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Full Length Research Paper

Comparative analysis of major constituents in *Viola yedoensis* Makino and different species from the Genus Viola by high-performance liquid chromatography with chemometrics methods

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The whole plant of *Viola yedoensis* Makino is used for the treatment of rhinitis, rheumatism, mastitis and acute pyogenic infections. A simple and accurate HPLC-UV method was developed for the simultaneous determination of six major constituents in the whole plant of *V. yedoensis*, and the comparison between its constituent profile and eight other species in the genus Viola was investigated. The optimal chromatographic conditions were achieved on a Hedera ODS-2 (250 mm×4.6 mm, i.d. 5 µm) column with gradient elution using 0.4% acetic acid aqueous and methanol at 354 nm. Calibration curves presented good linear regression (r > 0.9996) within test ranges. The overall recoveries ranged between 96.72 and 97.67% and the RSDs were less than 2.34%. The results indicated that esculetin was the major components in *V. yedoensis*. Principal component analysis and cluster analysis could evaluate the quality and distinguish *V. yedoensis* from eight other species in the genus *Viola*. The HPLC technique combined with chemometrics was a sensitive, selective, rapid and guiding method and could be applied to the further quality evaluation of complex materials.

Keywords: *Viola yedoensis*, coumarin, simultaneous determination, Viola, chemometrics method.

INTRODUCTION

Zi Hua Di Ding (*Viola yedoensis* Makino), is a popular medicinal plant indigenous to China, Japan and Korea (Ngan et al., 1988; Wang et al., 2008). In addition to its ornamental contribution of the flowers, *V. yedoensis* has been recorded in Chinese Pharmacopoeia for treating many health ailments, including pains, acute pyogenic infections, and inflammation such as rhinitis and mastitis (Pharmacopoeia Commission of People's Republic of China, 2005).

Phytochemical reports and our previous studies on *V. yedoensis* revealed that it contained coumarins, flavones, phenolic acids, fatty acid, etc (Xie et al., 2003; Zhou et al., 2009a). Among these, the first two types of compounds are major constituents, and modern pharmacological or clinical studies have shown that they possess anti-inflammatory, anti-infection, anti-microbial, anti-fungus, anti-tumor and anti-HIV activities (Chang and Yeung, 1988; Xie et al., 2004). Therefore, quantification of those constituents in *V. yedoensis* would be of great importance for the quality evaluation of the herb and the commercial crude drugs.

Until now, studies on constituents determination of *V. yedoensis* are few. Previous reports have determined total flavonoids or chlorogenic acid by ultraviolet spectrophotometry (Dong et al., 2004; Huang et al., 2009), or only the fingerprint chromatograms by HPLC (Chen et

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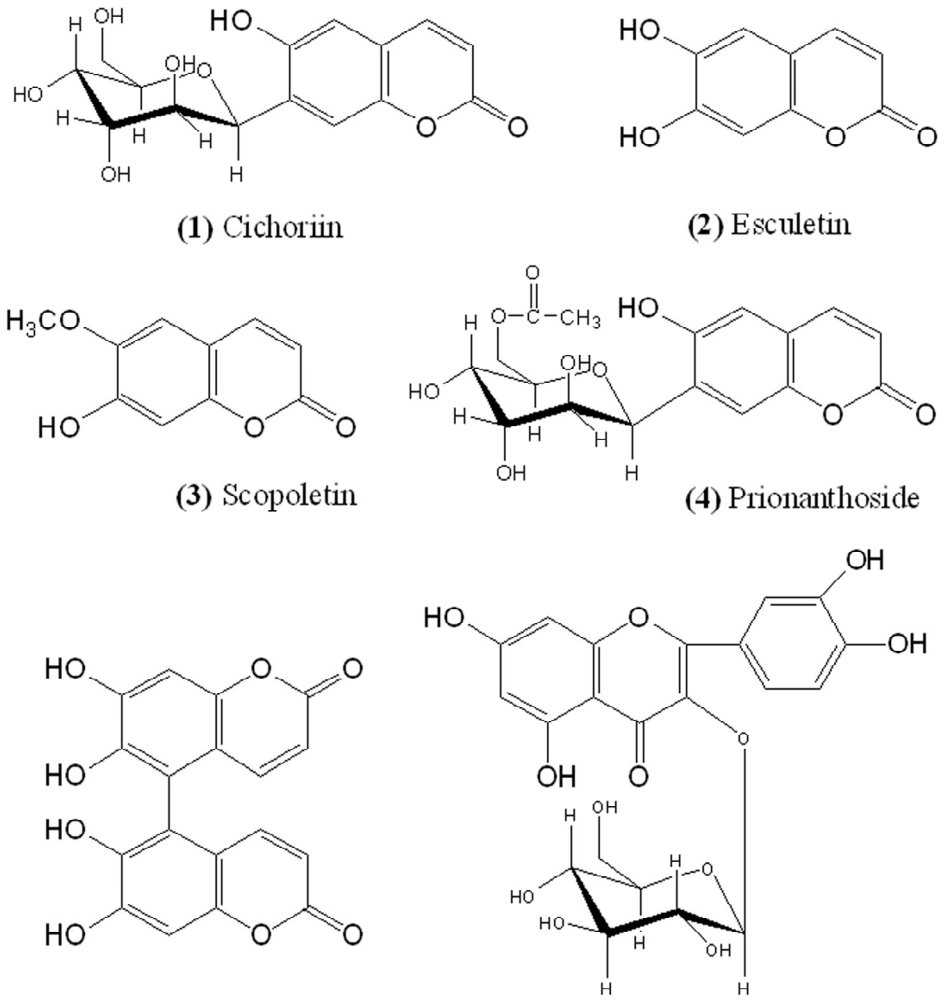

