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Chemical fingerprint analysis of rhizomes of *Gymnadenia conopsea* by HPLC–DAD–MS^n

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Abstract

A high-performance liquid chromatography–diode array detection–tandem mass spectrometry (HPLC–DAD–MS^n) method has been firstly developed for chemical fingerprint analysis of rhizomes of *Gymnadenia conopsea* R. Br. and rapid identification of major compounds in the fingerprints. Comparing the UV and MS spectra with those of reference compounds, seven main peaks in the fingerprints were identified as adenosine (**1**), 4-hydroxybenzyl alcohol (**2**), 4-hydroxybenzyl aldehyde (**3**), dactylorhin B (**4**), loroglossin (**5**), dactylorhin A (**6**) and militarine (**7**). Compounds **4**–**7** were succinate derivative esters and firstly discovered from this species. The Computer Aided Similarity Evaluation System (CASES) for chromatographic fingerprint of traditional Chinese medicine was employed to evaluate the similarities of 10 samples of the rhizomes of *G. conopsea* collected from Sichuan, Qinghai and Hebei provinces, Tibet autonomous region of China, and Nepal. These samples from different sources had similar chemical fingerprints. This method is specific and may serve for quality identification and comprehensive evaluation of this traditional Tibetan remedy.

Keywords: Chemical fingerprint; HPLC–DAD–MS^n; Identification; Succinate derivative ester; *Gymnadenia conopsea*

1. Introduction

Gymnadenia conopsea R. Br. is an orchid widely growing in China and Nepal. Its rhizome, called 'Wangla', has long been used as a traditional Tibetan remedy for coughs and asthma and as a tonic in Chinese folk medicine [1–3]. Previous studies indicated that it had antihepatitis B, radical scavenging effects [4,5] and that the major constituents of this plant are phenolic compounds and adenosine [6–8]. However, no HPLC method was established for analysis of this herbal medicine.

Chromatographic fingerprint technique is a useful method in the identification of herbal samples or their products, and hence has been widely used for studying traditional Chinese medicines (TCM) [9–12]. Moreover, high-performance liquid chromatography–diode array detection–tandem mass spectrometry (HPLC–DAD–MS^n) has grown into one of the most powerful analytical techniques available for analyzing TCM. Using the on-line combined technique, UV spectra and tandem mass spectra of separated ingredients can be obtained simultaneously, and comparing the spectra with those of the reference compounds, these ingredients can be identified.

In this article, using the HPLC–DAD–MS^n technique, a feasible and reliable fingerprint of the rhizomes of *G. conopsea* was established and seven "characteristic peaks" were rapidly identified as adenosine (**1**), 4-hydroxybenzyl alcohol (**2**), 4-hydroxybenzyl aldehyde (**3**), dactylorhin B (**4**), loroglossin (**5**), dactylorhin A (**6**) and militarine (**7**), respectively. Among them, succinate derivative esters (**4**–**7**, Fig. 1), which were reported beneficial for treating dementia, were firstly discovered from this species [8]. The Computer-Aided Similarity Evaluation System (CASES) for chromatographic fingerprint of traditional Chinese medicines, a chemometrics computer software recently developed by the Institute of Pharmacy Engineering (Zhejiang University, Hangzhou, China) and endorsed by the National Pharmacopoeia Committee of the People's Republic of China for similarity studies of chromatographic fingerprints of Chinese herbs [9,10,13,14], was also applied in this study. This software was employed to synchronize the chromatographic peaks and to

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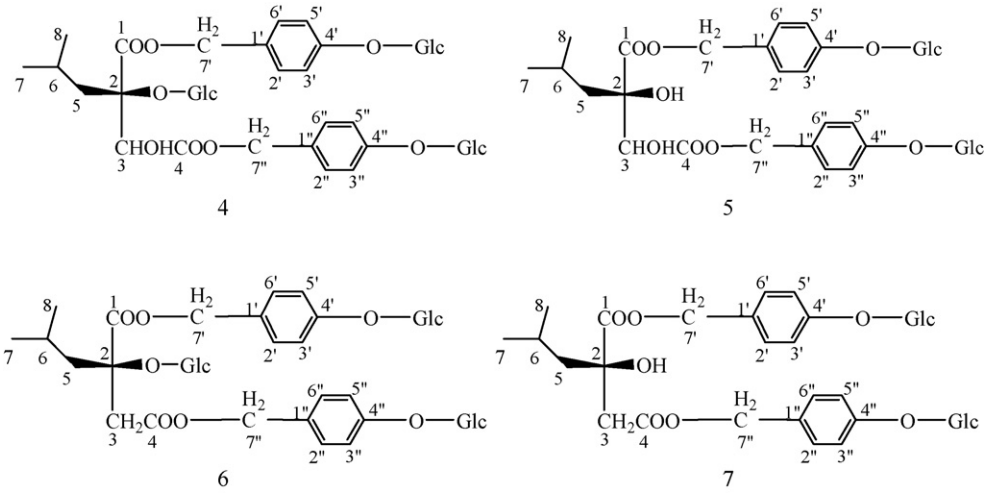


