Simultaneous Determination of Ten Flavonoids from *Viscum coloratum* Grown on Different Host Species and Different Sources by LC-MS

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A high-performance liquid chromatographic-mass spectrometric method was developed for the simultaneous determination of 10 flavonoids in *Viscum coloratum* obtained from different host species and different sources. *Viscum coloratum* was extracted with 50% methanol. The extracts were separated on a C₁₈ column with a gradient of 0.1% (v/v) formic acid and methanol. The flavonoids in the extracts were detected by negative electrospray ionization mass spectrometry in selective ion monitoring mode. The calibration curves showed good linearity ($r > 0.998$) within the test ranges (homoriodictyol: 0.149–8.940 $\mu\text{g/ml}$, homoriodictyol-7- O - β -D-glycoside: 0.230–13.80 $\mu\text{g/ml}$, homoriodictyol-7- O - β -D-apiose (1 $\rightarrow$ 2)- β -D-glycoside: 5.000–300.0 $\mu\text{g/ml}$, homoriodictyol-7- O - β -D-apiose (1 $\rightarrow$ 5)- β -D-apiose (1 $\rightarrow$ 2)- β -D-glycoside: 0.835–125.3 $\mu\text{g/ml}$, rhamnazin-3- O - β -D-glucoside: 0.064–3.840 $\mu\text{g/ml}$, rhamnazin-3- O - β -D-(6''- β -hydroxy- β -methylglutaryl)-glucoside: 1.435–86.10 $\mu\text{g/ml}$, isorhamnetin-3- O - β -D-glucoside: 0.930–55.80 $\mu\text{g/ml}$, 5-hydroxy-3,7,3'-trimethoxyflavone-4'- O - β -D-glucoside: 0.067–4.020 $\mu\text{g/ml}$, 5,7,4'-trihydroxy-3,3'-dimethoxyflavone: 0.270–16.20 $\mu\text{g/ml}$, pachypodol: 0.110–6.600 $\mu\text{g/ml}$). The limits of quantification were between 0.006–0.720 $\mu\text{g/ml}$. The assay was reproducible and the overall intra- and inter-day variations were less than 4.6%. The recoveries varied from 93.4 to 103.9% at three different concentration levels. The validation method was used to determine the contents of 10 flavonoids in *Viscum coloratum*. A one-way analysis of variance was applied to evaluate *Viscum coloratum*-host-source interactions. Compared with the host species, the sample source had a significant impact on the sample content.

Key words *Viscum coloratum*; determination; flavonoid; HPLC-MS

Viscum coloratum (KOM.) NAKAI (*V. coloratum*) is a perennial evergreen, semi-parasitic plant which grows on branches or stems of deciduous trees. It is known as Hujisheng in Chinese.¹⁾ *V. coloratum* is an important medicinal herb which has been used in various treatments such as cardiovascular diseases, cancer, hepatitis and hemorrhage.^{2,3)} It was the main ingredient in many formulae such as hujisheng injection, Huqi San, Hanbixiao keli etc.^{4–6)} *V. coloratum* has been attracting more and more attention for its therapeutic effects. However, Traditional Chinese medicine is a complex system. An herbal medicine may consist of hundreds of constituents, and their contents vary depending on climate, regions of cultivation and seasons of harvest, which make it difficult to ensure the batch-to-batch uniformity.⁷⁾ The quality control of standardized herbal extracts is essential for the therapeutic reproducibility, efficacy and safe application of extracts. Meanwhile, as a semi-parasitic plant, it is widely appreciated that the constituents of *V. coloratum* are not stable and depend not only on the biosynthesis pathway but also on the type of host plant. Therefore, host plant also is an influencing factor in the quality control of standardized herb extracts. But the ancient medical textbooks and Chinese pharmacopoeias do not give details of the host of *V. coloratum*. Luczkiewicz *et al.* have reported an HPLC method for comparative analysis of phenolic acids in *Viscum album* L. from various hosts.⁸⁾ They found that comparative chromatography revealed qualitative differences in the investigated compounds between the various plant materials. So in this paper we aim to control quality of *V. coloratum* through analyzing *V. coloratum*-host-source interactions.