Figure 1. Structures of major constutients in *Viola yedoensis* Makino. Standards compounds: Cichoriin (1), Esculetin (2), Scopoletin (3), Prionanthoside (4), Euphorbetin (5) and Quercetin-3-*O*-*β*-*D*-glucoside (6).

al., 2010). However, the simple quantification of total flavonoids or only one component in the herb could not represent its integral quality. Consequently, simultaneous quantitative analysis of the major active components is the most direct and important method for quality control of *V. yedoensis*.

Besides, due to the similarity in plant morphology of *V. yedoensis* with other species in the genus Viola, such as *V. prionantha*, *V. philippica* or *V. concordifolia*, the substitutions or misapplications of them are common in folk medicines in China (Xu, 1997; Li, 2008). Therefore, it is quite necessary to establish a method to determinate the constituents of *V. yedoensis* and its substitutes, in order to evaluate the quality of commercial crude drugs and investigate the reasonableness of substitutions.

In present study, we first developed and validated a simple and accurate HPLC method for simultaneous determination of six major components in *V. yedoensis*, including five coumarins (cichoriin, esculetin, scopoletin, prionanthoside and euphorbetin) and a flavonoid (quercetin-3-*O*-*β*-*D*-glucoside). The crude herb and commercial products were compared by their constituents profile for the first time. Furthermore, the chemometrics methods (principal component analysis and cluster analysis) were performed to develop a visual plot for evaluation of the resemblance and difference of *V. yedoensis* and other species in the genus Viola.

EXPERIMENTAL

Chemicals, reagents and materials

Six reference standards, including cichoriin (1), esculetin (2), scopoletin (3), prionanthoside (4), euphorbetin (5) and quercetin-3-*O*-*β*-*D*-glucoside (6) were isolated from the whole plant of *V. yedoensis* in our laboratory. The identifications were confirmed by ^{1}H-NMR, ^{13}C-NMR and MS spectral analysis (Zhou et al., 2009b). The chemical structures of standards were listed in Figure 1. The

Table 1. Description of the studied samples.

No.	Species	Collected location	Source	Collection time
S1	*V. yedoensis* Makino	Henan, China	Commercial products	Jun, 2007
S2	*V. yedoensis* Makino	Anhui, China	Commercial products	Aug, 2008
S3	*V. yedoensis* Makino	Hebei, China	Commercial products	Aug, 2008
S4	*V. yedoensis* Makino	Yunnan, China	Commercial products	Aug, 2008
S5	*V. yedoensis* Makino	Heilongjiang, China	Commercial products	Aug, 2008
S6	*V. yedoensis* Makino	Ningxia, China	Commercial products	Aug, 2006
S7	*V. yedoensis* Makino	Jiangsu, China	Commercial products	Aug, 2008
S8	*V. yedoensis* Makino	Shandong, China	Commercial products	Aug, 2008
S9	*V. yedoensis* Makino	Shanxi, China	Commercial products	Jun, 2006
S10	*V. yedoensis* Makino	Hebei, China	Original herb by field acquisition	Apr, 2008
S11	*V. prionantha* Bunge	Jiangsu, China	Original herb by field acquisition	Mar, 2008
S12	*V. concordifolia* C.J. Wang	Jiangsu, China	Original herb by field acquisition	Mar, 2007
S13	*V. inconspicua* Blume	Jiangsu, China	Original herb by field acquisition	Mar, 2008
S14	*V. variegate* Fisch. ex Link	Hebei, China	Original herb by field acquisition	Apr, 2008
S15	*V. patrinii* DC. ex Ging	Jiangsu, China	Original herb by field acquisition	Mar, 2008
S16	*V. betonicifolia* J.E. Smith	Jiangsu, China	Original herb by field acquisition	Mar, 2007
S17	*V. collina* Bess	Jiangsu, China	Original herb by field acquisition	Mar, 2007
S18	*V. grypoceras* A. Gray	Jiangsu, China	Original herb by field acquisition	Mar, 2007

purity of each compound was determined to be higher than 98% by normalization of the peak area detected by HPLC. Methanol was of HPLC grade (Merck, Darmstadt, Germany). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid and other reagents were of analytical grade from Hanbang Chemicals Co. Ltd. (Nanjing, China). All the standard substances and samples were filtered through a 0.45 µm Millipore membrane before injection.

