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Short communication

Simultaneous determination of nine major active compounds in *Dracocephalum rupestre* by HPLC

Dong-Mei Ren[a], Zhi Qu[b], Xiao-Ning Wang[a], Jing Shi[a], Hong-Xiang Lou[a,*]

[a] *Department of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University, 44 Wenhuaxi Road, Jinan 250012, PR China*
[b] *Shandong Physical Education College, 2 Wenhuaxi Road, Jinan 250012, PR China*

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ABSTRACT

A new method was developed for the simultaneous determination of nine major constituents in *Dracocephalum rupestre*, including 5,7-dihydroxychromone (**1**), eriodictyol-7-*O*-β-D-glucoside (**2**), luteolin-7-*O*-β-D-glucoside (**3**), naringenin-7-*O*-β-D-glucoside (**4**), apigenin-7-*O*-β-D-glucoside (**5**), eriodictyol (**6**), luteolin (**7**), naringenin (**8**) and apigenin (**9**). The quantitative determination was conducted by reversed phase high-performance liquid chromatography with photodiode array detector (LC–PDA). Separation was performed on an Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm i.d., 5 μm) with gradient elution of acetonitrile and 0.5% aqueous acetic acid. The components were identified by retention time, ultraviolet (UV) spectra and quantified by LC–PDA at 260 nm. All calibration curves showed good linearity ($r^2 > 0.999$) within test ranges. The reproducibility was evaluated by intra- and inter-day assays and R.S.D. values were less than 3.0%. The recoveries were between 95.15 and 104.45%. The limits of detection (LOD) ranged from 0.002 to 0.422 μg/ml and limits of quantification (LOQ) ranged from 0.005 to 1.208 μg/ml, respectively. The identity of the peaks was further confirmed by high-performance liquid chromatography with triple-quadrupole mass spectrometry system coupled with electrospray ionization (ESI) interface. The developed method was applied to the determination of nine constituents in 14 samples of *D. rupestre* collected at various harvesting times. Most compounds accumulated at much higher amounts in about June–July. The satisfactory results indicated that the developed method was readily utilized as a quality control method for *D. rupestre*.

1. Introduction

Dracocephalum rupestre Hance, which belongs to the plant family Labiatae, is a perennial herb that distributes in northern areas of China [1]. As a traditional Chinese medicine, *D. rupestre* has been employed in relieving headache, soothing sore throat, subsiding cough and preventing icterohepatitis [2]. Flavonoids and triterpenoids are the main constituents of *D. rupestre*. Our previous study has demonstrated that the total flavonoids from *D. rupestre* showed protection against oxidative cardiac cell injury [3–5]. The purification of the active fraction afforded mainly nine major components, namely 5,7-dihydroxychromone (**1**), eriodictyol-7-*O*-β-D-glucoside (**2**), luteolin-7-*O*-β-D-glucoside (**3**), naringenin-7-*O*-β-D-glucoside (**4**), apigenin-7-*O*-β-D-glucoside (**5**), eriodictyol (**6**), luteolin (**7**), naringenin (**8**) and apigenin (**9**), respectively. Among the components, **4** could ameliorate apoptosis of H9C2 cell induced by doxorubicin [6]. The eight other components possess various biological activities, such as antimicrobial, anti-inflammatory, antioxidant and antiatherogenic effects [7–14]. Hence, they could be considered as the "marker compounds" for the chemical evaluation or standardization of *D. rupestre*. The development of quality control methods for determination of the major flavonoids in *D. rupestre* was an essential issue for the effective clinical use of this medicinal herb. In this study, a simple and efficient LC–PDA method is proposed for simultaneous determination of nine major components. In order to validate the LC quantitative method, the specificity, linearity, accuracy, precision and limits of detection and quantification of the methodology were investigated. Each peak was further confirmed by high-performance liquid chromatography with mass spectrometry coupled with electrospray ionization interface (LC–ESI-MS). The established method was successfully applied to 14 samples collected at different harvesting seasons.

* Corresponding author. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H.-X. Lou).

