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An effective quantitative fingerprint method for evaluating the quality consistency of *Desmodium styracifolium*

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An HPLC method for combined quantification of five major constituents (vicenin-1, schaftoside, isoschaftoside, vicenin-3 and isovitexin) of *Desmodium styracifolium* was developed to evaluate the quality consistency of this medicinal herb. The chromatographic separation was accomplished on an Agilent 5 TC-C_{18} column (4.6 × 250 mm, 5 µm) with gradient elution using acetonitrile and 0.1 % aqueous formic acid (v/v). The column temperature was 30 °C and the detection wavelength was 272 nm. The chromatographic data were analyzed with a novel systematic quantitative fingerprint method (SQFM). The established quantitative fingerprint method was successfully applied to simultaneously determine the levels of the major constituents, and 39 common peaks were found. The samples collected in Guangdong province had a good quality consistency, in accordance with the traditional opinion that Guangdong province is the best region for cultivation of *D. styracifolium*. The fingerprint–antioxidant efficacy relationship of the different samples was investigated by examining the correlation between the common peaks and the antioxidant effect. Twenty-two common peaks were positively correlated with the antioxidant effect, while the others showed a negative correlation. This quantitative fingerprint method could be considered as a suitable approach to evaluate the quality of traditional Chinese medicines and herbal preparations. The SQFM was reliable and efficient for analysis of the chromatographic data.

1. Introduction

Dried aerial parts of *Desmodium styracifolium* (Osbeck.) Merr., family Leguminosae, are a popular traditional Chinese medicine. It is mainly distributed in southern China, with the Guangxi, Guangdong and Hainan provinces taking the lead (Li et al. 2017). Recent phytochemical and pharmacological studies confirmed that *D. styracifolium* has antilithic and antioxidant effects (Xiang et al. 2015; Cheng et al. 2017), and contains various chemical components such as flavonoids, saponins, organic acids, alkaloids and polysaccharides (Lin and Kong 2006; Phan et al. 2010; Su et al. 2013). The efficacy and bioactive compounds in medicinal plants differ significantly based on their geographical distribution (Salehi et al. 2008; Chen et al. 2010). Accordingly, it is necessary to evaluate the quality consistency of *D. styracifolium* collected from different geographical regions. Traditional Chinese medicines (TCMs) exert their pharmacological effects through their complex chemical compositions, which involve many different active molecules, and this poses an enormous challenge for controlling the quality of TCMs and clarifying their pharmacological mechanisms (Ma et al. 2015). According to the literature, quality evaluation of *D. styracifolium* has mainly been based on a method using HPLC with a UV detector, and the common peaks were limited to about 20. Moreover, these evaluation methods were based on the quantitation of one or two known active ingredients, and they were not reliable because they failed to present information about all the common compounds in herbal medicines (Cao et al. 2010; Zhou et al. 2012; Pu et al. 2014). In this study, a reliable quantitative fingerprint method was established to determine the identity, authenticity and quality consistency of herbal medicines.

As we metabolize food, our bodies constantly produce free radicals, which have both harmful and beneficial effects (Valko et al. 2007). At low/moderate concentrations, free radicals have a beneficial role in helping to kill viruses and bacteria, and in facilitating cellular signaling and the induction of mitogenic responses. However, at high concentrations, free radicals can have harmful effects by causing biological damage. High levels of free radicals are associated with diseases such as inflammation, cancer, aging, neurodegenerative disorders, blood disease and diabetes (Reuter et al. 2010; Cui et al. 2012; Losada-Barreiro and Bravo-Díaz 2017). Nowadays, screening plant extracts for antioxidants has become an area of interest. This led us to study the antioxidant activity of natural plant extracts.

The aim of the present study was to develop an effective quantitative fingerprint method for evaluating the quality consistency of *D. styracifolium* samples. We used linear regression, principal component regression and partial least squares (PLS) to investigate the relationship between chromatograph fingerprints and antioxidant activities. Our goal was to determine the degree of contribution of each common peak and provide more useful information for selecting plants with antioxidants (Zhou et al. 2017; Wang et al. 2017). The systematic quantitative fingerprint method (SQFM), a multivariate technique, was used to analyze fingerprint data from *D. styracifolium* samples from different areas.