The constituents of *V. coloratum* include flavonoids, alka-

loids, triterpenoids, lignans, diarylheptanoids and essential oils.^{2,9,10)} *V. coloratum* is used in treatments of cardiovascular disease that is linked to the presence of flavonoids.¹¹⁾ Homoriodictyol (Hedt-I), homoriodictyol-7- O - β -D-glycoside (Hedt-II), homoriodictyol-7- O - β -D-apiose (1 $\rightarrow$ 2)- β -D-glycoside (Hedt-III), and homoriodictyol-7- O - β -D-apiose (1 $\rightarrow$ 5)- β -D-apiose (1 $\rightarrow$ 2)- β -D-glycoside (Hedt-IV) are four dihydroflavones with anti-coagulant,¹²⁾ anti-inflammatory,¹³⁾ antioxidant,¹⁴⁾ anti-tumor¹⁵⁾ and anti-fungal activity.¹⁶⁾ Rhamnazin-3- O - β -D-glucoside (Rham-I), rhamnazin-3- O - β -D-(6''- β -hydroxy- β -methylglutaryl)-glucoside (Rham-II), isorhamnetin-3- O - β -D-glucoside (Isor), 5-hydroxy-3,7,3'-trimethoxyflavone-4'- O - β -D-glucoside (Httf), 5,7,4'-trihydroxy-3,3'-dimethoxyflavone (Tddf) and pachypodol (Pach) are six flavonols with the same effects.^{17–20)} Due to the importance of flavonoids, it could be considered as the 'marked compound' for the chemical evaluation or standardization of *V. coloratum*. To date, only a few studies of the quantitative determination of the flavonoids of *V. coloratum* using HPLC have been reported.^{21–23)} However, the constituents in *V. coloratum* are complex, and some of them usually in very low concentrations. Therefore, a more sensitive, selective method is required and the emergence of HPLC-MS makes this possible. It not only provides detailed structural information but also allows accurate and sensitive quantification of the multiple compounds present. For example, an HPLC-DAD-MSⁿ was developed for study of chemical and metabolic information of flavonoid extract of jujube seeds. This is of great importance in understanding the molecular mechanism of action and clinical effects of flavonoid extract of jujube seeds and facilitating further quantitative studies as well.²⁴⁾

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Table 1. Chemical Structures of 10 Flavonoids in *V. coloratum*

Marker component	Purity (%)	Structure	R ₁	R ₂	R ₃
1 Hedt-I	99.7		—H	—	—
2 Hedt-II	99.4		—β-D-Glc	—	—
3 Hedt-III	99.7		—β-D-Api-(1→2)-β-D-Glc	—	—
4 Hedt-IV	99.1		—β-D-Api-(1→5)-β-D-Api —(1→2)-β-D-Glc	—	—
5 Rham-I	99.2		—CH ₃	—β-D-Glc	—H
6 Rham-II	98.3		—CH ₃	β-D-(6''-β-Hydroxy-β-methylglutaryl)-Glc	—H
7 Isor	98.9		—H	—β-D-Glc	—H
8 Httf	98.1		—CH ₃	—CH ₃	—β-D-Glc
9 Tddf	98.7		—H	—CH ₃	—H
10 Pach	99.0		—CH ₃	—CH ₃	—H

In the present study, we aimed to develop and validate a sensitive and accurate HPLC-MS method for simultaneous determination of 10 flavonoids in *V. coloratum*, including Hedt-I (1), Hedt-II (2), Hedt-III (3), Hedt-IV (4), Rham-I (5), Rham-II (6), Isor (7), Httf (8), Tddf (9) and Pach (10). Their structures are listed in Table 1. During the method development, selective ion monitoring (SIM) was employed and an electrospray ionization source was operated in negative mode. *V. coloratum* growing on different host species and in different sources were determined using the developed method. A one-way analysis of variance was applied to evaluate *V. coloratum*-host-source interactions.

Experimental

Reagents and Standards Methanol was of HPLC grade (Fisher, U.S.A.). All other reagents were of analytical grade (Concord Tech Reagent Company, Tianjin, China). Deionized water was used throughout the study. The reference standards were isolated and purified in the laboratory from *V. coloratum*. The 10 flavonoids, Hedt-I, Hedt-II, Hedt-III, Hedt-IV, Rham-I, Rham-II, Isor, Httf, Tddf and Pach, were fully characterized using UV, NMR, MS, and the data compared with literature values.^{19,25–30} The purities of standards are listed in Table 1 (measured by HPLC).