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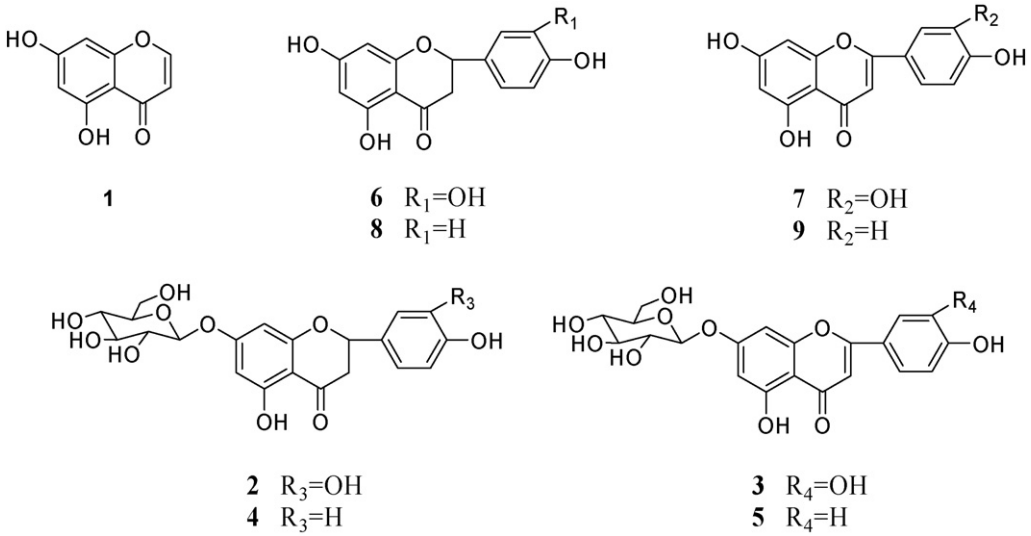

Fig. 1. Structures of compounds **1**–**9**: (**1**) 5,7-dihydroxychromone; (**2**) eriodictyol-7-*O*-β-D-glucoside; (**3**) luteolin-7-*O*-β-D-glucoside; (**4**) naringenin-7-*O*-β-D-glucoside; (**5**) apigenin-7-*O*-β-D-glucoside; (**6**) eriodictyol; (**7**) luteolin; (**8**) naringenin; (**9**) apigenin.

2. Experimental

2.1. Chemicals and materials

Acetonitrile used for HPLC was of chromatographic grade (Fisher Scientific, Fair lawn, NJ, USA). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals used in the study were of analytical grade (Laiyang Chemical Factory, Shandong, China).

The 14 samples of *D. rupestre* were collected from June to October in 2007 from the same field of Xiyang County, Shanxi Province, China. A voucher specimen of these collections has been identified and deposited in School of Pharmaceutical Sciences, Shandong University.

The standard compounds **1**–**9** were isolated by the authors from the aerial parts of *D. rupestre* [3–5]. Their purities were all proved to be above 98% by HPLC analysis. Structures of the nine compounds are shown in Fig. 1.

2.2. LC–PDA analysis

An Agilent 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA), equipped with a quaternary solvent delivery system, an autosampler and a photodiode array detector was used. An Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5 μm) at temperature 25 °C was applied for all analyses. UV absorption was measured at 260 nm. The eluents were A (acetonitrile) and B (0.5% aqueous acetic acid, v/v). The gradient program was used as follows: initial 0–30 min, linear change from A–B (9:91, v/v) to A–B (14:86, v/v); 30–48 min, linear change from A–B (14:86, v/v) to A–B (17:83, v/v); 48–70 min, linear change from A–B (17:83, v/v) to A–B (25:75, v/v) and then held for 10 min. Re-equilibration duration was 15 min between individual runs. The flow rate was 1.0 ml/min and aliquots of 10 μl were injected.

2.3. LC–ESI-MS analysis

As to HPLC–ESI-MS analysis, an API 4000 triple-quadrupole mass spectrometer (Applied Biosystem/MDS Sciex, Concord, ON, Canada) was connected to an Agilent 1100 HPLC instrument coupled with a binary pump, an autosampler and a diode array detector via an ESI interface. The chromatographic conditions were as described above. The API 4000 mass spectrometer was operated in positive ion mode with an ion spray voltage of 4.5 kV, curtain gas of 15 psi, nebulizer gas of 30 psi and auxiliary gas 30 psi. The ion source temperature was set at 200 °C. Ultrapure nitrogen was used as nebulizer, heater, curtain and collision-activated dissociation (CAD) gas. Data were collected and analyzed by the Analyst 1.3 data acquisition and processing software (Applied Biosystems/MDS Sciex).