2. Investigations, results and discussion

2.1. Optimization of extraction and chromatographic conditions

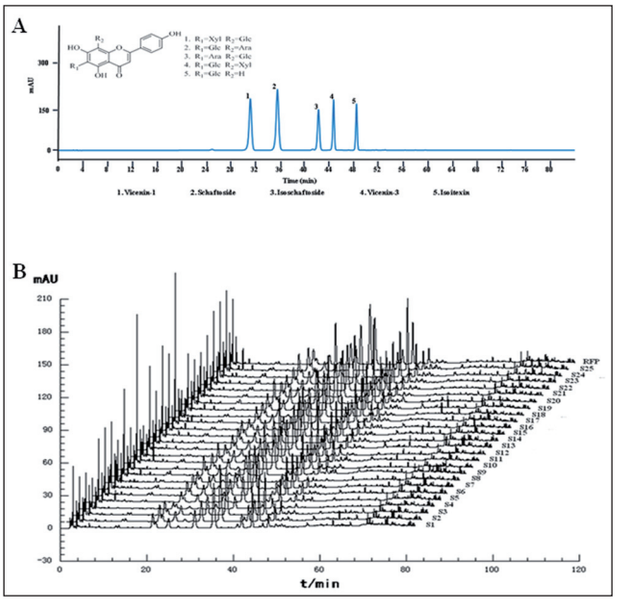
It is well known that the conditions used for extraction and chromatography play a very important role in the HPLC process (Cui et al. 2016). We therefore chose the ultrasonic extraction method (Zhou et al. 2012). In order to obtain better resolution in a shorter analysis time and more information about the components, the methanol concentration (v/v), the extraction time, the solvent volume and the absorption maximums of the major compounds were investigated. The index of the amount of fingerprint information (***I***), calculated by equation (1), was adopted to optimize the chromatographic conditions. ***I*** was the index that represents the signal size, signal homogeneity and the amount of information in the fingerprint (Sun et al. 2006). In Eq. (1), p_i represents the percentage of each peak area and A_i is the peak area. The bigger the ***I*** value, the better the conditions. We found that when we used a methanol concentration of 80 % (14.8), a solvent volume of 20 mL (14.6) and a detection wavelength of 272 nm (14.4), the ***I*** value was higher than under the other conditions. Since the difference between the ***I*** value at 30 min (14.6) and 60 min (14.7) was not significant, we chose the shorter time of 30 min to maximize the convenience of practical operation. Therefore, the optimized extraction solvent was 80% methanol, the ultrasonication time was 30 min, the detection wavelength was 272 nm and the solvent volume was 20 mL. The optimum HPLC conditions are listed in the "4.2. Instruments and chromatographic conditions" section.

$$I = -\sum_{i=1}^{n} p_i \ln p_i \ln A_i \quad (1)$$

2.2. Simultaneous quantitative analysis of the reference standards

2.2.1. Method validation

For each reference standard, seven different concentrations were used to evaluate the linearity of the developed method under the optimal separation conditions. The standard curves were established with the peak areas corresponding to the mixed standard solutions. Table 1 presented the regression equations, linear correlation coefficient (r^2), linear ranges, LOD and LOQ of the reference standards. The correlation coefficient (r^2) of each standard curve was greater than or equal to 0.9999, which meant a good linear calibration within the limits of the concentrations used.



The established method was demonstrated to have good precision for the five analytes, with intra- and inter-day variability RSD values less than 1.89 % and 1.06 %, respectively. According to the results, the RSD of the analytes was less than 2.72 %, which showed that the method had a good repeatability. The recovery values of the analytes were in a range of 96.96–99.10 %, and the RSD values were between 0.23 and 2.12 %, which showed that the developed method was accurate enough.

The stability of the sample solutions was evaluated by measuring changes in the standard composition content of the sample solutions after storage at room temperature for 0, 2, 4, 6, 8, 12 and 24 h. The results showed that the sample was stable within 24 h with the RSD (%) less than 1.21 %.

2.2.2. Sample analysis

The HPLC method was successfully established to simultaneously determine the concentrations of vicenin-1, schaftoside, isoschaftoside, vicenin-3 and isovitexin in 25 *D. styracifolium* samples. As shown in Fig. 1A, the peaks of the five standard compounds were well separated from each other. The measured concentrations (mg/g) of the five standard compounds are summarized in Table 2. The concentrations of the standard compounds in the 25 samples were obviously different from each other, particularly in samples from different regions. The concentrations of the five standard compounds in the samples from Guangdong province, Guigang city in Guangxi Zhuang Autonomous Region and Hainan province were higher than in the samples from the other regions. According to the survey, the samples which had higher concentrations of the five standard compounds were from regions at a lower elevation and closer to the ocean. The differences in content might be due to differences in the plant growth environment such as climate, altitude and terrain.