Fig. 1. Structures of compounds dactylorhin B (**4**), loroglossin (**5**), dactylorhin A (**6**) and militarine (**7**).

calculate the cosine values of vectorial angles among different chromatograms, as well as to compute the mean chromatogram as a representative standard fingerprint/chromatogram for a group of chromatograms. The closer the cosine values are to 1, the more similar the two chromatograms are. Furthermore, the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak related to the reference peak were calculated for quantitative expression of the chemical components in the chromatographic pattern of herbs. The generated data in this work provided valuable insights about the application of fingerprint analysis in comprehensive evaluation of *G. conopsea.*

2. Experimental

2.1. Instrumentation and chromatographic conditions

A TSP HPLC system consisting of a vacuum degasser, quaternary pump, autosampler and DAD detector (Thermo Separation Products Inc., Riviera Beach FL, USA) was used for acquiring chromatograms and UV spectra. For chromatographic analyses, an Inertsil C_{18} ODS-3 column (5 μm, 250 mm × 4.6 mm) with a suitable guard column (C_{18}, ODS, 5 μm, 4 mm × 3.0 mm) was used. HPLC separation was performed using a linear gradient at room temperature (20 °C) and a flow rate of 0.7 ml/min. The gradient elution started with a mixture (methanol:water 20:80, v/v) and the methanol content was increased to 100% within 60 min. The injection volume was 20 μl. DAD detector was set to scan from 200 nm to 800 nm, and 270 nm was used as detection wavelength for fingerprint analysis.

ThermoQuest Finnigan LCQ^{DECA} system equipped with an electrospray ionization source (ThermoQuest LC/MS Division, San Jose, CA, USA) was used for mass spectrometric measurements. The ESI MS^n spectra were acquired in both positive and negative ion modes. The mass spectrometry detector (MSD) parameters were as follows: nebulizer sheath gas, N_2 (80 uit); nebulizer auxiliary gas, N_2 (20 uit); capillary temperature, 350 °C; spray voltage, 4500 V in negative ion ESI mode, 5000 V in positive ion ESI mode; capillary voltage, −13 V in (−) ESI, 25 V in (+) ESI; lens voltage, 18 V in (−) ESI, −16 V in (+) ESI; isolation width for the MS^n experiments, 1.0 *m/z*; collision gas, He; and collision energy, 35%. Twenty-five percent triethylamine was introduced from the syringe pump into the column effluent of the LC by using Tee union at the rate of 3 μl/min, mixed with the separated ingredient and then allowed to enter the MSD to help the separated ingredient ionize. All data were processed by Finnigan Xcalibur^{TM} core data system Rev. 1.2 (ThermoQuest Corporation, San Jose, CA, USA).

2.2. Solvents and chemicals

HPLC grade methanol (Merck KGaA, Darmstadt, Germany) and deionized water obtained from a Milli-Q water system (Millipore Corp., Bedford, MA, USA) were used for preparation of mobile phase. All solvents were degassed by ultrasonic and on-line degassing system. Analytical grade triethylamine (Bodi Chemical Engineering Corporation, Tianjin, China) was used to help the separated ingredient ionize. Analytical grade methanol (Yili Chemicals Corporation, Beijing, China) was used for sample preparation.

2.3. Reference compounds

Compounds **1–7** were isolated from dried rhizomes of *G. conopsea* in our laboratory. These compounds were identified by HR-ESIMS, NMR spectral evidence. The ^{1}H NMR and ^{13}C NMR spectral data for compounds **4–7** were shown in Tables 1 and 2.

