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Short communication

Simultaneous determination of five main active constituents of *Erigeron multiradiatus* by HPLC-DAD–MS

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ABSTRACT

An HPLC-DAD–MS method was developed for simultaneous determination of the five major active constituents in *Erigeron multiradiatus* (Wall.) Benth, namely 6′-*O*-cafferylerigeroside (1), scutellarin (2), apigenin-7-*O*-β-D-glucuronide (3), apigenin (4) and kaempferol (5), respectively. They were identified by ESI-MS and comparisons with literature. A comprehensive validation of the method included tests of sensitivity, linearity, precision and accuracy. The linear regressions were acquired with $r > 0.999$. The precision was evaluated by intra- and inter-day assays, and relative standard deviation (R.S.D.) values were reported within 2.7%. The recovery studies for the quantified compounds were observed in the range of 95.3–102.4% with R.S.D. values less than 2.3%. The overall procedure may be suitable for the qualitative and quantitative analyses of a large number of *E. multiradiatus* samples. Hierarchical clustering analysis based on the characteristics of the 5 investigated compound peaks in HPLC profiles showed that 18 samples were divided into 2 main clusters. The clusters corresponded to their content. The five constituents in *E. multiradiatus* are generally regarded as an index for the quality assessment of this herb.

1. Introduction

Erigeron multiradiatus (Wall.) Benth, a kind of perennial herb of the family Compositae, is mainly distributed in alpine and subalpine meadow of West and Northwest China, at altitudes ranging from 2600 to 4300 m [1]. This plant has been used under "meiduoluomi" in folk medicine. In Himalayan region, people have used this plant for thousands of years to treat various diseases such as hypopepsia, enteronitis, diarrhea, hepatitis, adenolymphitis, rheumatism and hemiparalysis [2–4].

According to the existing literatures and our previous researches on phytochemistry of *E. multiradiatus*, some flavonoids compounds have been identified in *E. multiradiatus* [5–7] and they possess various remarkably pharmacological activities such as neuroprotective effects [8], and anticoagulant effect [9,10]. Hence, they could be considered as the marker compounds for the chemical evaluation or standardization of *E. multiradiatus*. The development of quality control methods for determination of the major flavonoids in *E. multiradiatus* was an essential issue for the effective clinical use of this medicinal herb. Until now, there is only one report of analytical method for the determination of scutellarin in *E. multiradiatus* [11]. However, the therapeutic effect of traditional herbal medicine is due to the synergic effect of its multiple chemical bioactive compounds [12–14]. Unfortunately, only one marker constituent is commonly difficult to stand for the complex herbal medicine [15]. Therefore, the analysis of multiple components is necessary to control the quality of herbal medicine.

In this study, an HPLC-DAD–MS method was developed for simultaneous determination of five major constituents, namely 6′-*O*-cafferylerigeroside (1), scutellarin (2), apigenin-7-*O*-β-D-glucuronide (3), apigenin (4) and kaempferol (5), respectively. Their structures were shown in Fig. 1.

2. Experimental

2.1. Materials and reagents

Samples of *E. multiradiatus* were collected from different natural growth sites in eastern Qinghai-Tibet Plateau of China. These herbs were authenticated by Professor Tianzhi Wang (West China School of Pharmacy, Sichuan University, Chengdu, PR China). Voucher specimens were deposited at Herbarium Center of West China, School of Pharmacy, Sichuan University. Specifications of the samples evaluated in the present study are shown in Table 1.

HPLC grade acetonitrile (TEDIA, Fairfield, OH, USA), HPLC grade water obtained from a deionized water treatment system (Millipore, Bedford, MA, USA) and analytical grade acetic acid (Huada,