The nine commercial crude herb samples (S1 to S9) were purchased from the local markets, while the nine crude herb samples (S10 to S18) from the genus Viola were collected from different geographic regions in China. The voucher specimens (no. 0703 to 0720) were authenticated by Prof. Min-Jian Qin and deposited in the Resources Science of Traditional Chinese Medicines, China Pharmaceutical University (Nanjing, China). The information of each sample is listed in Table 1.

Chromatographic conditions

Separation was performed on an Agilent Series 1100 series (Agilent Technologies, Palo, Alto, CA, USA) LC system equipped with a binary pump, micro degasser and a model 7725i injection valve (sample loop 20 µL). Chromatographic separation was carried out at 35°C on a Hedera ODS-2 (250 mm×4.6 mm, i.d.5 µm) column with Agilent zorbax high pressure reliance cartridge guard-column. The mobile phase consisted of 0.4% aqueous acetic acid (A) and methanol (B) using a gradient elution of 5 to 21% (v/v) B at 0 to 35 min, 21 to 35% B at 35 to 55 min and 35 to 70% B at 55 to 80 min. The flow rate was kept at 1.0 ml/min at 354 nm and the injection volume was 20 µL.

Preparation of solutions

Standard solutions

The concentrations of stock solutions were as follows: cichoriin, 0.73 mg/mL; esculetin, 1.6 mg/mL; scopoletin, 0.33 mg/mL; prionanthoside, 0.32 mg/mL; euphorbetin, 0.14 mg/mL and quercetin-3-*O*-*β*-*D*-glucoside, 0.56 mg/mL. The standard solutions were serially diluted with 80% (v/v) aqueous methanol to obtain working standard solutions at various concentration levels.

Sample preparation

The dry plant samples were ground to a fine powder using a pulveriser, and 1.0 g of powder was placed in a 50 ml conical flask. All crude was added and extracted with 25.0 ml of methanol at 80°C by refluxing for 1 h. The extracted solution was adjusted to the original weight by adding methanol, and then the supernatant was filtered through a syringe filter (0.45 µm) before HPLC injection.

Validation procedure

Calibration curves, limits of detection and quantification

The mixed standard stock solution was diluted to a series of appropriate concentrations for the construction of calibration curves. Six concentrations of the mixed standard solution were injected in triplicate. All calibration curves were constructed from peak areas of the reference compounds vs. their concentrations.

Limit of detection (LOD) and limit of quantification (LOQ) were determined by measuring the signal-to-noise ratio (S/N ratio) for each compound. The dilute solution was further diluted to a series of concentrations with 80% methanol, and then the LOD and LOQ were determined at the S/N ratio of 3 for LOD and S/N ratio of 10 for LOQ, respectively.

Precision, repeatability and stability

The instrument intra- and inter-precision was evaluated by analyzing the mixture standard solution in six replicate injections under the

optimal conditions. The intra-day precision was determined six replications within one day, while the inter-day precision was performed over three consecutive days. The RSD was taken as a measure of precision.

Stability was investigated with an extract solution of sample 1 (S1) and analysed every 12 h with 3 days. All solutions were stored at 4℃ before analysis.

Recovery

A recovery test was carried out to further evaluate the accuracy of the method. Three concentration levels (high, middle and low) of the standard solution were added to known amounts of *V. yedoensis* samples (S1). Triplicate samples at each level were extracted and analyzed as described by the foregoing methods. The average recoveries were calibrated by the formula:

Recovery (%) = (amount found - original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%.

Statistical analysis

Data transformation was performed using MS EXCEL. PCA and cluster analysis were carried out using SPSS software version 11.5 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Optimization of extraction and chromatographic condition

Four related extraction conditions were designed and evaluated, including extraction method (ultrasonication, reflux), methanol concentration (50, 70 and 100%, v/v), solvent volume (10, 25 and 30 ml) and extraction time (30 min, 1 and 2 h). By comparing the ultrasonicating with refluxing extraction, it was shown that coumarins cannot be extracted efficiently by ultrasonication, thus we employed refluxing extraction in our study. According to the summed numbers and areas of characteristic peaks in each chromatogram of different factors, the optimal condition for extraction was selected as 1.0 g powder of each dried sample extracted with 25.0 ml of 100% methanol by refluxing for 1 h.

To obtain optimum chromatographic conditions with a good separation within a short analysis time, the mobile phase, the gradient of mobile phase, flow rate and column temperature were optimized. Comparing with several mobile phases, including methanol – water, methanol – 0.4% aqueous formic acid, ACN – water, CAN – 0.4% aqueous formic acid and ACN – 0.4% aqueous acetic acid, the methanol – 0.4% aqueous acetic acid system was selected as the most appropriate mobile phase for the suitable resolution and good peak shapes of chromatogram. The temperature was investigated in the range between 25 and 45℃, and 35℃ was found to be optimal. The optimal flow rate was 1.0 ml/min.

