Resource evaluation and novel germplasm excavation of wild Chinese prickly ash in Qinling mountains

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Abstract: Wild Chinese prickly ash with elevated antioxidants is a valuable genetic resource for *Zanthoxylum bungeanum* Maxim improvement. There are rich wild germplasm resources in the Qinling Mountains. In a study with wild germplasm resources from different altitudes and six cultivated varieties, the phenolic and flavonoid compounds were analyzed by high performance liquid chromatography (HPLC). The chromatograms of them were basically the same, although their chemical composition content was greatly different. The thirty samples were divided into three categories through the hierarchical clustering analysis. And catechin, hyperoside and quercitrin were considered to be key compound for the quality evaluation, by contrast, the wild samples with an altitude of 2300±50 m (IV group) had the highest content of key compounds, and showed stronger antioxidant activity and antibacterial ability, indicating that these wild samples could be used as an excellent breeding resource. This is the first time to evaluate the quality of wild Chinese prickly ash in different altitude areas of Qinling Mountains. These excellent wild germplasm resources provided substantial potential accessions for use directly in Chinese prickly ash breeding programs.

Key words: Qinling mountains; Wild Chinese prickly ashes; HPLC fingerprint; Resource evaluation.

1. Introduction

Chinese prickly ashes, with Zanthoxylum genus belonging to the family of Rutaceae, is mainly distributed in some Asian countries [1, 2]. There are about 45 species and 13 varieties in China, and the peel is the main edible and medicinal parts of Chinese prickly ash [3, 4]. The Chinese prickly ash pericarps (CPP) contain natural flavonoids and phenolic compounds with outstanding pharmacological activities, such as antioxidant, anti-inflammatory, antibacterial and cardiovascular disease prevention and other beneficial human functions[5, 6].

Biodiversity research of germplasm resources, especially the preservation of wild germplasm, which were formed by the evolution of thousands of years, is extremely important [7, 8]. Wild germplasm resources, with unique quality traits and the characteristics to withstand natural disasters, are a valuable resource and the large variety provides potential for improvement; therefore, germplasm resources are the basis of breeding work [9, 10]. Rational development and utilization of wild germplasm resources can facilitate the development of new prickly ash cultivars. As early as the1960s, Japan began to study the preservation of prickly ash germplasm resources, established a germplasm resource library, and bred high yield, high quality, high oil content of *Zanthoxylum piperitum* and *Zanthoxylum piperitum f.inerme makino*. Korean Institute of Forestry Genetics has been working on excellent prickly ash clones with multiple spikes, large grains and no spines, and four superior prickly ash clones with excellent economic traits were preliminarily selected. Since the 1980s, China has carried out germplasm resource investigations and breeding of Chinese prickly ash. Currently, a number of excellent cultivated varieties have been released, such as ‘Fengxian Dahongpao’, ‘Hancheng Shizitou’, ‘Qin’an Yihao’, ‘Wudu Dahongpao’, ‘Xinong stingless’ and ‘Xiaohongguan’.
Qinling mountains are the abundant sources for wild Chinese prickly ashes, which provides an excellent source to promote the cultivation. A survey of the literature and additional work indicated that wild Chinese prickly ashes had been found at the altitudes of 1300-2500 m above sea level, which grew mainly along streams, in low forest or in stone heap. Unfortunately, the wild Chinese prickly ash resources are abundant and distributed widely in Qinling mountains, but there lacks a fine cultivar and strain, and their system breeding work has not been started. The studies of identification, preservation, and quality evaluation for wild Chinese prickly ash resources are of special importance.

Traditionally, only a few markers or bioactive components were used to assess the authenticity and quality of the medicinal germplasm resources. Nowadays, the quality evaluation and control of natural products and pharmaceuticals are essential, which is closely related to a variety of active ingredients and directly affects the products effect [11-13]. However, the traditional methods used for quality control of herbal medicines are inefficient, and a more efficient and accurate method is needed [14, 15]. HPLC fingerprinting combined with chemometrics is a comprehensive and quantifiable identification method, and has been successfully applied to food and crop analysis [16, 17]. It is, therefore, desirable to determine a reliable and accurate methodology to differentiate the samples collected from this area.

In this study, we aimed to adopt HPLC fingerprint technology combined with multivariate statistical methods to establish an effective set of CPP classification and quality evaluation of wild Chinese prickly ash samples collected at different altitudes in Qinling mountains, and understand how wild Chinese prickly ash differ from cultivated. The primary objective of this study was to characterize the flavonoids and phenolic compounds in wild clones and cultivated varieties, and screen out the excellent germplasm resources containing special physiological active ingredients, which provided valuable reference for the further research and improvement program Chinese prickly ash.