Plant Materials *V. coloratum* samples were collected from Changbai Mountain (CBM, Yanji, Jilin, China, temperate continental mountainous climate), Qian Mountain (QM, Anshan, Liaoning, China, warm temperate semi-humid continental monsoon climate), Xiyue Temple (XYT, Huayin, Shanxi, China, warm temperate semi-arid continental climate), Chengde Imperial Summer Resort (CISR, Chengde, Hebei, China, warm temperate semi-humid continental monsoon climate), Daqing Mountain (DQM, Bincounty, Heilongjiang, China, temperate semi-humid continental monsoon climate) and Xianxia Mountain (XXM, Quzhou, Zhejiang, China, subtropical humid monsoon climate). The collected plant materials were dried at room temperature in a well-ventilated room without light. Dr. Qishi Sun authenticated the plant material, and the voucher specimens were deposited in the state Key Laboratory of Traditional Chinese Medicine (Shenyang Pharmaceutical University, China).

Apparatus and Operating Conditions The HPLC system consisted of a DGU-14 AM degasser, two Shimadzu 10ADvp Pumps, a high pressure mixer and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface, and a Q-array-Octapole-Quadrupole mass analyzer (QoQ system) were used in the study.

Chromatographic separation of the extracts was performed on an ODS column (Synergi, 250 mm×4.6 mm i.d., particle size 4 μm, Phenomenex, U.S.A.), equipped with an ODS guard column (Security Guard, Phenomenex, U.S.A.). The mobile phase was 0.1% formic acid (A) and methanol (B). The gradient elution was: 0–20 min, a linear gradient of 40–52% B; 20–40 min, a linear gradient of 52–80% B, 40–45 min, 80% B; and then re-equilibrating the column with 40% B for 5 min. The flow-rate

Fig. 1. TIC Chromatograms in Negative Ionization Mode of *V. coloratum* (Host Species: *Acer monm* MAXIM.).

was 0.8 ml/min (split ratio 1 : 3) and the column temperature was 30 °C.

Mass spectrometric conditions were optimized to obtain maximum sensitivity. The extracts were ionized in the negative ESI interface of the mass spectrometer. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. The curve dissolution line (CDL) voltage was fixed at 10.0 kV, the interface temperature was 400 °C, the CDL temperature was 200 °C, the heat block temperature was 200 °C, the detector voltage was 1.75 kV and the nebulizing gas flow rate was 2.5 l/min. The flavonoids were monitored at *m/z* 301, 463, 595, 727, 491, 635, 477, 551, 329 and 343 for Hedt-I, Hedt-II, Hedt-III, Hedt-IV, Rham-I, Rham-II, Isor, Httf, Tddf and Pach, respectively. Data collection, peak integration, and calculations were performed using LC-MS Solution Version 3.0 software.

Preparation of Standard Solutions Stock solutions of Hedt-I (0.745 mg/ml), Hedt-II (0.460 mg/ml), Hedt-III (1.000 mg/ml), Hedt-IV (2.088 mg/ml), Rham-I (0.320 mg/ml), Rham-II (1.435 mg/ml), Isor (0.930 mg/ml), Httf (0.335 mg/ml), Tddf (0.540 mg/ml) and Pach (0.220 mg/ml) were prepared in methanol. Working standard solutions for calibration were prepared by diluting the stock solution with appropriate quantities of methanol. All working solutions were stored at 4 °C and brought to room temperature before use.

Preparation of Sample Solutions *V. coloratum* samples were powdered in an electric blender and then passed through a #4 mesh sieve. The powder was accurately weighed (1.00 g) in a round-bottom flask and 25 ml 50% (v/v) methanol was added. The flask was then weighed accurately and heated in a water bath for 30 min. The flask was then cooled and 50% methanol was added in order to compensate for the solvent loss. The extract was transferred to a centrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was then passed through a membrane filter (0.22 μm) prior to injection.

Fig. 2. (A) Mass Spectra of 10 Flavonoids Standard in Full Scan Mode; (B) Typical SIM Chromatograms of 10 Flavonoids Standard; (C) Typical SIM Chromatograms of 10 Flavonoids in *V. coloratum* (Host Species: *Acer monm* MAXIM.)

Results and Discussion

Optimization of LC-MS Conditions Since Pach has a low polarity compared with the other flavonoids, a different mobile phase with a different gradient elution of methanol–water or acetonitrile–water was tested. The results indicated that a mobile phase consisting of 0.1% aqueous formic acid-methanol with gradient elution provided high resolution for all the components in *V. coloratum* samples. The total ion current (TIC) chromatogram in negative ionization mode of a *V. coloratum* sample is shown in Fig. 1.