In order to obtain the maximum UV absorption wavelengths of analytes, UV spectra of the mixed standard solution were recorded from 190 to 400 nm and multiple chromatograms at different wavelengths were investigated. The wavelength 354 nm was selected for detection, at which both the total peak area and the numbers of detectable peaks reach a maximum in the chromatograms. The typical chromatograms of the mixed standard compounds and the extract of *V. yedoensis* obtained were shown in Figure 2.

Method validation

Linearity, LOD and LOQ

Linearity was evaluated using standard samples over six calibration points. Excellent linearity was observed for the analytes over the ranges of 1.670 to 1600 μg/mL, with correlation coefficients from 0.9996 to 0.9999. The LODs and LOQs for the compounds ranged from 0.3 to 2.2 ng and 1.0 to 5.6 ng, respectively. The results were collected in Table 2.

Precision, repeatability and stability

The precision of the chromatographic method was tested by performing intra - and inter - day multiple injections of the mixed standard solution. The percentage relative standard deviation (%RSD) of the peak area was calculated, and the values of intra- and inter-day for the analytes were less than 3.26 % (Table 3). The RSD values of six analytes for repeatability were less than 2.48%. The same real sample (S1) was analyzed over a period of 3 day for the stability test, and the solution was found to be stable with the RSD values less than 2.82%.

Recovery

Recovery of the standard substance from samples is generally used to evaluate the accuracy of this analytical method. The recovery of the investigated components ranged from 96.72 to 97.67%, and their RSD values were all less than 2.34% (Table 4). The results of recovery tests testified the reliability and accuracy of the measurement of these components by the developed method.

The results of linearity, LOD, LOQ, precision, repeatability, stability and recovery showed that, this analytical method for coumarins and flavonoid from *V. yedoensis* is precise, accurate and sensitive enough for simultaneous quantitative evaluation.

Quantitative determination of coumarins and flavonoid in *V. yedoensis* and different species of the *Viola spp.*