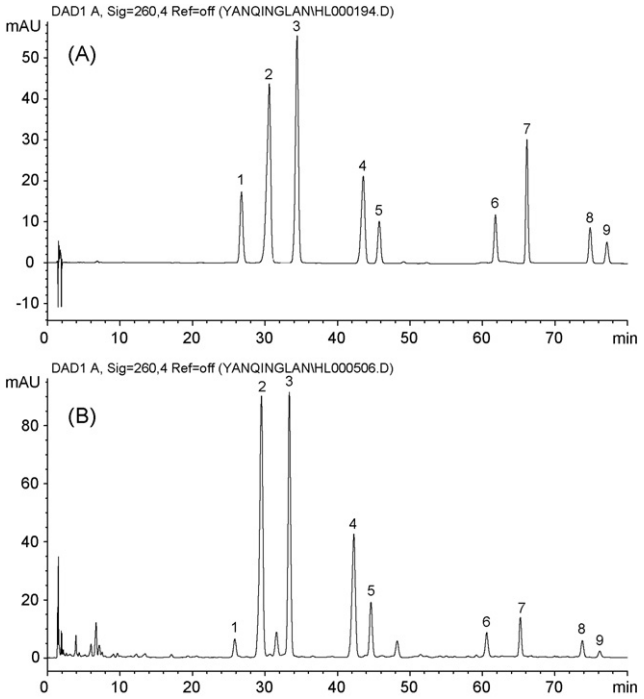

Fig. 2. HPLC chromatograms of standard mixture (A), *Dracocephalum rupestre* sample (B) detected at 260 nm. (**1**) 5,7-Dihydroxychromone; (**2**) eriodictyol-7-*O*-β-D-glucoside; (**3**) luteolin-7-*O*-β-D-glucoside; (**4**) naringenin-7-*O*-β-D-glucoside; (**5**) apigenin-7-*O*-β-D-glucoside; (**6**) eriodictyol; (**7**) luteolin; (**8**) naringenin; (**9**) apigenin.

Table 1
Identity confirmation of the nine major components in *D. rupestre*.

No.	Retention time (min)	Identification	UV λ_{max} (nm)	MS (*m*/*z*)
1	26.3	5,7-Dihydroxychromone	254.1, 295.0	179.1 ([M+H]$^+$)
2	29.8	Eriodictyol-7-*O*-β-D-glucoside	282.7	451.4 ([M+H]$^+$), 289.1 ([M+H-Glu]$^+$)
3	33.7	Luteolin-7-*O*-β-D-glucoside	253, 264.9, 346.2	449.3 ([M+H]$^+$), 287.2 ([M+H-Glu]$^+$)
4	42.9	Naringenin-7-*O*-β-D-glucoside	282.3	435.4 ([M+H]$^+$), 273.2 ([M+H-Glu]$^+$)
5	45.2	Apigenin-7-*O*-β-D-glucoside	265.1, 335.8	433.4 ([M+H]$^+$), 271.3 ([M+H-Glu]$^+$)
6	61.5	Eriodictyol	286.4	289.1 ([M+H]$^+$)
7	65.9	Luteolin	252.7, 262.4, 343.2	287.0 ([M+H]$^+$)
8	74.6	Naringenin	287	273.1 ([M+H]$^+$)
9	76.9	Apigenin	266.7, 336.5	271.2 ([M+H]$^+$)

Table 2
Linear regression data, LOD and LOQ of nine components in *D. rupestre*.

Analyte[a]	Regression equation[b]	r^2	Linear range (μg/ml)	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
1	$Y = 99.8x + 1.27$	0.9999	0.57–11.38	0.002	0.005
2	$Y = 318.3x + 56.33$	0.9990	51.75–1035.00	0.422	1.208
3	$Y = 318.3x + 37.57$	0.9990	14.79–295.80	0.037	0.142
4	$Y = 149.0x + 11.05$	0.9990	47.15–942.90	0.152	0.628
5	$Y = 57.31x + 1.92$	0.9990	2.90–58.00	0.056	0.206
6	$Y = 58.41x + 0.23$	0.9997	4.10–81.96	0.101	0.504
7	$Y = 127.04x + 1.12$	0.9999	0.86–17.25	0.055	0.185
8	$Y = 46.20x - 3.51$	0.9997	2.04–40.80	0.242	0.882
9	$Y = 27.75x + 0.19$	0.9996	0.23–4.58	0.055	0.176

[a] The notation for analyte refers to Fig. 1.
[b] *Y* is the peak area, *x* refers to the concentration of compound (μg/ml). Each regression equation included six data points.
[c] The limits of detection.
[d] The limits of quantification.