The 10 flavonoids were analyzed by MS in ESI negative ion mode without any problems. Full scan negative ion mass spectra (Fig. 2) of Hedt-I, Hedt-II, Hedt-III, Hedt-IV, Rham-I, Rham-II, Isor, Tddf and Pach formed strong deprotonated ions $[M-H]^-$ at m/z 301, 463, 595, 727, 491, 635, 477, 329 and 343, respectively. The adduct ion $[M+HCOOH-H]^-$ of Httf was more abundant than the corresponding deprotonated ions $[M-H]^-$, thus $[M+HCOOH-H]^-$ at m/z 551 was monitored for Httf. Typical SIM chromatograms of the 10 flavonoids in *V. coloratum* extracts had well-formed and fully separated peaks (Fig. 2). The compounds were identified by comparing their retention times and mass spectra with those of the pure isolated compounds.

Extraction Procedure Reflux and ultrasonication (all with methanol as extractants) were used to extract the marker constituents in *V. coloratum* and all the procedures were repeated twice. The results indicated that the extraction yield from reflux was markedly higher than from ultrasonication. Therefore, in this study, we used reflux extraction for the 10 flavonoids.

In order to obtain optimal extraction efficiency, different

extraction solvents (10%, 30%, 50%, 70%, 100% methanol aqueous solutions (v/v) and extraction times (10, 30, 60, 120 min) were tried. The results showed that the solvent containing 50% methanol had better extraction efficiency and produced fewer background peaks. In addition, the 10 flavonoids could be completely extracted within 30 min.

Method Validation. Calibration Curves, Limits of Quantification and Matrix Effects Standard stock solutions were prepared as described in ‘Preparation of Standard Solutions’ and diluted to appropriate concentrations to establish the calibration curves. At least seven different concentrations were used, and the calibration curves were then constructed by plotting the peak area *versus* the concentration of each analyte. The calculated results are given in Table 2. The correlation coefficients were all greater than 0.999 except for Rham-II and Pach, indicating a high degree of linearity.

The limit of quantification (LOQ) was calculated as the amount of the injected analyte which gave a signal-to-noise ratio of 10. The LOQ values of the method for the 10 flavonoids are also listed in Table 2.

To evaluate the matrix effect, a sample of *V. coloratum* (No. 14) was extracted as described in ‘Preparation of Sample Solutions’ and 1 ml of this solution was spiked with 1 ml of a standard mixture solution with a known concentration, while another 1 ml of sample solution was diluted to 2 ml with 50% methanol. The standard solution was also diluted with an equal volume of 50% methanol and analyzed. The corresponding peak areas of the analytes in the standard spiked sample solutions (*A*) minus those in sample solutions (*B*) were then compared with those of the standard solutions (*C*). The ratio $[(A-B)/C \times 100\%]$ was defined as the matrix

Table 2. Summary of the Method Validation of 10 Flavonoids in *V. coloratum*

Marker component	Retention times (min)	Quantitation ion (m/z)	Regression equation	r^2	Linear range ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)	Matrix effect (%)
Hedt-I	31.675	301.15 $[M-H]^-$	$y=9.43 \times 10^5 x + 1.02 \times 10^5$	0.9997	0.149–8.940	0.030	104.4
Hedt-II	17.250	463.30 $[M-H]^-$	$y=4.55 \times 10^4 x - 0.76 \times 10^4$	0.9991	0.230–13.80	0.105	98.8
Hedt-III	16.245	595.20 $[M-H]^-$	$y=7.31 \times 10^4 x + 0.82 \times 10^4$	0.9994	5.000–300.0	0.050	97.6
Hedt-IV	14.316	727.35 $[M-H]^-$	$y=3.53 \times 10^4 x - 0.68 \times 10^4$	1.0000	0.835–125.3	0.104	101.9
Rham-I	34.316	491.10 $[M-H]^-$	$y=8.80 \times 10^4 x - 0.22 \times 10^4$	0.9992	0.064–3.840	0.006	99.6
Rham-II	35.733	635.20 $[M-H]^-$	$y=6.17 \times 10^3 x + 1.61 \times 10^3$	0.9989	1.435–86.10	0.720	98.0
Isor	25.858	477.25 $[M-H]^-$	$y=2.70 \times 10^4 x + 0.67 \times 10^4$	0.9999	0.930–55.80	0.093	98.2
Httf	35.260	551.20 $[M+HCOOH-H]^-$	$y=5.20 \times 10^4 x + 0.02 \times 10^4$	0.9990	0.067–4.020	0.007	100.1
Tddf	38.341	329.10 $[M-H]^-$	$y=7.84 \times 10^4 x - 0.15 \times 10^4$	0.9994	0.270–16.20	0.054	98.0
Pach	44.213	343.15 $[M-H]^-$	$y=3.47 \times 10^5 x - 0.31 \times 10^5$	0.9986	0.110–6.600	0.110	100.5

y peak area, x concentration of the compounds ($\mu\text{g/ml}$), r^2 correlation coefficient of the equation, LOQ limit of quantification.