The established HPLC-UV method was successfully

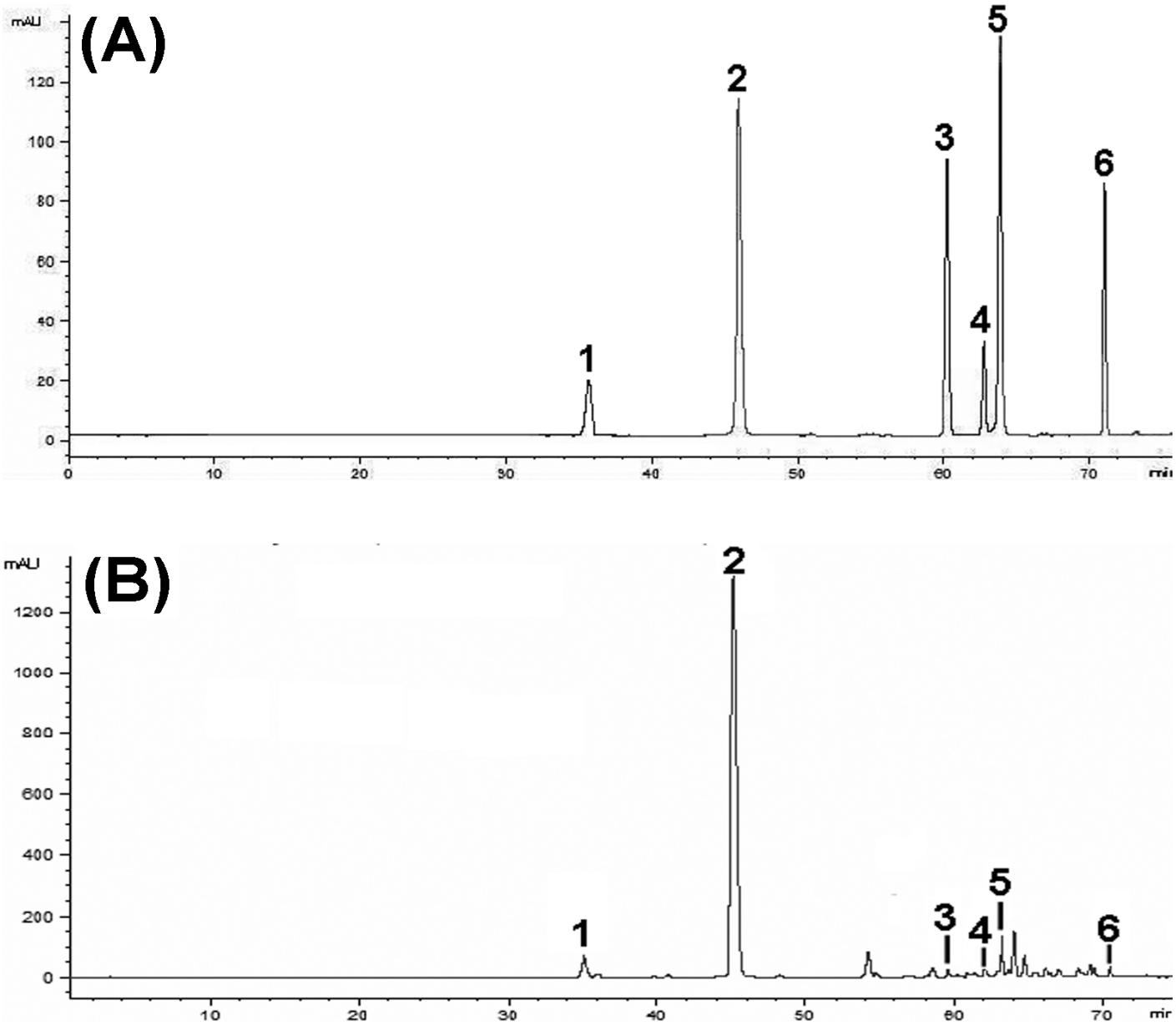

Figure 2. HPLC chromatograms recorded at 354 nm of the mixed standard compounds (A) The extract of *Viola yedoensis* (B). The standards: Cichoriin (1), Esculetin (2), Scopoletin (3), Prionanthoside (4), Euphorbetin (5) and Quercetin-3-*O*-*β*-*D*-glucoside (6).

Table 2. Regression data, LODs and LOQs for six major compounds obtained with the optimized HPLC method.

Analyte	Regression equation[a]	r	Linear range(μg/ml)	LOD[b](ng/ml)	LOQ[c] (ng/ml)
Cichoriin (1)	y=18292x+99.163	0.9998	3.650-730.0	1.8	2.9
Esculetin (2)	y=56256x-481.9	0.9998	8.000-1600	1.6	4.8
Scopoletin (3)	y=62102x+40.236	0.9999	1.700-326.0	0.3	1.0
Prionanthoside (4)	y=18920x-21.516	0.9997	1.670-320.0	1.0	1.6
Euphorbetin (5)	y=52332x-166.92	0.9998	2.800-140.0	1.4	2.1
Quercetin-3-*O*-*β*-*D*-glucoside (6)	y=29885x-79.260	0.9996	5.650-565.0	2.2	5.6

[a] y = peak area; x = concentration of compound (μg/mL). [b] LOD, limit of detection (S/N=3). [c] LOQ, limit of quantifi cation (S/N=10).

Table 3. Precision, repeatability and stability of the six major constituents assayed.

Analyte	Precision		Repeatability	Stability
	Intra-day (n = 6) RSD (%)	Inter-day (n = 3) RSD (%)	(n = 5) RSD (%)	(n = 5) RSD (%)
Cichoriin (1)	2.53	2.34	1.64	2.27
Esculetin (2)	0.62	0.83	0.76	1.31
Scopoletin (3)	2.65	2.70	1.57	2.82
Prionanthoside (4)	3.26	2.78	2.33	2.79
Euphorbetin (5)	1.91	2.05	2.48	2.15
Quercetin-3-O-β-D-glucoside (6)	1.63	1.96	1.81	1.90

Table 4. Recovery for the assay of the six major constituents assayed (n = 3).

Analyte	Original (μg)	Spiked (μg)	Determined (μg)	Recovery[a] (%)	Recovery average (%)	RSD (%)
Cichoriin (1)	4.218	2.920	7.031	96.34	97.33	1.13
	4.218	4.380	8.533	98.52		
	4.218	5.840	9.890	97.12		
Esculetin (2)	70.21	40.00	108.7	96.25	96.72	1.26
	70.21	80.00	148.7	98.10		
	70.21	120.0	185.2	95.80		
Scopoletin (3)	0.2924	0.1650	0.4495	95.21	97.01	2.20
	0.2924	0.3300	0.6107	96.45		
	0.2924	0.4950	0.7843	99.37		
Prionanthoside (4)	1.638	0.8000	2.404	95.75	97.67	1.70
	1.638	1.600	3.215	98.56		
	1.638	3.200	4.796	98.69		
Euphorbetin (5)	3.188	1.400	4.542	96.71	97.25	1.74
	3.188	2.800	5.873	95.89		
	3.188	4.200	7.352	99.14		
Quercetin-3-O-β-D-glucoside (6)	0.6692	0.5600	1.207	96.04	97.03	2.34
	0.6692	1.120	1.738	95.43		
	0.6692	1.680	2.343	99.63		

[a] Recovery (%) = ($amount_{determined}$ – $amount_{original}$)/$amount_{spiked}$ × 100%.

Table 5. Contents of the six major constituents determined in *V. yedoensis* and eight other species from genus *Viola*.