2. Materials and methods
2.1. Materials and chemicals
2.1.1 Plant Materials

A total of twenty-four raw herbs of wild germplasm resource of Chinese prickly ashes from the Qinling mountains were investigated and collected, and divided into group I (1400±50 m), group II (1700±50 m), group III (2000±50 m) and group IV (2300±50 m). Six cultivated varieties (‘Fengxian Dahongpao’, ‘Hancheng Shizitou’, ‘Hancheng Dahongpao’, ‘Wudu Dahongpao’, ‘Qin’an Yihao’ and ‘Xiaohongguan’) were collected from Fengxian, Hancheng, Hancheng, Wudu, Qin’an and Fuping, respectively (Table 1). With the prerequisite of protecting the local germplasm resources and ecological environment, representative plant samples were collected in replicates of three at each site, with a distance of more than 50 m between any two plants. All samples were collected in July to August 2020. Those wild samples were authenticated by Professor Zhenhai Wu (College of Life Sciences, Northwest Agriculture and Forestry University), and all voucher specimens were deposited at the College of Sciences, Northwest Agriculture and Forestry University, Yangling, China. The peels with no signs of mechanical damage or disease were dried in an oven at 45 °C until they reached a constant weight.

Table 1 The information of wild and cultivated varieties samples

| Samples | Varieties     | Location  | Longitude | Latitude | Elevation(m) | Slope       |
|---------|--------------|-----------|-----------|----------|-------------|-------------|
| S1      | *Zanthoxylum* L. | Taibai Shaanxi | 33.823    | 107.606  | 1395.40     | South Slope |
| S2      | *Zanthoxylum* L. | Zhouzhi Shaanxi | 34.053    | 106.962  | 1414.00     | North Slope |
| S3      | *Zanthoxylum* L. | Huxian Shaanxi | 33.690    | 108.287  | 1431.40     | North Slope |
| S4      | *Zanthoxylum* L. | Fengxian Shaanxi | 34.056    | 107.036  | 1440.40     | South Slope |
| S5      | *Zanthoxylum* L. | Fengxian Shaanxi | 33.816    | 107.641  | 1452.00     | South Slope |
|   | Species          | Location       | Latitude   | Longitude  | Altitude  | Slope      |
|---|-----------------|----------------|------------|------------|-----------|------------|
| S6 | *Zanthoxylum*   | Meixian Shaanxi| 34.052     | 106.960    | 1452.60   | North Slope|
| S7 | *Zanthoxylum*   | Meixian Shaanxi| 33.702     | 108.866    | 1650.20   | North Slope|
| S8 | *Zanthoxylum*   | Meixian Shaanxi| 34.027     | 107.867    | 1663.10   | North Slope|
| S9 | *Zanthoxylum*   | Zhouzhi Shaanxi| 33.610     | 106.730    | 1664.70   | North Slope|
| S10| *Zanthoxylum*   | Yueba Shaanxi  | 33.624     | 107.852    | 1688.20   | South Slope|
| S11| *Zanthoxylum*   | Ningshan Shaanxi| 33.682    | 108.203    | 1700.47   | South Slope|
| S12| *Zanthoxylum*   | Liuba Shaanxi  | 33.625     | 106.738    | 1740.52   | South Slope|
| S13| *Zanthoxylum*   | Foping Shaanxi | 33.803     | 108.394    | 1950.50   | South Slope|
| S14| *Zanthoxylum*   | Foping Shaanxi | 33.801     | 108.393    | 1979.20   | South Slope|
| S15| *Zanthoxylum*   | Ningshan Shaanxi| 33.801   | 108.392    | 1922.40   | South Slope|
| S16| *Zanthoxylum*   | Huxian Shaanxi | 33.690     | 108.392    | 1923.40   | South Slope|
| S17| *Zanthoxylum*   | Ningshan Shaanxi| 33.716   | 108.205    | 1956.00   | South Slope|
| S18| *Zanthoxylum*   | Huxian Shaanxi | 33.700     | 108.043    | 2055.80   | South Slope|
| S19| *Zanthoxylum*   | Huxian Shaanxi | 33.724     | 108.390    | 2254.50   | North Slope|
| S20| *Zanthoxylum*   | Huxian Shaanxi | 33.778     | 108.328    | 2274.40   | North Slope|
| S21| *Zanthoxylum*   | Huxian Shaanxi | 33.371     | 108.582    | 2340.70   | North Slope|
| S22| *Zanthoxylum*   | Ningshan Shaanxi| 33.801   | 108.329    | 2351.00   | South Slope|
| S23| *Zanthoxylum*   | Foping Shaanxi | 32.807     | 109.432    | 2363.90   | South Slope|
| S24| *Zanthoxylum*   | Ningshan Shaanxi| 33.811   | 108.330    | 2364.30   | South Slope|
| S25| Hancheng Dahongpao | Hancheng Shaanxi| 35.413 | 110.243    | 866.00    | ND         |
| S26| Hancheng Shizitou | Hancheng Shaanxi| 35.413 | 110.243    | 866.00    | ND         |
| S27| Fengxian Dahongpao | Fengxian Shaanxi| 33.985 | 106.656    | 1011.00   | South Slope|
| S28| Qin’an Yihao     | Qin’an Gansu   | 34.889     | 105.566    | 1621.00   | ND         |
| S29| Wudu Dahongpao   | Wudu Gansu     | 33.488     | 105.085    | 1634.00   | ND         |
| S30| Xiaohongguan     | Fuping Shaanxi | 34.939     | 110.394    | 378.00    | ND         |

2.1.2 Chemicals and Reagents

2,2-Diphenyl-1-picyrhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) diaminonium salt (ABTS), 2,4,6-tripryidyl-s-triazine (TPTZ), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, Miss., USA). Eight chemical standards (hyperoside, luteolin, kaempferol, quercitrin, catechin, rutinum, chlorogenic acid, quercetin) were brought from Beijing solarbio science & technology (Beijing, China). The structures of these chemical standards are shown in Fig.1. HPLC-grade methanol and trifluoroacetic acid were obtained from TEDA Chemical Co., Ltd. (Fairfield, Ohio, USA). Deionized water (18 MΩ cm) was used to prepare aqueous solutions.
Fig.1. Compounds identified in Chinese prickly ash.