Table 3. Results of Intra- and Inter-Day Precisions for 10 Flavonoids

Marker component	Theoretical concentration ($\mu\text{g/ml}$)	Intra-day ($n=6$)		Inter-day ($n=18$)	
		Detected ($\mu\text{g/ml}$)	RSD %	Detected ($\mu\text{g/ml}$)	RSD %
Hedt-I	1.490	1.512 ± 0.011	0.7	1.486 ± 0.064	4.3
Hedt-II	2.300	2.317 ± 0.106	4.6	2.324 ± 0.029	1.2
Hedt-III	50.00	49.41 ± 1.464	3.0	49.33 ± 0.030	0.6
Hedt-IV	20.88	20.48 ± 0.296	1.4	20.30 ± 0.443	2.2
Rham-I	0.640	0.633 ± 0.026	4.0	0.632 ± 0.015	2.3
Rham-II	14.35	14.31 ± 0.431	3.0	14.43 ± 0.258	1.8
Isor	9.300	9.253 ± 0.128	1.4	9.277 ± 0.154	1.7
Httf	0.670	0.644 ± 0.017	2.7	0.651 ± 0.026	4.0
Tddf	2.700	2.654 ± 0.104	3.9	2.628 ± 0.061	2.3
Pach	1.100	1.071 ± 0.044	4.1	1.084 ± 0.027	2.5

effect. The matrix effects of 10 flavonoids were ranged from 97.6 to 104.4% (Table 2), and these indicated that there were no significant matrix effects.

Precision Intra-day precision was examined by analyzing the standard solution within one day, and inter-day precision was determined on three independent days. The relative standard deviation (R.S.D.) was calculated as a measurement of method precision. The intra-day and inter-day precisions of the determination of the 10 flavonoids were not more than 4.6% and 4.3%. The results are shown in Table 3.

Recoveries The recoveries of the 10 flavonoids were determined by the standard addition method. Three concentrations (low, medium, high) of the mixed standard solutions were spiked with known amounts of *V. coloratum* sample (No. 14). Then, the resultant samples were extracted and analyzed by the proposed method and triplicate experiments were carried out at each level. The total detected amounts (A') minus the original amounts (B') were then compared with the added amounts (C'). The ratio $[(A' - B')/C' \times 100\%]$ was defined as the recovery. As shown in Table 4, the recoveries of the 10 flavonoids ranged from 93.4 to 103.9%, with an R.S.D. of no more than 4.7%. Considering the results of the recovery test, the method was considered to be accurate.

Repeatability and Stability Six individual samples from the same batch (No. 14) were extracted and analyzed by the proposed method. The R.S.D. value was calculated as a measurement of method repeatability. The R.S.D. values of the 10 compounds in these samples were not more than 5.1%, which showed the high repeatability of the method. Stability was tested with a standard solution and a sample so-

Table 4. Recoveries of 10 Flavonoids in *V. coloratum* ($n=9$)