No.	Sample name	Contents (mean±SD, mg/g)[a]					
		Cichoriin (1)	Esculetin (2)	Scopoletin (3)	Prionanthoside (4)	Euphorbetin (5)	Quercetin-3-*O*-*β*-*D*-glucoside (6)
S1	*V. yedoensis* Makino	1.054±0.017	17.552±0.404	0.073±0.003	0.409±0.009	0.797±0.026	0.167±0.006
S2	*V. yedoensis* Makino	0.820±0.023	10.255±0.178	0.063±0.002	0.359±0.012	0.598±0.024	0.245±0.009
S3	*V. yedoensis* Makino	1.364±0.043	8.902±0.118	0.087±0.003	0.440±0.011	0.612±0.019	0.364±0.012
S4	*V. yedoensis* Makino	2.364±0.034	9.233±0.197	0.055±0.001	0.466±0.015	0.831±0.027	0.314±0.010
S5	*V. yedoensis* Makino	0.088±0.002	1.245±0.043	0.012±0.0004	0.214±0.008	0.451±0.019	0.201±0.003
S6	*V. yedoensis* Makino	0.341±0.007	7.532±0.133	0.050±0.002	0.291±0.009	0.530±0.008	0.171±0.006
S7	*V. yedoensis* Makino	2.843±0.075	6.495±0.107	0.067±0.002	0.509±0.007	0.781±0.025	0.420±0.014
S8	*V. yedoensis* Makino	0.044±0.002	6.591±0.155	0.042±0.001	0.295±0.010	0.570±0.009	0.118±0.003
S9	*V. yedoensis* Makino	0.197±0.004	10.132±0.166	0.064±0.002	0.449±0.014	0.930±0.016	0.242±0.006
S10	*V. yedoensis* Makino	0.041±0.001	5.902±0.112	0.132±0.003	0.935±0.022	1.921±0.0519	0.148±0.004
S11	*V. prionantha* Bunge	tr[b]	0.2581±0.006	0.018±0.0006	0.256±0.005	0.944±0.030	0.267±0.006
S12	*V. concordifolia* C.J. Wang	nd[c]	tr	0.009±0.0003	0.681±0.020	0.325±0.008	0.141±0.004
S13	*V. inconspicua* Blume	nd	0.241±0.005	0.069±0.002	1.578±0.037	0.426±0.015	0.333±0.010
S14	*V. variegate* Fisch. ex Link	tr	0.313±0.008	0.183±0.005	0.111±0.004	1.271±0.021	0.897±0.022
S15	*V. patrinii* DC. ex Ging	nd	0.242±0.006	0.053±0.001	0.381±0.008	0.299±0.006	0.637±0.018
S16	*V. betonicifolia* J.E. Smith	nd	tr	0.034±0.001	0.273±0.007	0.241±0.008	0.252±0.009
S17	*V. collina* Bess	0.498±0.008	0.348±0.009	0.011±0.0003	nd	0.075±0.002	0.066±0.002
S18	*V. grypoceras* A. Gray	nd	tr	0.010±0.0004	tr	0.096±0.003	0.322±0.009

[a] Mean values (n = 3); RSD < 5%. [b] Trace (S/N>3 and <10). [c] Not detected (S/N<3).

applied to the simultaneous determination of the six compounds in 18 samples. The contents of the six marker compounds analyzed were shown in Table 5. It can be seen that the contents in different samples varied markedly. The highest content (17.552 mg/g) of esculetin was found in the commercial crude herb sample (S1) from Henan provience, and all the commercial crude herb samples showed higher contents of esculetin and cichoriin compared with eight other species in the genus *Viola*. In comparison with the content of flavonoid, quercetin-3-*O*-*β*-*D*-glucoside, there is no obvious difference between *V. yedoensis* and other *Viola* species. According to quantitative results, coumarins, especially esculetin and cichoriin, were the characteristic chemical constituents of *V. yedoensis*, which are much valuable for quality evaluation and distinction between *V. yedoensis* and its substitutes from other species in the genus *Viola*.

Comparative analysis by chemometrics methods

Principal component analysis

The contents of six compounds from *V. yedoensis* and eight other species in the genus *Viola* were analyzed by principal component analysis (PCA). The aim of this analysis was to see if the compounds can be used to group the differentquality of commercial crude herb samples and the other species of *Viola*, and which compound was most considerable for the differences (Lee et al., 1998, 2009; Cantarelli et al., 2010). As described in Figure 3A, the first and second PCs described 96.82 and 1.94% of the total variability in the original observations respectively, and both PCs accounted for 98.76% of the total variance. Thus, the first two PCs concentrated the multidimensional information into a 2-D dataset to cluster the samples.