2.2. Sample preparations

The dried peels were pulverized and sieved through a no. 60 mesh (< 0.250 mm). Each sample was accurately weighed at 1.0 g and extracted with 30 mL of 80% methanol at 50 °C for 40 min by ultrasonication and then centrifuged at 15000 rpm for 10 min. The supernatant was filtered through a 0.22μm membrane before injection.

2.3 Determination of total flavonoid content and total phenolic content

The total phenolic content of sample was detected by photometric method using Folin-Ciocalteu reagent assay [18]. Gallic acid standard solution (0 to 5 mg/L) was used for constructing the calibration curve (Y=0.0939X-0.0055, R^2=0.9991). Total flavonoid content was determined using NaNO2-Al(NO3)3 coloration assay [19]. The total flavonoid content was calculated by rutinum standard curve (Y=0.0184X+0.001, R^2 =0.9995) of different concentrations (0.0-20.0 mg/L). The results of total phenolic content and total flavonoid content were calculated as gallic acid equivalents per 100 grams of dry extract and rutinum equivalents per 100 grams of dried extracts, respectively.

2.4 HPLC analysis of eight compounds

The quantitative analysis of eight effective components in Chinese prickly ash peels was carried out by HPLC. Chromatographic separation was achieved on a Xterra MS C18 column (5μm, 4.6 × 250 mm, Agilent Technologies Inc., Santa Clara, CA, USA). The gradient elution system is consisted of (A) acetonitrile and (B) 0.05% trifluoroacetic acid as the mobile phase. The gradients were as follows: 0-5 min: 15-20% A, 85-80% B; 5-15 min: 20-40% A, 80-60% B; 15-20 min: 40-48% A, 60-52% B; 20-25 min: 48-55% A, 52-45% B; 25-26 min: 55-15% A, 45-85% B; 26-30 min: 15% A, 85% B. The flow-rate was 1.0 mL/min and the sample injection volume was 5μL. The temperature of the column was maintained at 28 °C. All samples were detected at 280 nm. The standard curve is shown in Table S1. All the relative standard deviation (RSD) values were less than 2.34%, which indicated that this HPLC method was stable and reliable.

2.5 Determination of antioxidant activity.

The DPPH radical scavenging capacities were measured using the methods described previously [20]. The Trolox and 80% methanol were used as positive control and control blank. Each extract was prepared to five different concentrations (0.15, 0.30, 0.45, 0.60, 0.75 mg/mL) by methanol. Each concentration of the solution 400 μL was added to 3.6 mL 0.11 mmol/L DPPH solution (80% ethanol preparation) and reacted at room temperature and controlled light for 30 min to measure the absorbance.
at 517 nm. A lower IC50 value represents the stronger DPPH radical scavenging activity. IC50 value was interpreted as a concentration of the sample at a DPPH radical scavenging rate of 50%, and was inversely proportional to the DPPH radical scavenging ability.

Ferric reducing antioxidant power (FRAP) activity was determined using the protocol of Benzie & Strain with some modifications [21]. The results of FRAP activity were expressed in terms of micromoles Trolox equivalent per gram dry extract weight (mmol equiv. Trolox/g). A higher FRAP value represents a stronger antioxidant activity.

The ABTS inhibitory ability was evaluated using the method of previous study [22]. One milligram per milliliter sample was reacted with ABTS•+ solution at 37 °C for 10 min. After that, the absorbance was measured at 734 nm. Trolox standard solution (0 to 600 µmol/L) was used for constructing a standard curve. The ABTS inhibitory ability was expressed as micromoles of Trolox equiv. per grams.

### 2.6 Determination of antibacterial activity

This study selected *Candida Albicans* (*C. Albicans*) as the representative of the fungi, *Staphylococcus aureus* (*S. aureus*) as the bacteria representative to determine the antibacterial activity. Antibacterial activity was tested using filter paper diffusion method. All the CPP extracts were diluted to 0.5, 0.8, 1.1, 1.4 and 1.7 mg/L with 80% methanol, using 80% methanol solution as blank control, and then soaked in a circular white filter for 24 h with a size of 5 mm × 5 mm.

The *C. Albicans* was cultured on rose-bengal medium, and the *S. aureus* was cultured on Buffer protein hydrophobic (BP) medium at 37 °C. In the ultra-clean bench, 0.5 mL of 10^6 cfu/mL–10^7 cfu/mL of bacterial fluid was absorbed to coat each plate, and then sterilized filter paper discs soaked in crude extracts were placed on the solidified petri dish surface. All strains were tested with 5 replicates and cultured in Biochemical incubator for 24 h at 37 °C. The diameter of the inhibition zone (DIZ) was measured using a cursor caliper using the cross-patch method, and the inhibition rate was calculated based on the diameter of the inhibition zone. Finally, the minimum inhibitory concentration (MIC50) was calculated according to the inhibition rate and the corresponding crude extracts concentration.