Table 1: Calibration data for five compounds in *Desmodium styracifolium*

Reference standards	Regression equation (y = ax + b)	Linear range (μg/mL)	r^2	LOD(μg/mL)	LOQ(μg/mL)
Vicenin-1	Y=16660X-0.6598	1.09-109.09	1.0000	0.19	0.55
Schaftoside	Y=16975X+0.2861	3.00-300.00	1.0000	0.27	0.75
Isoschaftoside	Y=11772X-2.5185	0.96-96.30	0.9999	0.18	0.48
Vicenin-3	Y=15363X+0.2571	1.75-175.00	1.0000	0.15	0.44
Isoitexin	Y=26473X-0.5125	0.36-35.71	1.0000	0.13	0.36

2.3. Evaluation of HPLC fingerprint profiles

A total of 25 *D. styracifolium* samples from different regions in Southern China were tested and their fingerprints were recorded using the optimum conditions. According to the fingerprints, 39 common peaks were detected, compared with the two dozen

Table 2: Contents (mg/g) of five reference standards and IC_{50} values (mg/mL) of *Desmodium styracifolium* collected from different regions

Sample ID	Vicenin-1	Schaftoside	Isoschaftoside	Vicenin-3	Isovitexin	IC_{50}
S1	1.175	2.987	0.975	1.590	0.276	0.3240
S2	1.326	3.518	1.108	1.879	0.357	0.3719
S3	1.236	3.074	1.053	1.788	0.276	0.4131
S4	0.298	0.724	0.278	0.406	0.019	0.4308
S5	0.757	1.689	0.523	0.840	0.163	0.2954
S6	1.155	2.914	0.942	1.565	0.279	0.3555
S7	1.092	2.902	0.976	1.419	0.221	0.3374
S8	0.825	1.772	0.942	1.037	0.071	0.2704
S9	1.470	3.370	1.015	1.785	0.424	0.2720
S10	1.479	3.449	1.042	2.028	0.368	0.4047
S11	1.688	4.225	1.188	2.476	0.480	0.3614
S12	0.889	2.328	0.771	1.265	0.224	0.4554
S13	1.119	2.872	0.865	1.496	0.273	0.3326
S14	1.249	3.075	1.016	1.745	0.275	0.2604
S15	1.307	3.276	1.014	1.715	0.331	0.2782
S16	0.885	2.221	0.720	1.117	0.228	0.2740
S17	1.195	3.429	1.033	2.009	0.419	0.4003
S18	1.123	2.999	1.084	1.632	0.274	0.4184
S19	1.425	3.533	1.123	2.133	0.435	0.3912
S20	2.313	5.425	1.694	3.334	0.571	0.3779
S21	1.164	3.039	1.027	1.603	0.282	0.3913
S22	1.183	3.003	0.989	1.601	0.268	0.3610
S23	1.296	3.199	1.032	1.671	0.291	0.3126
S24	1.283	3.682	1.034	1.879	0.688	0.3087
S25	1.831	4.218	1.188	2.839	0.387	0.3484

common peaks previously reported (Zhou et al. 2012). This is helpful for the authentication of *D. styracifolium* and for further research on this plant. The S4 and S20 samples were regarded as anomalies according to subsequent experiments. Therefore, the RFP was constructed by taking the average of the 25 sample chromatograms except for the two anomalous samples (S4 and S20). The results are shown in Fig. 1B. The fingerprints and the RFP were submitted to TCM Fingerprint software to calculate the values of S_m, P_m and α in order to evaluate the quality consistency of the samples. The values of the similarity parameters (S_m, P_m, α) and the quality grades of the 25 samples are summarized in Table 3. According to analysis results, S1, S3, S6, S14, S15, S18 and S21-S23 were assigned the best quality of grade 1, while S5 fell into the defective quality category of grade 7. A low value of P_m (<70%) was noticed for S5 and S8. Conversely, S20 had the lowest quality due to its extremely high P_m value (>150%). S4 was different from the other samples because it had the lowest P_m value of 27.4 and the highest α value of 0.27. In addition, the S_m values of all the samples were above 0.978, which means that the chemical components were very similar. However, the P_m values were in a range from 27.4 to 172.4 % with a high RSD of 27.47 %, which suggested that the overall concentrations of the compounds were significantly different between samples. As shown in Table 4, the five samples collected in Guangdong province (S17, S18, S19, S21, S22) were assigned to similar quality grades of 1-4, which indicated that the *D. styracifolium* from Guangdong province had a good quality consistency, while the samples from Guangxi and Hainan provinces were assigned to a variety of different quality grades. Usually, samples in the grade of 1-5 are considered as eligible ones. A total of six samples failed to qualify because their contents were too low or too high. As we know, patients may be immune to a dose that is too low, while a dose that is too high may have harmful effects. These results are in accordance with the traditional opinion that Guangdong province is the best region for cultivation of *D. styracifolium*.