The scores plot and loadings plot obtained from PCA are shown in Figure 3B to D. As shown in the

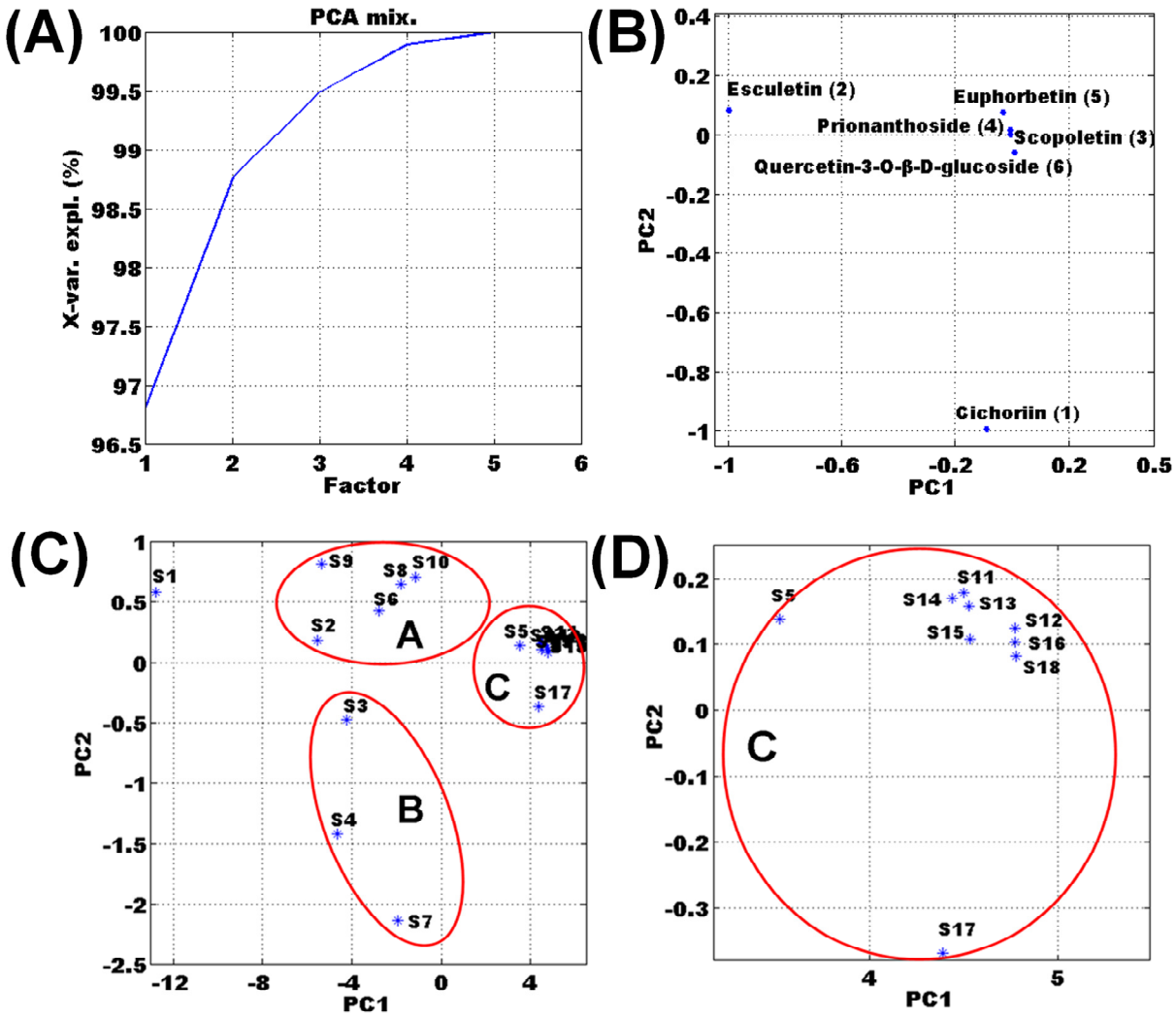

Figure 3. The principal component analysis for quality evaluation of *Viola yedoensis* and eight species from the genus *Viola*. The accumulative contribution rates of PCs (A) The loadings plot of variables (B) Distribution of samples on the scores plot (C) The zoomed view of group C (D). The numbers in this figure are related to the sample numbers (S1-S18) in Table 1 and the compounds (1-6) in Figure 1.

loading plots (Figure 3B), PC1 mostly explained esculetin negatively, and PC2 significantly explained cichoriin negatively, which two influenced the cluster in a top-down order. Therefore, cichoriin and esculetin were the marker components to assess the quality of *V. yedoensis*. However, the loading plots of scopoletin, prionanthoside, euphorbetin and quercetin-3-*O*-*β*-*D*-glucoside were almost zero, indicating that these constituents have equal or very low influences on the distinction. According to the scores plot (Figure 3C), the samples were clustered into three different groups. The samples in group A and group B were from the samples of *V. yedoensis*, and group C were from eight other species in the genus *Viola* (except sample 5). Meanwhile, the loading plots of PC1 versus PC2 could explain which components influenced the cluster more significantly. Goup A was mostly characterized by negative values of PC1 and positive values of PC2, which were with high content of esculetin. All samples in the group A came from *V. yedoensis*, including commercial products and original herb by field acquisition (sample 10). Furthermore, group B was mainly characterized and separated from group A by negative values of PC2, which indicated the higher contents of cichoriin in group B than other groups. Group C was characterized essentially by positive values of PC1 and positive values of PC2 (except sample 17). As shown in Figure 3C and the amplified view in Figure 3D, most of the samples in group C came from eight other species in the genus *Viola*. However, sample 5 was clustered in group C, because the contents of the major constituents were low, which was more similar to other species in the genus *Viola*. According to the constituents profile by PCA, it was suggested that sample 5 might be substitutes from the other species in the genus *Viola*. In conclusion, through