### 2.7 Statistical analysis

Chemometric analyses, such as hierarchical cluster analysis, principal component analysis, were performed to systematically analyze the difference of the flavonoids and phenolic compounds between wild and cultivated varieties peels. Hierarchical cluster analysis and principal component analysis were generated using the Origin software for statistical and computing (Origin Pro 2020b, Origin Lab, USA). The significant difference was calculated by SPSS one-way ANOVA followed by Duncan’s test, values <0.05 were considered to be significant (SPSS 24.0 for Windows, SPSS Inc., Chicago, IL, USA).

### 3. Results

#### 3.1. Total flavonoids contents (TFC) and total phenolic contents (TPC)

As shown in Table 2, the mean values of TFC ranged from 55.644±3.012 to 146.190±4.027 mg/g and TPC from 41.821±0.461 to 99.041±4.141 mg/g. The accumulation of TFC and TPC in pericarp of wild Chinese prickly ash grown at different altitudes were significantly different (p<0.05), but there was no significant difference in the TFC and TPC at each group. The TFC and TPC contents of all wild varieties were lower than those of cultivars (*‘Fengxian Dahongpao’*, ‘Hancheng Shizitou’, ‘Hancheng Dahongpao’ and ‘Xiaohongguan’). The TFC and TPC of S30 (*Xiaohongguan*) were the highest. The Fig.2(A) and Fig.2(B) showed that the average TFC and TPC of 4 groups at different altitudinal belts decreased and then rose with the increase of altitude, respectively; and the group IV had the highest TFC and TPC among the wild samples, which were close to those of S28 (*‘Qin’an Yihao’*) and S29 (*‘Wudu Dahongpao’*).
3.2 Quantification of the bioactive metabolites of Chinese prickly ash

The reliable and replicable HPLC method was used to simultaneously determine eight flavonoid and phenolic components in 30 populations of Chinese prickly ash peels (Fig. 2(C)). The compounds were extracted and analyzed in triplicate (summarized in Table 2). Significant differences (P < 0.05) were observed in the contents of the eight active ingredients of Chinese prickly ash peels. The contents of hyperoside, quercitrin and catechin were significantly higher than that of other substances, and they were the main compounds of CPP at different altitudes, which was consistent with our previous research results. The $Y_{HY}$ was the most abundant component, ranging from 9.754 to 27.840 mg/g. The next most plentiful compounds were $Y_{QI}$ (8.956±0.364 to 36.275±0.424mg/g), $Y_{C}$ (4.775±0.057 to 19.367±0.100mg/g), $Y_{CA}$ (0.433±0.009 to 14.535±0.191mg/g) and $Y_{RU}$ (0.437±0.009 to 13.065±0.191mg/g). The content of $Y_{LU}$ (0.468±0.009 to 2.335±0.063mg/g), $Y_{KP}$ (0.011±0.003 to 2.097±0.021mg/g) and $Y_{QU}$ (0.197±0.031 to 3.015±0.275mg/g) was lower.

By comparison, the content of $Y_{HY}$, $Y_{QI}$ and $Y_{CA}$ of S30 (‘Xiaohongguan’) was kept in front of all the samples. The eight bioactive metabolites contents of six cultivated varieties were higher than them of group I, group II and group III. The content of $Y_{QI}$ of group I was kept at a higher level than that of other wild samples. The contents of $Y_{HY}$, $Y_{LU}$, $Y_{QI}$, $Y_{C}$, $Y_{RU}$ and $Y_{CA}$ of group IV were the highest among the wild samples. Moreover, the contents of $Y_{C}$ and $Y_{QI}$ were close to that of cultivars, and the $Y_{HY}$
content was even higher than that of cultivars, which indicated that the group IV might be the excellent germplasm resource.

Table 2. Content (mg/g) of investigated compounds in Chinese prickly ash peel samples.