2.4. Materials and sample preparation

The tested samples of rhizomes of *G. conopsea* were collected from the Chinese provinces of Sichuan, Qinghai, Hebei and Tibet as well as from Nepal, and were authenticated by Professor Zuocheng Zhao (Chengdu Institute of Biology, Chinese Academy of Sciences). The sources of samples are shown in Table 3.

Table 1
^{1}H NMR spectral data for compounds **4**–**7**

Number	**4**	**5**	**6**	**7**
H_A-3	4.48s	4.26s	2.99d (17.7)	2.57d (15.3)
H_B-3			3.11d (17.7)	2.90d (15.3)
H_A-5	1.69m	1.57m	1.70m	1.57m
H_B-5	2.11m	1.77m	1.74m	1.70m
H-6	1.73m	1.57m	1.73m	1.58vm
H_3-7	0.91d (6)	0.72d (6)	0.75d (6)	0.79d (6.6)
H_3-8	0.76d (6)	0.85d (6)	0.85d (6)	0.91d (6.6)
1-*O*-(4-β-D-Glucopyranosyloxy)benzyl moiety				
H_A-7′	4.93d (11.9)	4.82d (12.3)	4.92d (12.0)	4.95d (11.9)
H_B-7′	5.10d (11.8)	4.90d (12.4)	5.12d (11.9)	5.02d (11.9)
H-2′,6	7.28d (9.2)	7.21d (8.4)	7.25d (8.7)	7.25d (8.4)
H-3′,5′	7.10d (8.1)	7.01d (8.4)	7.03d (8.4)	7.06d (8.4)
Glc-1	4.89d (7.4)	4.86d (7.3)	4.86d (7.5)	4.86d (7.2)
Glc-2	3.46m	3.23m	3.24m	3.26m
Glc-3	3.46m	3.27m	3.23m	3.32m
Glc-4	3.39m	3.17m	3.40m	3.21m
Glc-5	3.43m	3.33m	3.24m	3.28m
Glc-6	3.87m	3.68m	3.46m	3.46m
	3.70m	3.46m	3.68m	3.70m
4-*O*-(4-β-D-Glucopyranosyloxy)benzyl moiety				
H_A-7″	5.14d (11.8)	4.91d (12.4)	4.90d (12.1)	4.92d (12.0)
H_B-7″	4.87d (12.0)	5.02d (12.1)	5.05d (12.0)	4.89d (11.8)
H-2″,6″	7.29d (9.0)	7.28d (8.4)	7.29d (8.6)	7.29d (8.6)
H-3″,5″	7.10d (8.1)	7.01d (8.2)	7.03d (8.1)	7.02d (8.1)
Glc-1	4.87d (7.8)	4.86d (7.5)	4.86d (7.5)	4.85d (7.5)
Glc-2	3.46m	3.23m	3.24m	3.24m
Glc-3	3.46m	3.27m	3.22m	3.29m
Glc-4	3.39m	3.17m	3.39m	3.20m
Glc-5	3.46m	3.33m	3.29m	3.26m
Glc-6	3.87m	3.68m	3.66m	3.70m
	3.70m	3.46m	3.46m	3.44m
2-*O*-β-D-Glucopyranosyloxy benzyl moiety				
Glc-1	4.57d (7.1)		4.67d (7.5)	
Glc-2	3.19m		2.96m	
Glc-3	3.16m		3.09m	
Glc-4	3.35m		3.17m	
Glc-5	2.81m		3.35m	
Glc-6	3.68m		3.42m	
	3.62m		3.52m	

The assignment was based on ^{1}H-^{1}H COSY, HMQC and HMBC. Compound **4** was measured in CD_3OD and compounds **5**,**6** and **7** were measured in DMSO-d_6 at 600 MHz.