2.4. Antioxidant activity detection by PLS

D. styracifolium has good antioxidant activity, but the antioxidant activities of each of the common components are not fully understood. In this study, the antioxidant activities of all the chemical compounds corresponding to the common peaks of *D. styracifolium* were analyzed for the first time. To identify the antioxidant compounds in the medicinal herb, a PLS model was established to analyze the relationship between the fingerprint data of 25 samples and their respective IC_{50} values (see Table 2) of antioxidant activity. The data were mean centered, a t[1]–t[2] score was constructed, while two samples (S4 and S20) were identified as outliers. These two abnormal samples were omitted,

Table 4: Results of evaluation of 25 *Desmodium styracifolium* samples

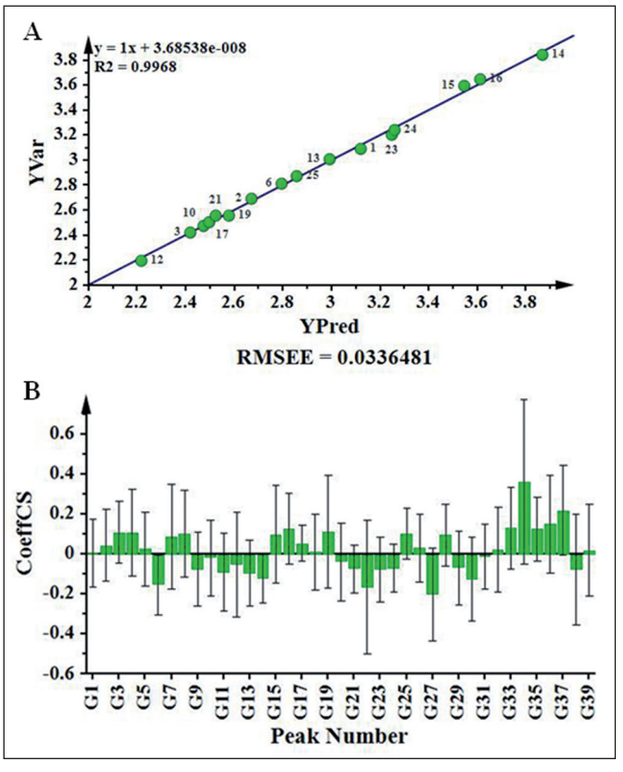
Para.	S1	S2	S3	S5	S6	S7	S8	S9	S10
S_m	0.991	0.987	0.982	0.96	0.991	0.972	0.956	0.971	0.979
P_m %	96.6	111.5	99.9	59.8	95.8	92.5	62.2	110.4	110.7
α	0.009	0.017	0.011	0.146	0.025	0.006	0.062	0.008	0.047
Grade	1	3	1	7	1	2	6	3	3
Quality	Best	Good	Best	Defective	Best	Better	Common	Good	Good

Para.	S11	S12	S13	S14	S15	S16	S17	S18	S19
S_m	0.98	0.987	0.983	0.989	0.989	0.982	0.978	0.982	0.979
P_m %	134.4	75.2	94.1	99.7	104.5	73.4	110.6	95.8	115.8
α	0.041	0.009	0.045	0.012	0.012	0.048	0.001	0.011	0.006
Grade	6	5	2	1	1	5	3	1	4
Quality	Common	Moderate	Better	Best	Best	Moderate	Good	Best	Fine