| Samples | TPC (mmolRU/100 g) | TFC (mmolGAE/100 g) | Content (mg/g) |
|---------|---------------------|----------------------|----------------|
|         | Y_\text{G}         | Y_\text{G}          | Y_\text{G}    |
| S1      | 94.78±2.37^a        | 67.840±0.55^a       | 17.85±0.16^a  |
| S2      | 92.65±2.18^a        | 64.552±0.19^a       | 18.74±0.49^a  |
| S3      | 97.18±1.50^a        | 61.114±0.07^a       | 19.83±0.19^a  |
| S4      | 94.78±1.63^a        | 68.030±0.82^a       | 20.53±0.05^a  |
| S5      | 97.18±1.50^a        | 63.465±0.44^a       | 18.64±0.73^a  |
| S6      | 95.84±1.88^a        | 60.713±0.15^a       | 20.81±0.54^a  |
| S7      | 62.56±1.13^a        | 44.821±0.41^a       | 11.20±0.07^a  |
| S8      | 64.16±0.75^a        | 47.17±0.83^a        | 11.44±0.35^a  |
| S9      | 64.43±0.37^a        | 45.258±1.24^a       | 11.72±0.01^a  |
| S10     | 65.22±0.25^a        | 47.255±1.76^a       | 10.22±0.05^a  |
| S11     | 64.69±0.73^a        | 44.688±1.24^a       | 11.73±0.02^a  |
| S12     | 55.64±0.27^a        | 44.514±1.72^a       | 9.75±0.15^a   |
| S13     | 75.34±0.45^a        | 56.345±0.57^a       | 15.13±0.13^a  |
| S14     | 73.46±0.33^a        | 53.995±0.70^a       | 13.84±0.04^a  |
| S15     | 74.54±0.16^a        | 53.152±0.23^a       | 14.56±0.08^a  |
| S16     | 73.83±0.38^a        | 53.139±0.28^a       | 13.23±0.02^a  |
| S17     | 72.95±0.31^a        | 52.500±0.34^a       | 13.62±0.20^a  |
| S18     | 72.15±0.20^a        | 55.911±0.45^a       | 14.15±0.41^o  |
| S19     | 105.96±0.82^a       | 75.631±1.45^a       | 22.84±0.06^a  |
| S20     | 111.02±0.59^a       | 77.038±2.37^a       | 24.47±0.32^a  |
| S21     | 106.23±0.27^a       | 72.052±0.42^a       | 23.52±0.30^a  |
| S22     | 104.63±0.61^a       | 74.669±0.76^a       | 24.15±0.42^a  |
| S23     | 107.02±1.12^a       | 72.052±0.31^a       | 22.96±0.39^a  |
| S24     | 103.02±1.36^a       | 74.307±0.95^a       | 22.88±0.07^a  |
| S25     | 129.44±1.01^a       | 88.658±1.50^a       | 18.55±0.19^a  |
| S26     | 123.36±0.71^a       | 72.875±1.88^a       | 22.87±0.06^d  |
| S27     | 139.82±1.36^a       | 91.321±0.73^a       | 14.22±0.80^a  |
| S28     | 92.18±0.48^a        | 63.099±1.50^a       | 15.90±0.21^a  |
| S29     | 110.95±1.64^a       | 68.424±1.06^a       | 13.815±0.06^a |
| S30     | 146.19±0.42^a       | 99.042±1.41^a       | 27.84±0.38^a  |

NOTE: mmol equiv. RU/100 g, millimole rutin equivalent per 100 g; mmol equiv. GAE/100 g, millimole gallic acid equivalent per 100 g. All units was mg/g. Values are mean ± SD (n=3). Means with different letters within a column are significantly different (P < 0.05). Y_\text{H}-Hydroxypaeonol, Y_\text{L}-Luteolin, Y_\text{K}-Kaempferol, Y_\text{QI}-Quercitrin, Y_\text{C}-Catechin, Y_\text{R}-Rutinum, Y_\text{CA}-Chlorogenic acid, Y_\text{QK}-Quercetin.

3.3. HCA and PCA analysis

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed, so as to analyze the correlation between multiple compounds of Chinese prickly ash peels. The HCA was used to sort samples into groups by applying the inter-group connection, which used the pearson
correlation as the measurement standard. The Z-score method was used to standardize the related variables to obtain the clustering diagram. The results of the HCA were shown in Fig. 3(A). It was clear that the thirteen varieties were classified into three clusters (C I - C III). Firstly, the C I consists of group I, group II and group III. Both of them were wild samples and the TFC and TPC were lower than the remaining samples. Samples S25 ('Hancheng Dahongpao'), S26 ('Hancheng Shizitou') and S30 ('Xiaohongguan') were grouped together in C III. Both of them came from Shaanxi Province and the content of Y卢, YK, YQI and YCA were higher than that of all other samples. Samples S19, S20, S21, S22, S23, S24, S27 ('Fengxian Dahongpao'), S28 ('Qin’an Yihao') and S29 ('Wudu Dahongpao') were clustered into a larger group (C II) and the total flavonoid contents and total phenolic contents in these samples was higher. Besides, the content of YHY, YQI, YC and YCA were close. According to these results, the clustering results were closely related to the concentration of secondary metabolites and the quality of Chinese prickly ashes.

In the PCA, three principal components were constructed, the first three main components (PC1, PC2, and PC3) was 63.790%, 14.016%, 12.281% respectively, and the cumulative contributory ratio was 90.087%, indicating that the PC1, PC2, and PC3 could reflect the most information on raw data. As the first two principal components represented 77.806% of the total variance, two-dimensional score plots were generated (Fig.3(B)), in which each sample is represented by a marker. In the 2D score plot, the tested samples were separated as relatively independent clusters on PC1, PC2 and PC3, which were approximately in accordance with the HCA.

