consistent with the present finding. Tannin, flavonoids, and rutin contain ortho-dihydroxylated structure and exhibit ultraviolet absorption, which is the reason why *P. fruticosa* can endure the strong ultraviolet radiation at higher altitude areas.

Besides germplasm and genetic factors, the metabolism and accumulation of active ingredients are closely affected directly or indirectly by environmental factors of their growth. Different types of active ingredients are regulated by different environmental factors. The dominant factors significantly affecting tannin content were annual average precipitation (X_{15}), pH (X_8), rapidly-available phosphorus (X_2) and annual sunshine duration (X_{16}) (Tables 4 and 5). The dominant factors significantly affecting flavonoids content were pH (X_8), January average temperature (X_{10}) and annual sunshine duration (X_{16}) (Tables 4 and 5). The dominant factors significantly affecting rutin content were pH (X_8), July average temperature (X_{11}), annual average precipitation (X_{15}) and annual sunshine duration (X_{16}) (Tables 4 and 5). For radical scavenging property, the dominant factors that had significant influence were rapidly available phosphorus (X_2), pH (X_8) and annual sunshine duration (X_{16}) (Tables 4 and 5). pH (X_8) and annual sunshine duration (X_{16}) were common dominant factors that had significant positive influence on active ingredients and the radical scavenging property. Soil pH, which indicates the acid-base type of soil, has a large influence on soil fertility and plant growth. Various medicinal plants have special requirements for soil pH, with most medicinal plants thriving better in slightly acidic or neutral soil (Tang & Chen, 2011). pH has a very important impact on the effectiveness of nutrients in the soil; for instance, the validity of phosphorus in the neutral soil is good, and in alkaline soil, the effectiveness of microelements (manganese, copper, zinc, and so on) is poor (Bao, 2000). Thus, selecting or creating a suitable soil pH for the growth of medicinal *P. fruticosa* is an important condition to obtain high-quality resource. For example, Huzhu Northern Mt., Taibai Mt. and Zibai Mt. were screened and considered to be good production locations for harvesting *P. fruticosa* that is rich in tannin, flavonoids and rutin when the yields of other active ingredients were not considered. Illumination affects the synthesis and accumulation of secondary metabolites in medicinal plants. To some plants, the increase of illumination time can increase the contents of secondary metabolites. For example, the amount of flavonoids in *Arabidopsis* increased after long time illumination (Fuglevand *et al.*, 1996). The contents of ginsenogsides in *Panax quinquefolium* were positively correlated to the annual sunshine duration (Zhu *et al.*, 2001). *P. fruticosa* is a heliophilous plant, locations with long time sunshine would be favorable for its growth, resulting in adequate substrate to synthesize secondary metabolites. MLSRA and PA showed that the content of active ingredients (tannin, total flavonoids and rutin) and IC_{50} values were highly associated to sunshine duration, significant positive correlation between them was observed (Tables 4 and 5), indicating that annual sunshine duration is a key environmental factor to the synthesis, accumulation and property of these phytochemicals. Annual average precipitation (X_{15}) also had a noteworthy role. Rainfall is the main source of moisture for wild plants, whereas moderate rainfall is propitious to plant growth, development, and organic matter generation (Huang & Guo, 2009). Notably, annual average precipitation (X_{15}) played a negative direct role in the accumulation of tannin and flavonoids, and further studies should be conducted to determine its mechanism of action.

The ecological conditions in the study areas had significant differences, and the distributions of light, temperature, and moisture were extremely uneven. Multiple differences in the hydrothermal conditions restricted the productivity (yield and quality) of *P. fruticosa*. Therefore, the effect of environmental factors as well as their interaction on active ingredient contents and radical scavenging property should be considered in selecting the best provenance for wild high-quality *P. fruticosa* herbs. Additionally, no systematic and in-depth study on the relationship between environmental factors and quality of TCHM has been conducted. A comprehensive and in-depth understanding of the relationship between TCHM quality and environmental factors should be established from the perspective of key enzymes of secondary metabolites, gene expression and regulation associated with ecological methodologies, which would be conducive to the breeding and efficient cultivation of TCHM to improve the active ingredient contents and produce more effective medicinal components used in the treatment of human diseases.

In the pharmaceutical practice related to TCHM, the general view is that herbs with high active ingredient contents and radical scavenging property would be of high quality. However, this perspective was not evident in this study. *P. fruticosa* from Huzhu Northern Mt. did not have a high radical scavenging property but had high active ingredient contents. This could be because that radical scavenging property does not likely depend on

active ingredient contents alone but also on the constituents, types, structures, and different anti-oxidative mechanisms (Pourmorad *et al.*, 2006; Bai *et al.*, 2007; Al-Juhaimi & Ghafoor, 2013). Radical scavenging property could also depend on the regulation of plant hormones in the secondary metabolism process controlled by internal (germplasm resources and gene) and external factors (complex mechanism of various ecological factors) (Lu *et al.*, 2006). It remains unclear whether the relationship between contents of active ingredients and radical scavenging property has regularity or not. Therefore, further studies are needed to determine the correlation between them.

Conclusions

This study investigated the differences of active ingredient contents and radical scavenging property of *P. fruticosa* sampled from different growing locations all over China. The contents of tannin, total flavonoids, rutin and radical scavenging property were quantified and varied within the range of 7.64±0.43~10.68±0.67%, 2.29±0.34~5.37±0.36%, 0.19±0.053~0.79±0.125% and 7.24±0.423 to 17.23±0.551 μg mL^{-1} (IC_{50} values), respectively. These data revealed that there were significant variations in phytochemicals and radical scavenging property among all samples. Moreover, a series of analysis viz. PCA, MLSRA, and PA were further introduced to evaluate the influence of environmental factors on these variations. The results showed that environmental factors (soil factors and climate factors) had significant impact on the active ingredient contents and radical scavenging property ($p<0.05$). The dominant soil and climate factors for each active ingredient and radical scavenging property were screened and their influence extent was quantified. From the view of the contents of active ingredients and radical scavenging property, leaf extracts from *P. fruticosa* could become useful supplements for pharmaceutical products as a new antioxidant agent. Huzhu Northern Mt. in Qinghai Province is a favorable location for obtaining *P. fruticosa* containing higher contents of tannin, flavonoids, and rutin. For *P. fruticosa* with higher radical scavenging property, E-mei Mt. in Sichuan Province could be selected.

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