2.4. Preparation of samples

The dried powders of *D. rupestre* samples (0.2 g, 40 mesh) were accurately weighed and extracted by refluxing with 10 ml methanol for 60 min. Then the resultant mixture was adjusted to the original weight and the supernatant were filtered through 0.45 μm membrane prior to HPLC injection. All samples were prepared in triplicate.

2.5. Preparation of standard solutions

A mixed methanolic stock solution of standards, containing 5,7-dihydroxychromone (11.38 μg/ml), eriodictyol-7-*O*-β-D-glucoside (1035.00 μg/ml), luteolin-7-*O*-β-D-glucoside (295.80 μg/ml), naringenin-7-*O*-β-D-glucoside (942.90 μg/ml), apigenin-7-*O*-β-D-glucoside (58.00 μg/ml), eriodictyol (81.96 μg/ml), luteolin (17.25 μg/ml), naringenin (40.80 μg/ml) and apigenin (4.58 μg/ml),

Table 3
Recoveries of the nine constituents in *D. rupestre* ($n = 3$).

Compound	Original (μg/ml)	Spiked (μg/ml)	Detected (μg/ml)	Recovery (%)	R.S.D. (%, $n = 3$)
1	0.76	0.61	1.36	98.36	2.87
		0.73	1.46	95.89	2.15
		0.92	1.64	95.65	1.91
2	381.72	305.25	694.78	102.59	3.28
		366.30	759.41	103.11	3.52
		457.88	828.75	97.63	2.89
3	59.91	48.36	107.88	99.19	1.63
		58.03	118.49	100.95	1.23
		72.54	131.52	98.72	1.70
4	103.73	80.10	185.05	101.52	2.07
		96.12	203.27	103.56	1.84
		120.15	226.93	102.54	1.16
5	4.73	3.55	8.16	96.62	1.67
		4.26	8.85	96.71	1.52
		5.33	10.05	99.81	1.38
6	19.42	15.26	34.49	98.75	2.10
		18.31	37.18	97.00	1.07
		22.89	41.20	95.15	1.11
7	2.85	2.20	5.08	101.36	2.21
		2.64	5.55	102.27	1.02
		3.30	6.29	104.24	0.95
8	9.80	8.02	17.88	100.75	2.15
		9.62	19.34	99.17	2.05
		12.03	21.70	98.92	1.73
9	0.53	0.45	1.00	104.45	2.52
		0.54	1.09	103.70	2.19
		0.68	1.23	102.94	2.61

was prepared. The standard stock solution was further diluted with methanol to make six different concentrations including 1, 1/2, 1/4, 1/5, 1/10 and 1/20 of the original concentration. All solutions were stored in a refrigerator at 4 °C before analysis.

2.6. Identification

The peaks in samples were assigned by comparison of the retention time and the UV spectra with the standard compounds with LC–PDA method. Identified peaks were further confirmed by spiking sample with standard mixtures using LC–PDA method and by mass spectra in LC–ESI-MS method.

3. Results and discussion

3.1. Extraction procedure

Various extraction methods, solvents and times were evaluated to obtain the best extraction efficiency. The results revealed that refluxing was better than ultrasonic bath extraction, so the further experiments were carried out with refluxing. Various solvents including water, 10, 20, 40, 50 and 100% methanol were screened. The results showed that pure methanol was the most suitable extraction solvent, which allowed extraction of all the major constituents in high yields. The influence of the extraction time on the efficiency of extraction was also investigated. Powdered samples were extracted with methanol for 20, 30, 40 and 60 min, respectively. The results suggested that the nine constituents were almost completely extracted within 60 min.