Additionally, the loading of principal component was important to evaluate the contribution of each component for the separation of clusters. YHY (1.562) showed higher weights in the first principal component (PC1), and YC (1.889) was the chief indexes of the second component (PC2). YQI (1.861) loaded highly in the third principal component (PC3). And the loading of each principal component was also visualized in a 3D loading plot (Fig. 3 (C)). Therefore, YHY, YC and YQI were key compounds and had significant influence on the quality evaluation of different varieties due to these loadings of characteristic peaks were far away from other loadings. Because the contribution rate of PC1 was higher than that of PC2 and PC3, it was inferred that the varieties in the first quadrant of the 2D scoring map had high quality. Therefore, group IV, S25 ('Hancheng Dahongpao'), S26 ('Hancheng Shizitou') and S30 ('Xiaohongguan') were rated as the best variety, but the quality of group II and group III were the last, which was in agreement with the results of flavonoids compositions. It meant that these samples of group IV were the excellent wild germplasm resource and were thus screened for further analysis.

### 3.4 Dimensionality reduction analysis

In order to simplify the analysis and reduce the identification difficulty, three key chemical compounds with higher loading values such as YHY, YQI, and YC were selected for dimensionality reduction analysis. The PCA results after dimension reduction are shown in Fig. 3 (D). Compared with the common pattern with eight compounds, the analysis results were consistent. It meant that reducing characteristic peaks from eight to three was still satisfactory for analytical effects. That was to say, YHY, YQI, and YC could be optimized as markers of different locations and all of them might be suitable for evaluating the quality of Chinese prickly ash.
3.5 Antioxidant activity and antibacterial activity

In order to further evaluate the quality of wild germplasm resources, we measured the antioxidant capacity and antibacterial ability, and compared with the six cultivated varieties.

3.5.1 Antioxidant activity

The antioxidant activity of Chinese prickly ashes peels was evaluated by integrating the results of these three methods (DPPH, ABTS, FRAP). According to Table 3, all the samples exhibited the high efficiency of scavenging free radical and chelating metal ions. Among them, the antioxidant activity of S30 (‘Xiaohongguan’) was the strongest with the relatively lowest DDPH IC$_{50}$ value (18.835±0.280 μg/ml), and the highest values of ABTS (1250.662±11.144 μmol Trolox/g) and FRAP (950.493±26.024 μmol Trolox/g). Among them, the antioxidant activity of CPP at the altitude of 1700±50m was lower than that of other varieties. However, the samples (S19, S20, S21, S22, S23, S24) exhibited stronger antioxidant activity than the remaining wild samples, and exceeded cultivated varieties S28 (‘Qin’an Yihao’) and S29 (‘Wudu Dahongpao’). In addition, the samples with strong antioxidant activity may be related to the high contents of the key compounds (Y$_{HY}$, Y$_{C}$ and Y$_{QI}$).

Table 3 Antioxidant activities of wild and cultivated varieties peels

| Samples | DPPH IC$_{50}$(μg/ml) | FRAP (μmol Trolox/g) | ABTS (μmol Trolox/g) |
|---------|------------------------|----------------------|----------------------|
| S1      | 32.517±0.395$^c$       | 701.230±2.307$^f$    | 554.441±20.008$^f$   |
| S2      | 34.760±0.343$^c$       | 704.692±3.077$^f$    | 528.142±18.839$^f$   |
| S3      | 29.993±0.586$^c$       | 686.231±2.692$^f$    | 560.250±26.859$^f$   |
3.5.2 Antibacterial activity

The antibacterial activity of 30 crude extracts of CPP against *C. Albicans* and *S. aureus* was studied by paper disc diffusion method. The size of the antibacterial ring was measured by the cursor caliper using the cross-method, and the antibacterial rate was calculated according to the diameter of the antibacterial circle. The minimum antibacterial concentration was calculated according to the antibacterial rate and the corresponding crude content concentration, represented by the DIZ and MIC values respectively.

As were shown in the table 4, all crude extracts had antibacterial activity against the bacterial and fungal for test, and the antibacterial rate was also increasing with the increase of the concentration of extracts. The DIZ value of *C. Albicans* was between 4.93±0.13 mm (S8) to 13.20±0.23 mm (S30), and
the DIZ value of *S. aureus* was between 5.98±0.33 mm (S8) to 12.57±0.37 mm (S30). It could be seen that CPP extract had certain inhibitory effect on *C. Albicans* and *S. aureus*. The group IV at the altitude of 2300±50 m exhibited the best antibacterial activity, with the best inhibition zone diameters in the wild samples, and its antibacterial ability was lower than S25('Hancheng Dahongpao'), S26 ('Hancheng Shizitou'), S27('Fengxian Dahongpao'), S30('Xiaohongguan'), but was higher than S28('Qin’an Yihao') and S29 ('Wudu Dahongpao').