Marker component	Detected amount (μg)	Original amount (μg)	Added amount (μg)	Recoveries (%)	Mean $\pm$ S.D. (%)	RSD (%)
Hedt-I	100.8	65.00	37.25	96.1	98.7 $\pm$ 2.9	3.0
	140.9		74.50	101.9		
	174.5		111.8	98.0		
Hedt-II	179.6	113.0	69.00	96.4	98.9 $\pm$ 2.5	2.5
	249.3		138.0	98.8		
	322.8		207.0	101.4		
Hedt-III	2976	1970	1000	100.6	100.4 $\pm$ 0.4	0.4
	3983		2000	100.7		
	4968		3000	99.9		
Hedt-IV	2838	1862	939.6	103.9	99.4 $\pm$ 3.9	4.0
	3677		1879	96.6		
	4613		2819	97.6		
Rham-I	10.30	5.500	4.800	99.4	99.1 $\pm$ 3.0	3.1
	14.70		9.600	95.9		
	20.20		14.40	102.0		
Rham-II	299.9	195.5	100.5	103.9	100.9 $\pm$ 3.5	3.5
	399.9		200.9	101.7		
	488.0		301.4	97.1		
Isor	293.9	204.0	93.00	96.7	98.6 $\pm$ 2.9	3.0
	393.7		186.0	102.0		
	475.1		279.0	97.2		
Httf	3.000	2.000	1.005	100.8	100.8 $\pm$ 1.6	1.6
	4.100		2.010	102.4		
	5.000		3.015	99.3		
Tddf	93.40	62.50	32.40	95.5	98.8 $\pm$ 3.4	3.5
	128.8		64.80	102.3		
	158.4		97.20	98.7		
Pach	35.30	24.00	11.00	102.6	98.0 $\pm$ 4.6	4.7
	44.60		22.00	93.4		
	56.40		33.00	98.1		

Table 5. Contents of 10 Flavonoids in *V. coloratum* Grown on Different Host Species and Different Sources (mg/g)

No.	Host species	Sources	Hedt-I	Hedt-II	Hedt-III	Hedt-IV	Rham-I	Rham-II	Isor	Httf	Tddf	Pach	Total amounts
1	<i>Populus ussuriensis</i> KOM. ^{△△}	CBM	0.113	0.073	1.462	0.039	0.007	0.047	0.021	N.D.	0.041	0.018	1.821
2	<i>Populus ussuriensis</i> KOM. ^{△△}	CBM	0.013	0.092	5.813	0.182	0.008	0.563	0.474	N.D.	0.016	0.012	7.173
3	<i>Populus ussuriensis</i> KOM. ^{△△}	CBM	0.156	0.195	1.962	0.429	0.008	0.067	0.037	N.D.	0.048	0.018	2.920
4	<i>Ulmus pumila</i> L. [△]	CBM	0.041	0.070	1.568	3.906	0.020	0.434	0.142	0.006	0.046	0.013	6.245
5	<i>Ulmus pumila</i> L. [△]	CBM	0.012	0.060	2.244	3.923	0.004	0.256	0.258	N.D.	0.057	0.023	6.837
6	<i>Ulmus pumila</i> L. [△]	CBM	0.005	0.037	2.464	4.670	0.005	0.271	0.188	N.D.	0.035	0.017	7.692
7	<i>Ulmus pumila</i> L. [△]	CBM	0.028	0.088	3.918	0.183	0.014	0.340	0.121	0.003	0.044	0.022	4.760
8	<i>Tilia amurensis</i> RUFR. [△]	CBM	0.013	0.080	5.558	0.128	0.005	0.437	0.147	0.002	0.032	0.016	6.417
9	<i>Tilia amurensis</i> RUFR. [△]	CBM	0.129	0.110	3.148	1.151	0.015	0.163	0.155	0.003	0.100	0.032	5.006
10	<i>Tilia amurensis</i> RUFR. ^{△△}	CBM	0.040	0.027	1.558	3.385	0.004	0.352	0.232	N.D.	0.019	0.014	5.631
11	<i>Betula platyphylla</i> SUK. [△]	CBM	0.008	0.049	6.252	0.149	0.009	0.515	0.174	0.002	0.053	0.018	7.227
12	<i>Betula platyphylla</i> SUK. [△]	CBM	0.011	0.046	1.291	2.538	0.006	0.245	0.073	N.D.	0.011	0.008	4.230
13	<i>Pyrus ussuriensis</i> MAXIM.	CBM	0.026	0.093	3.911	0.055	0.005	0.231	0.211	0.003	0.057	0.029	4.621
14	<i>Acer monm</i> MAXIM.	CBM	0.130	0.226	3.940	3.724	0.011	0.391	0.408	0.004	0.125	0.048	9.007
15	<i>Syrigu reticulata</i> (BLUME) HARA var. <i>mandshurica</i>	CBM	0.018	0.096	2.160	1.419	0.006	0.553	0.264	N.D.	0.023	0.015	4.554
16	<i>Populus ussuriensis</i> KOM. [△]	QM	0.063	0.177	2.136	0.018	0.005	0.079	0.240	0.003	0.018	0.009	2.748
17	<i>Populus ussuriensis</i> KOM. [△]	QM	0.026	0.248	4.958	0.340	0.025	1.258	0.494	0.005	0.062	0.014	7.426
18	<i>Populus ussuriensis</i> KOM. [△]	XYT	0.126	0.099	2.849	1.082	0.014	0.152	0.143	0.003	0.097	0.033	4.598
19	<i>Populus ussuriensis</i> KOM. [△]	XYT	0.106	0.075	2.049	1.056	0.009	0.172	0.191	0.002	0.055	0.013	2.758
20	<i>Populus ussuriensis</i> KOM. [△]	CISR	0.003	0.044	0.648	0.021	0.005	0.028	0.036	N.D.	0.006	0.005	0.796
21	<i>Populus ussuriensis</i> KOM. [△]	CISR	0.007	0.048	0.834	0.018	0.004	0.068	0.062	N.D.	0.012	0.005	1.058
22	<i>Populus ussuriensis</i> KOM. [△]	CISR	0.040	0.022	0.933	0.022	0.004	0.111	0.036	N.D.	0.015	0.007	1.190
23	<i>Populus ussuriensis</i> KOM. [△]	CISR	0.070	0.012	2.278	0.044	0.025	0.633	0.316	0.002	0.026	0.008	3.414
24	<i>Ulmus pumila</i> L.	CISR	0.040	0.018	1.452	0.044	0.005	0.304	0.027	N.D.	0.004	0.005	1.899
25	<i>Salix babylonica</i> L.	CISR	0.013	0.039	2.829	0.030	0.003	0.127	0.124	N.D.	0.039	0.022	3.226
26	<i>Populus ussuriensis</i> KOM. [△]	DQM	0.012	0.053	2.749	3.382	0.005	0.962	0.125	0.003	0.052	0.029	7.372
27	<i>Populus ussuriensis</i> KOM. [△]	DQM	0.004	0.032	1.854	2.387	0.007	0.439	0.109	N.D.	0.015	0.009	4.855
28	<i>Melia azedrach</i> L.	XXM	0.001	0.013	0.232	0.085	0.048	0.133	0.159	0.102	0.049	0.092	0.914
29	<i>Melia azedrach</i> L.	XXM	N.D.	0.012	0.089	0.082	0.017	0.047	0.039	0.013	0.060	0.060	0.419
30	<i>Melia azedrach</i> L.	XXM	N.D.	0.016	0.288	0.034	0.047	0.143	0.192	0.028	0.149	0.188	1.085