3.2. Optimization of separation conditions

Different compositions of mobile phase were tried to obtain chromatograms with good resolution of adjacent peaks. Various mixtures of water and methanol were used as mobile phase but separation was not satisfactory. However, when methanol was replaced by acetonitrile, the situation was greatly improved and satisfactory resolution was obtained. Addition of acid in mobile phase was found to enhance the resolution and eliminate the peak tailing of target compounds. So the type and concentration of acids (0.5% formic acid, 0.5% acetic acid and 0.02% phosphoric acid) were examined. As a result, acetonitrile and water containing 0.5% acetic acid were chosen, as the eluting solvent system that gave desired separation. And gradient elution was applied.

It was also suggested that separation was better when column temperature was kept at 25 °C than 20, 30, 35 and 40 °C.

According to the absorption maxima of nine standards on the UV spectra with three-dimensional chromatograms of HPLC–PDA detection, the monitoring wavelength was set at 260 nm. Representative HPLC chromatograms are shown in Fig. 2.

3.3. Identification of nine constituents in D. rupestre

Utilizing the PDA makes it possible to obtain the UV spectra. The compounds in samples were confirmed by overlapping their spectra with those of the standards at same retention time. Spiking sample with reference compounds performed a further confirmation assay. LC–ESI-MS experiments were performed to further confirm the identity of peaks 1–9 using the conditions described in Section 2.3. For ESI-MS analysis, positive ionization mode was used. Positive MS spectra were dominated by the $[M+H]^+$ ion for all the nine compounds (Table 1). The retention times and mass spectra of products exactly matched with the corresponding standard compounds, which were shown in Table 1.

Table 4
Content of nine major constituents in different *D. rupestre* samples (mean ± S.D., $n = 5$).

No.	Collection time	Content (mg/g)									
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	Total
1	1 June 2007	0.052 ± 0.001	28.461 ± 0.711	4.132 ± 0.121	14.104 ± 0.428	0.525 ± 0.012	1.356 ± 0.043	0.117 ± 0.003	1.121 ± 0.033	0.026 ± 0.000	49.894 ± 1.266
2	10 June 2007	0.076 ± 0.002	38.172 ± 0.920	5.991 ± 0.135	10.373 ± 0.307	0.473 ± 0.010	1.942 ± 0.057	0.285 ± 0.007	0.980 ± 0.032	0.053 ± 0.001	58.345 ± 1.470
3	19 June 2007	0.040 ± 0.001	33.701 ± 0.755	4.433 ± 0.139	19.432 ± 0.576	0.597 ± 0.015	1.096 ± 0.036	0.128 ± 0.004	1.110 ± 0.033	0.032 ± 0.001	60.569 ± 1.622
4	29 June 2007	0.038 ± 0.001	28.955 ± 0.641	4.557 ± 0.130	12.154 ± 0.331	0.529 ± 0.015	0.886 ± 0.027	0.087 ± 0.003	0.634 ± 0.020	0.029 ± 0.001	47.869 ± 1.258
5	9 July 2007	0.100 ± 0.003	28.537 ± 0.663	4.543 ± 0.138	13.350 ± 0.353	0.561 ± 0.016	1.562 ± 0.047	0.511 ± 0.013	1.232 ± 0.035	0.090 ± 0.002	50.486 ± 1.456
6	19 July 2007	0.170 ± 0.003	27.368 ± 0.650	4.689 ± 0.144	12.057 ± 0.330	0.577 ± 0.017	1.475 ± 0.042	0.429 ± 0.013	0.834 ± 0.026	0.081 ± 0.002	47.680 ± 1.255
7	29 July 2007	ND[a]	30.260 ± 0.799	5.571 ± 0.153	16.182 ± 0.501	1.011 ± 0.030	1.230 ± 0.039	0.152 ± 0.005	0.893 ± 0.028	0.100 ± 0.003	55.399 ± 1.487
8	9 Aug 2007	0.067 ± 0.002	25.121 ± 0.545	4.985 ± 0.150	10.423 ± 0.299	0.526 ± 0.017	1.194 ± 0.038	0.196 ± 0.006	0.401 ± 0.012	0.110 ± 0.003	43.023 ± 1.244
9	19 Aug 2007	0.060 ± 0.002	20.094 ± 0.517	2.962 ± 0.095	9.754 ± 0.303	0.583 ± 0.018	0.937 ± 0.029	0.162 ± 0.005	0.644 ± 0.020	0.100 ± 0.003	35.296 ± 1.115
10	29 Aug 2007	0.037 ± 0.001	22.428 ± 0.622	4.399 ± 0.147	6.315 ± 0.210	0.467 ± 0.012	0.856 ± 0.027	0.085 ± 0.003	0.328 ± 0.011	0.091 ± 0.002	35.334 ± 1.112
11	8 Sep 2007	0.034 ± 0.001	24.171 ± 0.614	3.620 ± 0.109	8.702 ± 0.253	0.673 ± 0.018	0.791 ± 0.025	0.068 ± 0.002	0.390 ± 0.012	0.140 ± 0.004	38.589 ± 1.202
12	18 Sep 2007	0.052 ± 0.002	27.352 ± 0.705	4.031 ± 0.120	6.290 ± 0.207	0.262 ± 0.006	0.714 ± 0.022	0.122 ± 0.003	0.491 ± 0.013	0.075 ± 0.002	39.389 ± 1.119
13	28 Sep 2007	0.071 ± 0.003	11.442 ± 0.357	3.974 ± 0.119	6.465 ± 0.205	0.497 ± 0.014	1.046 ± 0.035	0.343 ± 0.011	0.967 ± 0.033	0.171 ± 0.004	24.976 ± 0.827
14	8 Oct 2007	ND	15.561 ± 0.408	2.861 ± 0.100	6.970 ± 0.202	0.182 ± 0.006	0.374 ± 0.011	0.056 ± 0.002	0.182 ± 0.004	0.026 ± 0.000	26.212 ± 0.831