**Table 4** Antibacterial activities of wild and cultivated varieties peels

| Samples | Inhibition zone diameter (mm) | MIC<sub>50</sub> (mg/ml) |
|---------|-------------------------------|--------------------------|
|         | *C. Albicans*                  | *S. aureus*              | *C. Albicans* | *S. aureus* |
| S1      | 8.85±0.59<sup>b</sup>          | 8.36±0.39<sup>f</sup>    | 6.73          | 6.37        |
| S2      | 8.50±0.38<sup>b</sup>          | 8.45±0.49<sup>f</sup>    | 6.55          | 6.43        |
| S3      | 8.77±0.43<sup>b</sup>          | 8.53±0.12<sup>f</sup>    | 6.67          | 6.45        |
| S4      | 8.90±0.59<sup>b</sup>          | 8.25±0.35<sup>f</sup>    | 6.55          | 6.38        |
| S5      | 8.80±0.65<sup>b</sup>          | 8.63±0.11<sup>f</sup>    | 6.52          | 6.50        |
| S6      | 8.64±0.39<sup>b</sup>          | 8.42±0.32<sup>f</sup>    | 6.57          | 6.65        |
| S7      | 5.82±0.19<sup>j</sup>          | 6.25±0.16<sup>i</sup>    | 7.53          | 7.18        |
| S8      | 4.93±0.13<sup>j</sup>          | 5.98±0.33<sup>i</sup>    | 7.83          | 7.42        |
| S9      | 5.42±0.17<sup>j</sup>          | 6.13±0.13<sup>j</sup>    | 7.65          | 7.30        |
| S10     | 5.28±0.11<sup>kl</sup>         | 6.09±0.22<sup>i</sup>    | 7.70          | 7.33        |
| S11     | 5.49±0.06<sup>b</sup>          | 6.15±0.28<sup>i</sup>    | 7.63          | 7.28        |
| S12     | 6.27±0.42<sup>¢</sup>          | 6.38±0.43<sup>i</sup>    | 7.38          | 7.08        |
| S13     | 8.28±0.10<sup>d</sup>          | 7.49±0.33<sup>b</sup>    | 7.17          | 6.57        |
| S14     | 8.14±0.35<sup>d</sup>          | 7.34±0.55<sup>b</sup>    | 7.16          | 6.58        |
| S15     | 8.24±0.37<sup>d</sup>          | 7.28±0.19<sup>b</sup>    | 7.29          | 6.57        |
| S16     | 7.99±0.41<sup>d</sup>          | 7.19±0.28<sup>b</sup>    | 7.23          | 6.65        |
| S17     | 8.10±0.27<sup>d</sup>          | 7.23±0.18<sup>b</sup>    | 7.02          | 6.62        |
| S18     | 8.21±0.32<sup>d</sup>          | 7.37±0.31<sup>b</sup>    | 7.11          | 6.58        |
| S19     | 10.49±0.15<sup>f</sup>         | 9.65±0.36<sup>c</sup>    | 5.07          | 5.02        |
| S20     | 10.64±0.16<sup>f</sup>         | 9.57±0.40<sup>c</sup>    | 5.02          | 4.98        |
| S21     | 10.38±0.25<sup>f</sup>         | 9.63±0.11<sup>c</sup>    | 5.03          | 5.08        |
| S22     | 9.92±0.14<sup>f</sup>          | 9.75±0.21<sup>c</sup>    | 5.18          | 5.22        |
| S23     | 9.82±0.43<sup>b</sup>          | 9.54±0.25<sup>c</sup>    | 5.22          | 5.19        |
| S24     | 10.06±0.22<sup>b</sup>         | 9.52±0.42<sup>c</sup>    | 5.13          | 5.12        |
| S25     | 11.07±0.34<sup>e</sup>         | 10.69±0.30<sup>d</sup>   | 4.69          | 4.70        |
| S26     | 10.85±0.19<sup>e</sup>         | 10.18±0.28<sup>d</sup>   | 4.75          | 4.81        |
| S27     | 12.58±0.25<sup>d</sup>         | 11.24±0.39<sup>e</sup>   | 4.04          | 4.48        |
| S28     | 8.81±0.09<sup>b</sup>          | 7.98±0.38<sup>f</sup>    | 6.69          | 6.38        |
| S29     | 9.72±0.15<sup>e</sup>          | 9.50±0.29<sup>c</sup>    | 5.25          | 4.97        |
| S30     | 13.20±0.23<sup>c</sup>         | 12.57±0.37<sup>b</sup>   | 3.98          | 4.39        |
| Tetracycline | 16.59±0.35<sup>b</sup>   | 18.65±0.46<sup>a</sup>   | 1.49          | 1.74        |
| Penbritin | 27.15±0.51<sup>a</sup>        | 18.29±0.51<sup>a</sup>   | 0.86          | 0.93        |

Note: Values are mean ± SD (n=3). Means with different letters within a column are significantly different (P < 0.05).
The minimum inhibitory concentration (MIC\textsubscript{50}) referred to the minimum standard of antibacterial activity at the antibacterial rate of 50%. We used MIC\textsubscript{50} to further evaluate its antibacterial activity. The MIC\textsubscript{50} of \textit{C. Albicans} ranged from 3.98 to 7.83 mg/mL and the \textit{S. aureus} was 4.39 to 7.42 mg/mL. Consistent with the DIZ results, S30 had the highest antibacterial activity against \textit{C. Albicans} and \textit{S. aureus} with the lowest MIC\textsubscript{50} at 3.98 mg/ml and 4.39 mg/ml.

Compared with the results of TFC and TPC, the flavonoid-rich CPP demonstrated better antioxidant activity. The antioxidant activity and antibacterial activity were positively related to the TFC, indicating that antibacterial activity was closely related to the content of active substances. These results suggested that Chinese prickly ashes of group IV can be used as the potential germplasm resources for breeding.