N.D.: Lower than LOQ; CBM: Changbai Mountain, QM: Qian Mountain, XYT: Xiyue temple, CISR: Chengde Imperial Summer Resort, DQM: Da Qing Mountain, XXM: Xianxia Mountain. [△] A group, ^{△△} B group.

Table 6. Summary of A Group (Same Source: CBM, Different Host Species: No. 1—12) ANOVA Results

Marker component	Sources of variation	SS	df	MS	F	Sig.	Marker component	Sources of variation	SS	df	MS	F	Sig.
Hedt-I	Between groups	0.013	3	0.004	1.775	0.229	Rham-II	Between groups	0.032	3	0.011	0.322	0.810
	Within groups	0.019	8	0.002				Within groups	0.266	8	0.033		
	Total	0.032	11					Total	0.299	11			
Hedt-II	Between groups	0.008	3	0.003	1.590	0.266	Isor	Between groups	0.005	3	0.002	0.085	0.966
	Within groups	0.014	8	0.002				Within groups	0.153	8	0.019		
	Total	0.022	11					Total	0.158	11			
Hedt-III	Between groups	2.429	3	0.810	0.187	0.902	Tddf	Between groups	0.001	3	0.000	0.294	0.829
	Within groups	34.692	8	4.336				Within groups	0.005	8	0.001		
	Total	37.121	11					Total	0.006	11			
Hedt-IV	Between groups	15.561	3	5.187	1.999	0.193	Pach	Between groups	0.000	3	0.000	0.668	0.595
	Within groups	20.761	8	2.595				Within groups	0.000	8	0.000		
	Total	36.322	11					Total	0.000	11			
Rham-I	Between groups	0.000	3	0.000	0.256	0.855	Total amounts	Between groups	10.339	3	3.446	1.059	0.419
	Within groups	0.000	8	0.000				Within groups	26.043	8	3.255		
	Total	0.000	11					Total	36.383	11			

The content of Httf was too low to allow statistical analysis.