[a] Not detected.

3.4. Method validation

3.4.1. Linearity, limits of detection and quantification

Linear regression analysis for each compound was performed by plotting the peak area versus concentration by the external standard method. The LOD was determined at a signal-to-noise (S/N) ratio of 3, and LOQ was determined at a S/N ratio of 10. The results were reported in Table 2.

3.4.2. Precision and stability

The intra- and inter-day precisions were determined by analyzing known concentrations of the nine analytes in five replicates during a single day and by duplicating the experiments on 3 successive days. The relative standard deviation (R.S.D.) was taken as a measure of precision. The results indicated that the intra- and inter-day R.S.D. values of the nine compounds were all less than 3.0%.

Stability of sample solution was tested at room temperature. The sample solution was analyzed in triplicate every 12 h within 3 days, and the analytes were found to be rather stable in methanol solution within 72 h (R.S.D. < 3.0%).

3.4.3. Recovery

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of *D. rupestre* samples. The resultant samples were then extracted and analyzed by using the proposed procedure. As shown in Table 3, the recoveries of the nine compounds were in the range of 95.15–104.45%, with R.S.D. less than 3.6%.

3.5. Sample analysis

The established analytical method was applied to determine the contents of the 9 constituents in 14 *D. rupestre* samples collected at various harvesting times. The contents of the nine compounds in the samples were quantified and the results are shown in Table 4.

Among the analyzed compounds, eriodictyol-7-*O*-β-D-glucoside, naringenin-7-*O*-β-D-glucoside and luteolin-7-*O*-β-D-glucoside were dominant compounds, with the concentration of 11.442–38.172, 6.291–19.432 and 2.861–5.991 mg/g, respectively. The samples analyzed contained much lower level of the amount of other flavonoids and chromone.

It could also be seen that the total contents of the nine compounds varied with time remarkably. All compounds accumulated at much higher amounts in June–July. This would be helpful to determine the appropriate harvesting time of *D. rupestre*. In the collecting place, the plant began to grow in June. Growth was highest in late July to early August, in September and October it was markedly retarded. The flower stage was from late July to early August. Based on the results mentioned above, it seemed that the best harvesting time was in June and July. But in June, the plant was relatively dwarfed, and the yield would be lower if it was harvested during this period of time. Between the flower bud stage and early flower stage, the plant was flourishing and the amount of analyzed compounds was much higher. It suggested that the most appropriate time to harvest *D. rupestre* for use as a crude drug was during this period of time, that is, from late July to early August.

4. Discussion and conclusion

An LC–PDA method has been developed to simultaneously determine nine major active components in *D. rupestre*. The method showed a good linearity, precision and accuracy, so it was suitable for quality evaluation of *D. rupestre*. The LC–ESI-MS experiment was performed to further confirm the identity of peaks of samples. In addition, 14 samples collected at various harvesting times have been analyzed. The results were helpful to further discussion of the appropriate harvesting seasons of *D. rupestre*.

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