Table 2
^{13}C NMR spectral data for compounds **4**–**7**

Number	**4**	**5**	**6**	**7**
C-1	172.4s	173.5s	172.6s	174.6s
C-2	84.7s	79.9s	79.9s	75.2s
C-3	74.4d	76.6d	42.0t	44.8t
C-4	170.7 s	171.4s	170.3s	170.2s
C-5	45.4t	44.4t	46.7t	48.0t
C-6	23.5d	23.9d	23.6d	23.7d
C-7	23.3q	23.9q	23.8q	23.3q
C-8	22.7q	24.7q	24.8q	24.7q
1-*O*-(4-β-D-Glucopyranosyloxy)benzyl moiety				
C-7′	67.1t	66.4t	66.7t	66.3t
C-1′	128.9(s)	129.3s	129.4s	129.5s
C-2′,6′	130.1(d)	130.1d	130.3d	129.9d
C-3′,5′	116.6d	116.6d	116.6d	116.4d
C-4′	158.0s	157.8s	157.5s	157.7s
Glc-1	100.9d	100.8d	100.8d	100.9d
Glc-2	73.5d	73.7d	73.6d	73.5d
Glc-3	76.7d	77.1d	76.9d	76.6d
Glc-4	70.0d	70.2d	70.7d	70.0d
Glc-5	76.5d	77.5d	77.2d	76.7d
Glc-6	61.1t	61.2t	61.3t	61.1t
4-*O*-(4-β-D-Glucopyranosyloxy)benzyl moiety				
C-7″	67.0t	66.3t	66.1t	65.8t
C-1″	128.9s	129.4s	129.4s	129.6s
C-2″,6″	130.7d	130.3d	130.3d	129.9d
C-3″,5″	116.5d	116.5d	116.6d	116.4d
C-4″	158.1s	157.8s	157.7s	157.8s
Glc-1	100.9d	100.8d	100.8d	100.9d
Glc-2	73.5d	73.7d	73.6d	73.5d
Glc-3	76.7d	77.1d	76.9d	76.6d
Glc-4	70.0d	70.2d	70.2d	70.0d
Glc-5	76.5d	77.5d	77.2d	76.7d
Glc-6	61.1t	61.2t	61.3t	61.1t
2-*O*-β-D-Glucopyranosyloxy benzyl moiety				
Glc-1	98.1d		98.6d	
Glc-2	74.1d		74.2d	
Glc-3	76.5d		76.6d	
Glc-4	68.8d		69.7d	
Glc-5	75.8d		77.2d	
Glc-6	60.2t		61.3t	

The assignment was based on DEPT, HMQC and HMBC. Compound **4** was measured in CD_3OD and compounds **5**,**6** and **7** were measured in DMSO-d_6 at 125 MHz.

An accurately weighed sample of 5 g grinded powder was introduced into the flask, extracted with 80 ml methanol at a constant temperature of 70 °C for 2 h. The extraction process was repeated three times. The extracts were combined, filtered and evaporated under vacuum, and then diluted to volume with methanol in a 25 ml volumetric flask. A volume of 2 ml of the solution was filtered through a 0.45 μm filter into an HPLC sample vial before analysis. An aliquot of 20 μl solution was injected for HPLC–MS analysis.

2.5. Data analysis of chromatogram

The cosine values of vectorial angle of entire chromatographic patterns among samples were calculated and the simulative mean chromatogram was calculated using the CASES. The

Table 3
Sources of samples

Sample number	Sources
1	Jingchuan, Sichuan, China
2	Kangding I, Sichuan, China
3	Kangding II, Sichuan, China
4	Wenchuan, Sichuan, China
5	Anguo, Hebei, China
6	Qinghai I, China
7	Qinghai II, China
8	Tibet I, China
9	Tibet II, China
10	Nepal

Table 4
The retative retention time (RRT) and relative peak area (RPA) of characteristic peaks

Peak number	Compound	RRT	RPA
1	Adenosine	0.841 ± 0.0013	0.962 ± 0.5299
2	4-Hydroxybenzyl alcohol	1.000	1.000
3	4-Hydroxybenzyl aldehyde	1.808 ± 0.0076	1.302 ± 1.1474
4	Dactylorhin B	2.084 ± 0.0100	0.583 ± 0.1348
5	Loroglossin	2.211 ± 0.0108	0.359 ± 0.0574
6	Dactylorhin A	2.476 ± 0.0149	0.418 ± 0.0680
7	Militarine	2.778 ± 0.0197	0.148 ± 0.0113

The data of RRT and RPA are the radio of t_R and peak area of each characteristic peak to those of peak 2, respectively. The value is mean ± S.D. ($n = 10$).

similarities of the entire chromatographic profiles were analyzed among tested samples. The RRT and RPA of each characteristic peak to reference peak were calculated in the chromatograms.

3. Results and discussion

3.1. Selection of chromatographic conditions

As no HPLC method has been reported for analyzing the chemical constituents of this herb, in order to obtain the optimal elution conditions for the separation and determination of the constituents, various linear gradients of aqueous solution and methanol at a flow rate of 0.7 ml/min were investigated. By the optimal gradient elution, all of the seven main peaks could be well separated.