4 Discussion

Crop wild relatives are an invaluable reservoir of productivity enhancement related characters having resilience to climate change and farming system, and are source of novel traits [23]. The accurate evaluation of crop wild relatives is a prerequisite for identifying interested target traits, and then they are infiltrated into the background of cultivated varieties to improve genetic gains [24-26]. Any new varieties are modified, machined, and improved on the basis of the original plant resources through the methods of selection, hybridization, backcrossing, and mutation [27, 28]. The precise evaluation of crop wild relatives is pre-requisite to identify target traits of interest followed by their introgression into the background of cultivated varieties for enhancing genetic gains [9, 29]. Viera et al. characterized wheat germplasm based on phenotypic traits and obtained distances up to 196.61. The report of existing variability among germplasm accessions provides an idea about the expected heterosis for starting breeding programme [30]. A study found that a broad range of Euclidean distances obtained showed the importance of wild lentil accessions as a source of diverse heterotic material [31]. Liu et al. analyzed the wild germplasm resource of \textit{Ophiopogon Japonicus} from Sichuan Basin, China by RP-HPLC coupled with hierarchical cluster analysis, and chose excellent sources from these wild resources [32]. A wide range of variation in wild Chinese prickly ash accessions was observed against the target traits flavonoids, antioxidant activity and antibacterial activity suggesting diverse genetic makeup and geographical origins of wild Chinese prickly ashes collections.

The wild germplasm resources in high altitude areas are known to be resistant against major biotic and abiotic stresses [33]. Those promising resources belonging to different taxa and ecological niches may be useful accessions to enhance genetic gains of cultivated varieties. Collection of new germplasm in stress-prone areas will augment the sources of new genes for abiotic stress tolerance in developing stress-tolerant varieties [27, 34]. Screening germplasm resources rich in flavonoids with strong resistance can effectively improve the medicinal value of Chinese prickly ash pericarp and provide high-quality resources for the cultivation of Chinese prickly ash varieties.

The flavonoids and phenolic compounds are the important bioactive component of Chinese prickly ash pericarps, and its content determines its medicinal value and quality [35, 36]. From the results, it can be seen that the content of flavonoids of CPP varied greatly with the change of altitude. The TFC of group IV (2300±50 m) was significantly higher than those at other altitudes. Through the comparison of wild samples with the cultivated varieties, it was found that the content of quercetin and catechin of wild samples at the altitude of 2300±50m was close to that of cultivated varieties, and the content of hyperoside was even higher than that of some excellent cultivated varieties, such as ‘Hancheng Shizitou’, ‘Hancheng Dahongpao’and ‘Fengxian Dahongpao’. Furthermore, wild taxa of Chinese prickly ash showed excellent adaptability, stress resistance, antioxidant activity and antibacterial activity. Moreover, according to the results of HCA and PCA, those wild samples were clustered with the cultivated varieties...
S27 (‘Fengxian Dahongpao’), S28 (‘Qin’an Yihao’) and S29 (‘Wudu Dahongpao’). Therefore, they are the carrier of important characters and have potential value in diversification of cultivated gene pool for enhancing genetic gains.

In our study, these wild samples at the altitude of 2300±50m can be used in breeding programs to produce cultivars with high antioxidiant properties. The germplasm resources rich in flavonoids and phenolic compounds are particularly valuable, and in the next step, the flavonoids content traits should be mapped to find QTLs closely linked to the flavone content traits, providing a theoretical basis for fine mapping and cloning in the future, which has far-reaching significance for the quality breeding and improvement of Chinese prickly ash.

5 Conclusions

In the present study, the HPLC fingerprint analysis method for flavonoids and phenolic compounds of Chinese prickly ash was established, and 24 batches of Chinese prickly ash from different altitude in Qinling mountains and six cultivated varieties were analyzed using this method. The wild germplasm resources of Chinese prickly ash from different altitude in the Qinling mountains, with significantly different contents of flavonoids and phenolics, provided plentiful variations. The 30 samples were classified into 3 categories by hierarchical cluster analysis. In addition, according to the results of PCA, the catechin, hyperoside and quercitrin were considered to be the key compounds for the quality evaluation of Chinese prickly ash peels. The chemometric analysis demonstrated that the contents of some functional components of individual samples, such as the samples from group IV (2300±50m), are higher than other wild samples. As a result, superior resources can be selected from these wild resources and used to cultivate new varieties of high quality. Taken together, these wild Chinese prickly ash with rich flavonoids and phenolic compounds provide new germplasm resources for prickly ash breeding, further broaden the number of prickly ash parents, and provide a new way to enrich prickly ash varieties.

Author Contribution Statement

Tao Zheng and Shu-Ming Liu conceived and designed the experiments, Tao Zheng analyzed the data, modified the picture and wrote the paper, all authors have read and approved the manuscript for publication.

Competing interest statement

All authors have declared that they have no conflict of interest.

Funding statement

This article was supported by the project “The demonstration and promotion of efficient cultivation and management techniques of Zanthoxylum bungeanum in Weibei dry plateau” [2017]18.

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