Para.	S21	S22	S23	S24	S25	RFP	S4	S20
S_m	0.978	0.991	0.985	0.982	0.977	1	0.917	0.966
P_m %	97	96.8	103.2	117.4	139.3	100	27.4	172.4
α	0.012	0.003	0.003	0.008	0.027	0	0.27	0.077
Grade	1	1	1	4	6	1	8	8
Quality	Best	Best	Best	Fine	Common	Best	Inferior	Inferior

and the normal samples were divided randomly into two groups of calibration and prediction sets to structure the PLS model. A calibration model with three latent variables was selected in terms of cross-validation criteria, achieving an explained variance (R^2) of 0.9968 for Y variables (antioxidant activity), a predictive ability (Q^2) of 0.61, and a root mean square error of estimation value of 0.0336. As shown in Fig. 2A, the predicted values were in keeping with the experimental values, indicating that the present model was appropriate. According to the plot of standardized regression coefficients (Fig. 2B), it was evident that 22 common peaks are positively correlated with the antioxidant activity, while the other common peaks are negatively correlated.

A comparison of experimental and predicted values of antioxidant activity for both the calibration and prediction models showed that the predicted values agreed well with the experimental data, which demonstrated that the developed model was valid.



2.5. Conclusions

In this study, a method of HPLC fingerprinting coupled with quantitative determination of multiple chemical compounds was successfully established to evaluate the quality consistency of 25 samples of the herb *D. styracifolium* from different regions in China. The analysis of the fingerprints also indicated that quantitative analysis of reference standards might be necessary for assessment of this TCM. Furthermore, a PLS model was constructed to analyze the fingerprint–efficacy relationship and to identify potential antioxidants in the herb samples using their fingerprint profiles. The established method could be used to quickly and systematically evaluate the quality consistency of *D. styracifolium*. It lays the foundation for evaluating the authenticity of medicinal materials in a multi-dimensional evaluation system which combines active constituents, biological activity and environment. This method will also be helpful for the quality control of other medicinal materials and herbal preparations.

4. Experimental

4.1. Materials and reagents

The 25 samples were collected from different regions in Guangxi, Guangdong, and Hainan provinces in China and identified as *Desmodium styracifolium* (Osb.) Merr. by Professor Li-ming Pan (School of Chinese Materia Medica, Guangdong Pharmaceutical University). All samples were labeled and stored at the Guangdong Pharmaceutical University Herbarium. All other chemical reagents used were of analytical grade or better.

Reference standards: Vicenin-1 was purchased from the Renjie Biotech Co., Ltd (Shanghai, China). Schaftoside and isoschaftoside were purchased from the Feiyu Biotech Co., Ltd (Nantong, China). Vicenin-3 and isovitexin were purchased from the Pufei De Biotech Co., Ltd (Chengdu, China). The purities of vicenin-1, schaftoside, isoschaftoside and isovitexin were greater than or equal to 98%. The purity of vicenin-3 was greater than or equal to 96%.

4.2. Instruments and chromatographic conditions

HPLC analysis was performed on an Agilent Infinity 1260 II HPLC. The chromatographic separation was carried out on an Agilent 5 TC-C_{18} column (4.6 × 250 mm, 5 μm) with the column temperature kept at 30 °C. The detection wavelength was 272 nm, the flow rate was 1.0 mL/min and the injection volume was 10 μL. The mobile phase was composed of acetonitrile (A) and 0.1% aqueous formic acid (v/v) (B). The separation was achieved using a gradient elution as follows: 12 % A at 0-15 min, 12 % A-14 % A at 15-35 min, 14 % A-18 % A at 35-45 min, 18 % A-20 % A at 45-50 min, 20 % A-35 % A at 50-65 min, 35-100 % A at 65-80 min, 100 % A at 80-82 min.

4.3. Preparation of the standard solution

Each reference standard was dissolved in 80% methanol, and diluted to a concentration of 1 mg/mL. The individual standard stock solutions were mixed together to make a mixed standard solution, which was further diluted with 80 % methanol to prepare a series of concentrations for the standard curve. All the standard solutions were stored at 4 °C.

4.4. Preparation of the sample solutions

About 0.5 g of each sample was weighed accurately, and then extracted with 20 mL of 80 % methanol in an ultrasonic water bath for 30 min. After the ultrasonic extraction, the sample was weighed again and the lost liquid was replenished with 80 % methanol. The sample solution was filtered through a 0.45 μm membrane filter prior to injection into the HPLC system.