Selection of detection wavelength was one of the key factors contributing to reliable and reproducible HPLC fingerprints. It was observed that the UV absorption maximum for compounds **1** and **2** were located at 258 nm and 273 nm. The UV absorption maximum for compounds **4**, **5**, **6** and **7** were located at 269 nm. It was also observed that most compounds in the chromatograms possessed strong UV absorbance at 270 nm. Hence, characteristic chromatographic patterns were obtained by using 270 nm as detection wavelength.

The injection precision was evaluated by replicated injection of the same sample solution six times in a day. The R.S.D. of peak areas of seven main peaks were found in the range of 0.13–1.73%, respectively. The repeatability was assessed by analyzing six independently prepared samples. The R.S.D. of peak areas of seven main peaks were found in the range of 1.5–2.9%, respectively. The detection limits (S/N = 3) of the compounds **1**–**7** ranged from 2.21 ng to 40.5 ng. All the results indicated that conditions for the fingerprint analysis were satisfactory.

Table 5
The Similarities of 10 chromatograms

Sample number	Similarities
1	0.97
2	0.99
3	0.97
4	0.88
5	0.90
6	0.99
7	0.84
8	0.95
9	0.90
10	0.95

3.2. Optimization of the MS conditions

The instrumental parameters (sheath gas flow-rate, spray voltage, capillary temperature, capillary voltage, lens voltage)

Table 6
The on-line detected chromatographic and spectrometric data

Peak number	t_R (min)	λ_{max} (nm)	Positive ions in MS (*m/z*)	Negative ions in MS (*m/z*)	Identification
1	9.57	259	–	266$[M-H]^-$ 326$[M+CH_3COO]^-$	Adenosine
2	11.38	273	–	123$[M-H]^-$	4-Hydroxybenzyl alcohol
3	20.57	284	–	135$[M-H]^-$	4-Hydroxybenzyl aldehyde
4	23.70	269	927$[M+Na]^+$, 1006$[M+NH(CH_2CH_3)_3]^+$ 1831$[2M+Na]^+$, 1910$[2M+NH(CH_2CH_3)_3]^+$	903$[M-H]^-$ 963$[M+CH_3COO]^-$ 1807$[2M-H]^-$	Dactylorhin B
5	25.15	269	765$[M+Na]^+$, 844$[M+NH(CH_2CH_3)_3]^+$ 1507$[2M+Na]^+$, 1586$[2M+NH(CH_2CH_3)_3]^+$	741$[M-H]^-$ 801$[M+CH_3COO]^-$ 1483$[2M-H]^-$	Loroglossin
6	28.16	269	911$[M+Na]^+$, 990$[M+NH(CH_2CH_3)_3]^+$ 1799$[2M+Na]^+$, 1878$[2M+NH(CH_2CH_3)_3]^+$	887$[M-H]^-$ 947$[M+CH_3COO]^-$ 1775$[2M-H]^-$	Dactylorhin A
7	31.60	269	749$[M+Na]^+$, 828$[M+NH(CH_2CH_3)_3]^+$ 1475$[2M+Na]^+$, 1554$[2M+NH(CH_2CH_3)_3]^+$	725$[M-H]^-$ 785$[M+CH_3COO]^-$ 1451$[2M-H]^-$	Militarine

were optimized for maximum abundance of the ions of interest by analyzing compound **2**. Triethylamine was added to the solution of compound **2**, leading to the intensified MS response. Therefore, triethylamine was introduced from the syringe pump into the column effluent of the LC by using Tee union at the rate of 3 μl/min to help compounds ionize.

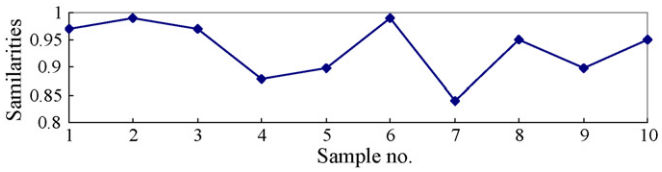


Fig. 2. The similarities of 10 samples of different sources.