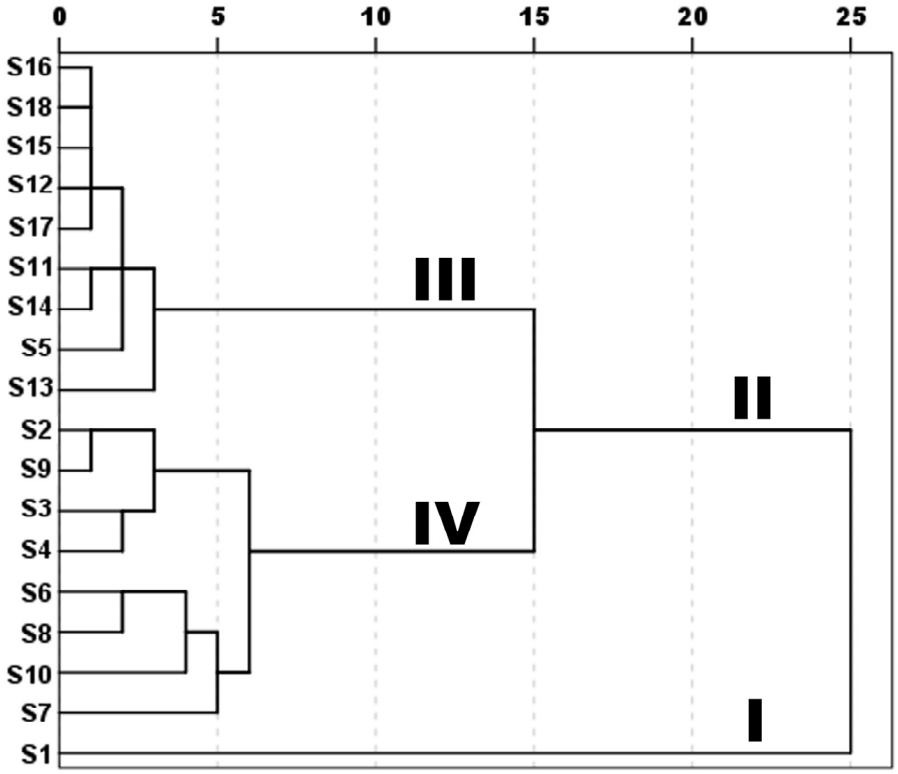

Figure 4. Dendrogram resulting from average linkage cluster analysis. The numbers in the figure are related to the sample numbers (S1-S18) in Table 1.

the scores and loadings plots, different chemical properties of each group were easily identified. The PCA results indicated that, the quality of most commercial products from *V. yedoensis* are similar (except for sample 5), and this traditional medicinal plant can be distinguished from other species in the genus *Viola* by PCA.

Cluster analysis

Cluster analysis could be used to derive groups from the autoscaled dataset and to predict the sample membership based on observed characteristics of each case. In this study, average linkage was used and Euclidean distances were calculated. As presented in Figure 4, group IV clustered commercial products from *V. yedoensis*. Furthermore, the subsets gave some information on the geographical distribution. S2, S3 and S9 were from Anhui, Hebei and Shanxi province, which are located in the warm-temperate zone. Group III clustered the eight other species in the genus *Viola*, however, sample 5 was included in this group because of the low contents of constituents. The distance between sample 1 and others was relatively far, because of the extremely high content of esculetin in this sample. The cluster analysis provided some support to the results of PCA, further confirming that the chemometrics methods established in this paper are reliable for quality evaluaion of *V. yedoensis* and other species in the genus *Viola*.

Conclusion

A HPLC method was developed for the simultaneous determination of six bioactive components including coumarins and flavonoid in *V. yedoensis* and eight other species from the genus *Viola* firstly. Besides, chemometrics methods could accomplish quality evaluation and the identification more directly. It was shown that the contents of constituents in *V. yedoensis* and its substitutes were different. The substitutions of the other *Viola* species were not suitable according to their chemical profiles. However, it needs further pharmacological research to confirm this view. In conclusion, the HPLC method combined with chemometrics methods was demonstrated to be simple, flexible and reliable for the quality evaluation of *V. yedoensis* and other species from the genus *Viola*.

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