Table 7. Summary of B Group (Same Host Species: *Populus ussuriensis* KOM., Different Sources: No. 1—3, No. 16—23, No. 26—27) ANOVA Results

Marker component	Sources of variation	SS	df	MS	F	Sig.	Marker component	Sources of variation	SS	df	MS	F	Sig.
Hedt-I	Between groups	0.013	3	0.004	2.150	0.182	Rham-II	Between groups	0.357	3	0.119	0.752	0.555
	Within groups	0.015	7	0.002				Within groups	1.108	7	0.158		
	Total	0.028	10					Total	1.465	10			
Hedt-II	Between groups	0.046	3	0.015	8.632	0.009	Isor	Between groups	0.088	3	0.029	0.929	0.475
	Within groups	0.012	7	0.002				Within groups	0.221	7	0.032		
	Total	0.058	10					Total	0.309	10			
Hedt-III	Between groups	10.018	3	3.339	1.351	0.333	Tddf	Between groups	0.005	3	0.002	4.478	0.047
	Within groups	17.308	7	2.473				Within groups	0.003	7	0.000		
	Total	27.326	10					Total	0.008	10			
Hedt-IV	Between groups	1.532	3	0.511	27.407	0.000	Pach	Between groups	0.000	3	0.000	3.968	0.061
	Within groups	0.130	7	0.019				Within groups	0.000	7	0.000		
	Total	1.663	10					Total	0.001	10			
Rham-I	Between groups	0.000	3	0.000	0.307	0.820	Total amounts	Between groups	19.374	3	6.458	1.369	0.328
	Within groups	0.001	7	0.000				Within groups	33.013	7	4.716		
	Total	0.001	10					Total	52.386	10			

The content of Httf was too low to allow statistical analysis.

lution that were stored at room temperature and analyzed every 12 h over 3 d, and the analytes were found to be stable over 72 h (R.S.D. <5%).

Sample Assay The optimized chromatographic method was used to determinate the 10 flavonoids in *V. coloratum*. In Fig. 2, the peaks of the 10 flavonoids showed that the separations were good. The contents of the 10 flavonoids in *V. coloratum* samples growing on different host species and at different sources are summarized in Table 5. The predominant compounds in all examined samples were Hedt-III and Hedt-IV, which ranged from 6.252 to 0.089 mg/g and from 4.670 to 0.018 mg/g, respectively. These results correspond to the pharmacological investigation of Shanghai mistletoe Group.⁴⁾ They found that the dihydroflavones contribute in cardiovascular diseases treatment. Wang *et al.* have reported an HPLC method for the simultaneous determination of 4 flavonoids (Hedt-I, Tddf, Pach and ombuine) in *V. coloratum*.²¹⁾ However, the detectable contents were low in comparison with our results. The discrepancy could be a result of the complex extraction method as to cause the loss of flavonoids.

In order to evaluate *V. coloratum*-host-source interactions, one-way analysis of variance was performed on the A group

(same source: CBM, different host species: No. 1—3, *Populus ussuriensis* KOM.; No. 4—7, *Ulmus pumila* L.; No. 8—10, *Tilia amurensis* RUPR.; No. 11—12, *Betula platyphylla* SUK.) and the B group (same host species: *Populus ussuriensis* KOM., different sources: No. 1—3, CBM; No. 16—17, QM; No. 18—19, XYT; No. 20—23, CISR; No. 26—27, DQM), respectively. The results from the A group (Table 6) showed that there was no significant difference among samples, while in the B group (Table 7), 3 of the 10 individual flavonoids level varied significantly at $p \leq 0.05$. Therefore, compared with the host species, the sample source has a significant impact on the sample content. In short, the host species was not the main factor in affecting the quality of *V. coloratum*. The reason for this may be that *V. coloratum* is semi-parasitic plant and can photosynthesizes itself. *V. coloratum* only acquires mineral nutrition and water from their host through a root connection. This could explain why the ancient medical textbooks and Chinese pharmacopoeias do not give details of the host of *V. coloratum*. As far as flavonoids are concerned, we proposed that *V. coloratum* could be used directly as a medicine without knowledge of the host species.

Conclusion

A novel sensitive and selective HPLC-MS method was developed and validated to simultaneously determine 10 flavonoids from *V. coloratum* grown on different host species and different sources. A one-way analysis of variance was used to evaluate *V. coloratum*-host-source interactions. In accounting the contents of flavonoids, the source of *V. coloratum* could be a significant impact on the sample content. In contrast, the host did not play significant role on the chemical composition. Here, we proposed that *V. coloratum* could be used directly as a medicine without knowledge of the host species.

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