3.3. HPLC fingerprints of rhizomes of G. conopsea

To standardize the fingerprint, 10 samples of rhizomes of *G. conopsea* were analyzed. Peaks that existed in all 10 samples were assigned as "characteristic peaks" for rhizomes of *G. conopsea*. There are seven characteristic peaks (from peak 1 to peak 7) in the fingerprint. CASES was used to evaluate these chromatograms. Peaks 2 and 3 were chosen as markers to match peaks and peak 2 was chosen to calculate RRT and RPA. RRT and RPA of characteristic peaks in 10 samples are shown in Table 4. The similarities of chromatograms of 10 samples comparing with the reference fingerprint are close to 1, which are shown in Table 5 and Fig. 2 (The reference fingerprint was

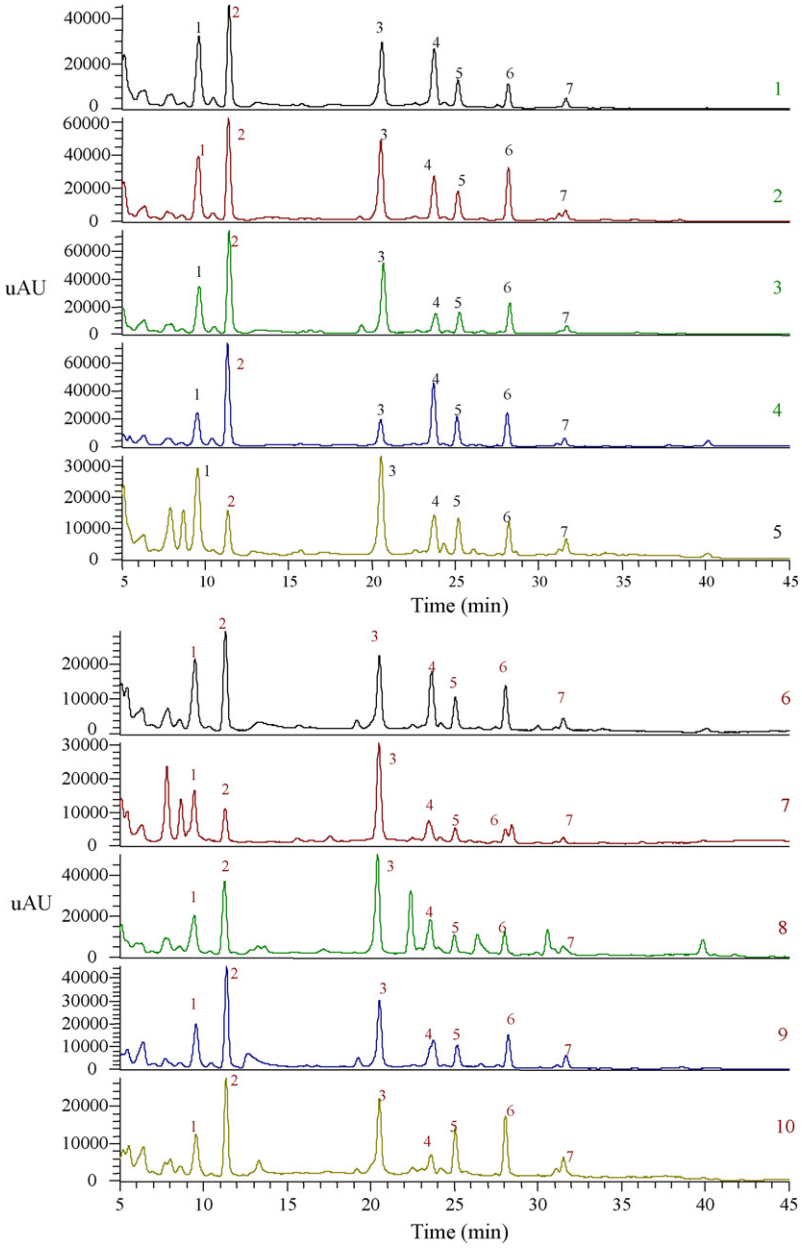
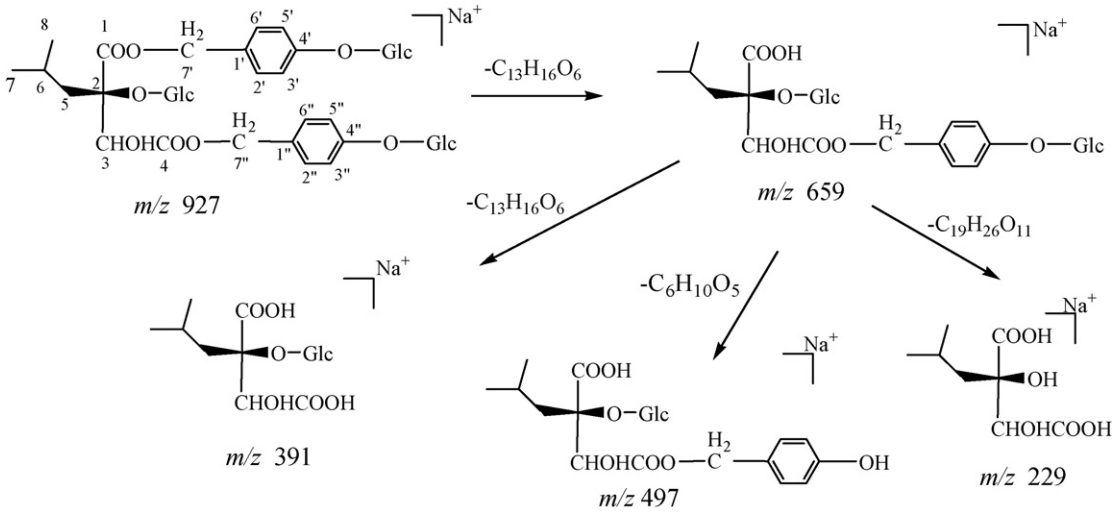