4.5. Validation of the HPLC method

To validate the method, data were collected on linearity, limit of detection (LOD) and limit of quantification (LOQ), precision (inter- and intra-day precision), repeatability, accuracy and stability.

4.5.1. Calibration, LOD and LOQ

Stock solutions of the reference standards were further diluted with 80 % methanol to appropriate concentrations ranges for establishing calibration curves. Seven different concentrations were injected in triplicate. The peak areas corresponding to each concentration were used to generate the standard curve, and the standard curve was then used to calculate the concentrations of the standard compounds in the samples. The correlation coefficient (r^2) of each standard curve was greater than or equal to 0.9999, which meant a good linear calibration within the appropriate concentration range. The limits of detection (LOD) and quantification (LOQ) were measured at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

4.5.2. Precision

Inter-and intra-day precision was evaluated using the relative standard deviation (RSD). Standard solutions (low, medium and high concentration) were analyzed to evaluate the precision of the instrument. To calculate the RSD for inter-day precision, the standard solutions were measured in triplicate for three successive days. To calculate the RSD for intra-day precision, six replicates of the standard solutions were measured in one day.

4.5.3. Repeatability

The repeatability was evaluated with six individual sample solutions and the calculated results showed that the RSD was less than 3 %.

4.5.4. Accuracy

The standard addition method was used to perform the recovery test to examine accuracy. The specific operations are as follows: three concentration levels (80, 100 and 120 %) of the reference standards were added into sample solutions (the amount of analytes was known), and then the mixed samples were determined in triplicate for each concentration level.

4.5.5. Stability

To evaluate the stability of the sample solutions, the samples were left at room temperature for 0, 2, 4, 8, 12 and 24 h, and were then analyzed.

4.6. The theory of SQFM

The reference fingerprint (RFP) and the sample fingerprint (SFP) are defined as a RFP vector $\vec{y}=(y_1,y_2,...,y_n)$ and a SFP vector $\vec{x}=(x_1,x_2,...,x_n)$, where x_i and y_i represent the peak areas in the SFP vector and RFP vector, respectively. The linear regression ($\vec{X}=a+b\vec{Y}$) between the SFP vector and RFP vector is analyzed and a basic qualitative similarity parameter ($\boldsymbol{S}_F$) that equates with the linear correlation coefficient is obtained, as shown in Eq. (2) below. The parameter $\boldsymbol{S}_F$ accurately describes the distribution proportion similarity between the experimental samples and the reference samples, but it is significantly affected by large peaks. To exclude this effect, the fingerprint vectors are transformed inversely as $\vec{Y}'=\left(\frac{1}{y_1},\frac{1}{y_2},...,\frac{1}{y_n}\right)$ and $\vec{X}'=\left(\frac{1}{x_1},\frac{1}{x_2},...,\frac{1}{x_n}\right)$, and the reverse correlation coefficient between the two reversed vectors is calculated to get the qualitative ratio similarity ($\boldsymbol{S}'_F$), as shown in Eq. (3). The average value of $\boldsymbol{S}_F$ and $\boldsymbol{S}'_F$ is used as the linear qualitative similarity parameter (S_m), as shown in Eq. (4), which is applied to describe both the number and distribution of chemical components between the SFP and RFP. The slope of the linear regression line ($\vec{X}=a+b\vec{Y}$) is defined as a quantitative parameter named $\boldsymbol{C}$, as shown in Eq. (5), which reflects the similarity of total chemical component contents between the RFP and SFP under the obvious influence of large peaks. Therefore, another quantitative parameter named $\boldsymbol{P}$ is defined as shown in Eq. (6). To integrally reflect the similarity of overall chemical component contents in the SFP and RFP, a macro quantitative ratio similarity parameter ($\boldsymbol{P}_m$) is defined by calculating the two quantitative parameters $\boldsymbol{C}$ and $\boldsymbol{P}$ based on Eq. (7). Note that the parameter named $\boldsymbol{P}_m$ should be revised using a correction factor (f_i) equating to the ratio of the weight of RFP (m_{RFP}) and the weight of the sample (m_i). To monitor the difference in fingerprint uniformity between the SFP and RFP, the fingerprint uniformity variation coefficient, $\boldsymbol{\alpha}$, is calculated according to Eq. (8). To evaluate the quality grades of TCM and herbal preparations, a method combining the above parameters ($\boldsymbol{S}_m$, $\boldsymbol{P}_m$ and $\boldsymbol{\alpha}$) was developed and termed as SQFM. The eight quality grades of TCM and herbal preparations are ranked depending on the values of $\boldsymbol{S}_m$, $\boldsymbol{P}_m$ and $\boldsymbol{\alpha}$. The classification criteria for each grade can be seen in Table 5. Lower grade numbers mean that the TCM and herbal preparations are of higher quality (Li et al. 2015; Yang and Sun 2017).