Fig. 3. HPLC chromatograms of 10 samples of different source: (1) Jingchuan, Sichuan, China; (2) Kangding I, Sichuan, China; (3) Kangding II, Sichuan, China; (4) Wenchuan, Sichuan, China; (5) Anguo, Hebei, China; (6) Qinghai I, China; (7) Qinghai II, China; (8) Tibet I, China; (9) Tibet II, China; (10) Nepal.



Scheme 1. Main fragmentation patterns proposed for dactylorhin B (**4**).

developed with the median of all chromatograms). The result indicated that their chromatographic patterns were generally consistent, although the absorption intensities of some peaks were different. Despite the similarity of the main peaks, some differences existed among these samples. The peak of 22.45 min only existed in sample 8. Those peaks eluting before compound **1** also showed variations. The seven common peaks and distinct peak of sample 8 were identified. Those peaks eluting before compound **1** are unknown at present. The chromatograms are shown in Fig. 3.

3.4. Identification of chemical compounds in fingerprint chromatograms

HPLC–DAD–MS^n method was employed to analyze the components in the dried rhizomes of *G. conopsea*. The on-line detected chromatographic and spectrometric data of the common peaks **1**–**7** in the HPLC chromatograms are given in Table 6. Comparing the retention time of UV and ESI MS^n spectra with those of reference compounds, they were unequivocally identified.

Under the optimized MS conditions, both positive and negative modes were used to identify the peaks. The deprotonated molecules $[M-H]^-$ were found in the ESI MS spectra for all compounds. The ions $[M+CH_3COO]^-$, $[2M-H]^-$, $[M+Na]^+$, $[M+NH(CH_2CH_3)_3]^+$, $[2M+Na]^+$ and $[2M+NH(CH_2CH_3)_3]^+$ were also found in the ESI MS spectra for compounds **4**–**7**. The varieties of ions are useful for deducing the molecular mass of each compound.

Compounds **4**–**7** had the same UV maximal absorption wavelengths: 269 nm. They also had the similar fragmentation patterns. MS^2 and MS^3 mass data of compounds **4**–**7** are shown in Fig. 4. The proposed representative fragmentation patterns of compound **4** are shown in Scheme 1. As shown in Scheme 1 and Fig. 4, the positive fragment ions at *m/z* 659 $[M+Na–C_{13}H_{16}O_6]^+$, 497$[M+Na–C_{13}H_{16}O_6–C_6H_{10}O_5]^+$, 391$[M+Na–2C_{13}H_{16}O_6]^+$ and 229 $[M+Na–2C_{13}H_{16}O_6–C_6H_{10}O_5]^+$ are characteristic fragment ions for compound **4**. All these fragment ions indicated the glucopyranosyloxy-benzyl moiety was easily lost. For compound **5**, the strong positive fragment ion at *m/z* 497 $[M+Na–C_{13}H_{16}O_6]^+$ in MS^2 spectrum and the fragment ion at *m/z* 229 $[M+Na–2C_{13}H_{16}O_6]^+$ are also in accordance with the fragment ions of compound **4**. Thus, compound **4** was deduced to be an analogue of compound **5**. The NMR spectral data confirmed that both compounds **4** and **5** were 2, 3-dihydroxy dicarboxylic esters. Meanwhile, compound **6** showed the same fragmentation patterns and produced the characteristic fragment ions at *m/z* 643 $[M+Na–C_{13}H_{16}O_6]^+$, 481 $[M+Na–C_{13}H_{16}O_6–C_6H_{10}O_5]^+$, 375 $[M+Na–2C_{13}H_{16}O_6]^+$ and 213 $[M+Na–2C_{13}H_{16}O_6–C_6H_{10}O_5]^+$. Compound **7** had the same fragment ions at *m/z* 481 $[M+Na–C_{13}H_{16}O_6]^+$ and 213 $[M+Na–2C_{13}H_{16}O_6]^+$ as compound **6**. It indicated that

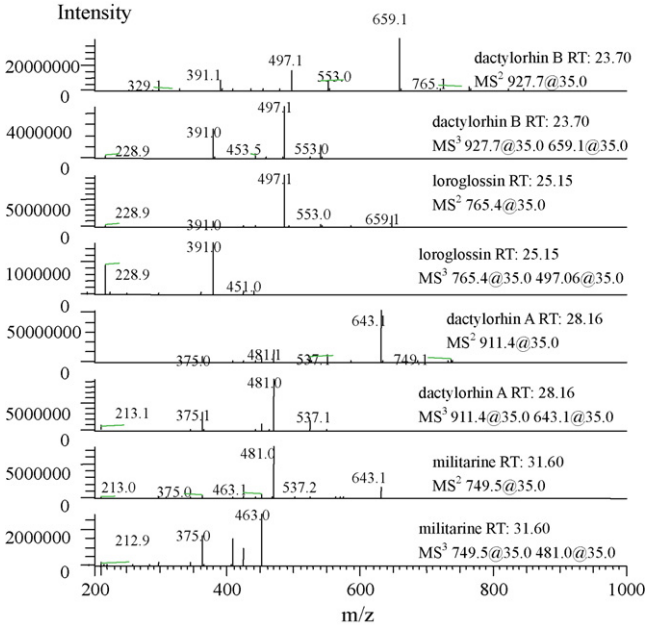


Fig. 4. MS^2 and MS^3 mass spectra of dactylorhin B (**4**), loroglossin (**5**), dactylorhin A (**6**), militarine (**7**).

compound **7** was an analogue of compound **6**. The NMR spectral data confirmed that both compounds **6** and **7** were 2-dihydroxy dicarboxylic esters. The distinct peak in the chromatogram of sample 8 eluted at 22.45 min had the UV maximal absorption wavelength at 274 nm; the deprotonated molecule at *m/z* 135 $[M-H]^-$ indicated that its molecular mass is 136 Da; the negative fragment ions at *m/z* 93 $[M–CH_3CO]^-$ and 121 $[M–CH_3]^-$ were found in MS^2. Comparing these data with those of the reference compound, this peak was unequivocally identified as 4-hydroxyacetophenone.

4. Conclusions

A HPLC–DAD–MS^n method was firstly developed for fingerprint analysis of the rhizomes of *G. conopsea*, a traditional Tibetan remedy and a tonic in Chinese folk medicine. Although there are differences in peak areas of chemical constituents for 10 batches of samples, the chromatograms of different samples were evaluated by CASES to be generally consistent. The consistency in chromatograms of these representative samples reflects the similar chemical constituents. Seven main peaks are the common peaks of the 10 chromatograms and were unequivocally identified by HPLC–DAD–MS^n technique. Compounds **4**, **5**, **6** and **7** are succinate derivative esters and firstly discovered from this species. Under this HPLC condition, characteristic compounds were well separated and had good limits of detection. The technique was proved useful in studies on chemical constituents of the rhizomes of *G. conopsea*.

Previous chemical investigations indicated that some species of Orchidaceous plants contain these succinate derivative esters [15–19]. Furthermore, *Coeloglossum viride* var. *bracteatum* is recorded to be used as the substitute of *G. conopsea* [3]. The main constituents of the rhizomes of *C. viride* var. *bracteatum* were reported to be succinate derivative esters [15,16], which were also found as the major constituents in the rhizomes of *G. conopsea* in this article. The similarity of chemical constituents between the two species may provide an explanation for the substitution of usage.

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