Table 5: Quality grades identified by the LQFM

Grade	1	2	3	4	5	6	7	8
$S_m \geq$	0.95	0.9	0.85	0.8	0.7	0.6	0.5	<0.50
P_m /%	95~105	90~110	80~120	75~125	70~130	60~140	50~150	0~∞
$\alpha \leq$	0.05	0.1	0.15	0.2	0.3	0.4	0.5	>0.50
Quality	Best	Better	Good	Fine	Moderate	Common	Defective	Inferior

$$\boldsymbol{S}_F=\cos\theta=\frac{\sum_{i=1}^{n}x_iy_i}{\sqrt{\sum_{i=1}^{n}x_i^2}\sqrt{\sum_{i=1}^{n}y_i^2}} \quad (2)$$

$$\boldsymbol{S}'_F=\cos\theta'=\frac{\sum_{i=1}^{n}\frac{x_i}{y_i}}{\sqrt{n\sum_{i=1}^{n}\left(\frac{x_i}{y_i}\right)^2}} \quad (3)$$

$$\boldsymbol{S}_m=\frac{1}{2}\left(\boldsymbol{S}_F+\boldsymbol{S}'_F\right)=\frac{1}{2}\left(\frac{\sum_{i=1}^{n}x_iy_i}{\sqrt{\sum_{i=1}^{n}x_i^2}\sqrt{\sum_{i=1}^{n}y_i^2}}+\frac{\sum_{i=1}^{n}\frac{x_i}{y_i}}{\sqrt{n\sum_{i=1}^{n}\left(\frac{x_i}{y_i}\right)^2}}\right) \quad (4)$$

$$\boldsymbol{C}=\frac{\sum_{i=1}^{n}x_iy_i}{\sum_{i=1}^{n}y_i^2}\times 100\% \quad (5)$$

$$\boldsymbol{P}=\frac{\sum_{i=1}^{n}x_i}{\sum_{i=1}^{n}y_i}\boldsymbol{S}_F\times 100\% \quad (6)$$

$$P_m = \frac{1}{2}(C + P)f_i = \frac{1}{2}\left(\frac{\sum_{i=1}^{n} x_i y_i}{\sum_{i=1}^{n} y_i^2} + \frac{\sum_{i=1}^{n} x_i}{\sum_{i=1}^{n} y_i} S_F\right)\frac{m_{RFP}}{m_i} \times 100\% \quad (7)$$

$$\alpha = \left|1 - \frac{P}{C}\right| \quad (8)$$

4.7. Data analysis

An internally developed software called Digitized Evaluation System for Super-Information Characteristics of TCM Chromatographic Fingerprints 4.0 (Software certificate no. 0407573, China) was used to analyze fingerprint similarity and evaluate the quality consistency. SIMCA–P+ 13.0 software (Umetrics, Umea, Sweden) was used for PLS analysis.

4.8. Antioxidant activity

A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.04 mg/mL) was prepared with anhydrous ethanol (v/v). DPPH solution (150 µL) and 25 µL of sample solution were added into 96-well plates and incubated at room temperature away from light, and the absorbance was measured at 517 nm after 30 min. Each sample solution was analyzed in triplicate (in three DPPH-treated wells) along with one blank well that contained 25 µL of sample solution and 150 µL of anhydrous ethanol. The blank control group was set up by adding 25 µL of 80% methanol and 150 µL of DPPH solution into 3 wells of each 96-well plate.

Control solution: the vitamin C solution (1 mg/mL) was diluted to the concentration of 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 50 µg/ml with 80% methanol (v/v).

The free radical scavenging activity was calculated by the following equation:

DPPH scavenged (%) $=[A_0-(A_1-A_2)]/A_0\times100\%$

where A_0 = absorbance of the control group, A_1= absorbance of the sample solution and A_2 = absorbance of the sample blank well.

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Conflicts of Interest: The authors declare no conflict of interest.

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