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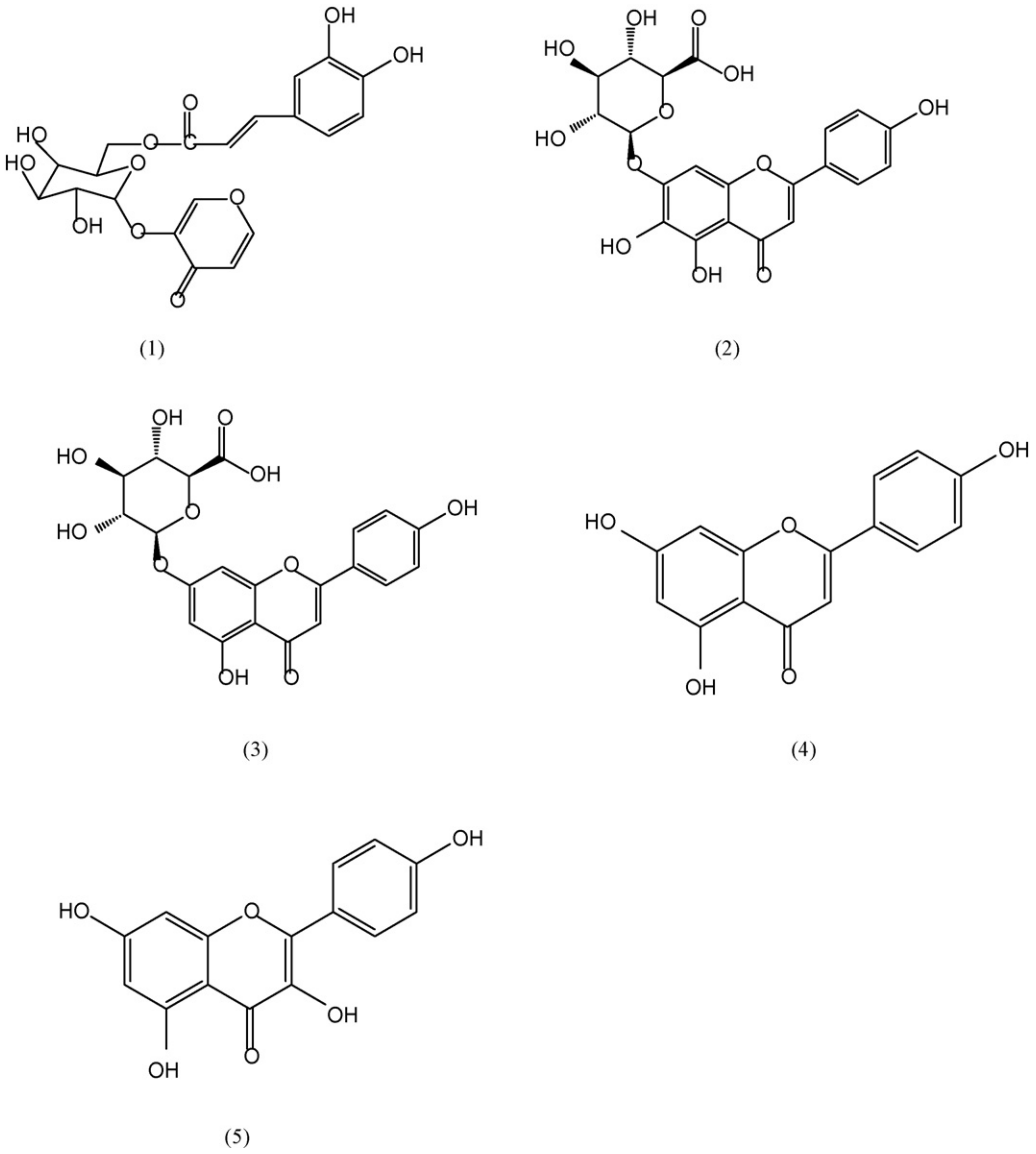

Fig. 1. Structures of five marker compounds isolated and identified from *E. multiradiatus*. 6′-*O*-Cafferylerigeroside (1), scutellarin (2), apigenin-7-*O*-β-D-glucuronide (3), apigenin (4) and kaempferol (5).

Table 1
The specifics and contents of five constituents of *Erigeron multiradiatus* in natural habit

No.	Source and altitude of harvest	Contents of five constituents (%)				
		1	2	3	4	5
01	Sihuochang, Kangding, Sichuan (2750 m)	0.10 ± 0.00	1.30 ± 0.02	0.69 ± 0.01	0.20 ± 0.01	0.18 ± 0.01
02	Haizigou, Kangding, Sichuan (2850 m)	0.04 ± 0.00	0.48 ± 0.00	0.29 ± 0.00	0.07 ± 0.00	0.07 ± 0.00
03	Zheduotang, Kangding, Sichuan (2950 m)	0.05 ± 0.00	0.63 ± 0.01	0.73 ± 0.01	0.10 ± 0.00	0.09 ± 0.00
04	Zhongdian, Yunnan (3400 m)	0.07 ± 0.00	0.72 ± 0.01	0.42 ± 0.00	0.09 ± 0.00	0.11 ± 0.00
05	Yulin, Kangding, Sichuan (2600 m)	0.08 ± 0.00	0.98 ± 0.01	0.69 ± 0.01	0.15 ± 0.00	0.13 ± 0.00
06	Simaqiao, Kangding, Sichuan (2600 m)	0.04 ± 0.00	0.51 ± 0.00	0.30 ± 0.00	0.08 ± 0.00	0.07 ± 0.00
07	Keka, Daofu, Sichuan (3000 m)	0.08 ± 0.00	1.10 ± 0.01	0.76 ± 0.00	0.17 ± 0.00	0.15 ± 0.00
08	Huiyuan temple, daofu, Sichuan (3200 m)	0.01 ± 0.00	0.16 ± 0.00	0.15 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
09	Shangbai, daofu, Sichuan (3200 m)	0.05 ± 0.00	0.62 ± 0.00	0.61 ± 0.00	0.10 ± 0.00	0.08 ± 0.00
10	Shuiqiao, Kangding, Sichuan (3700 m)	0.12 ± 0.01	1.50 ± 0.03	0.44 ± 0.00	0.23 ± 0.01	0.20 ± 0.01
11	Xinduqiao, kangding,Sichuan (3500 m)	0.05 ± 0.00	0.64 ± 0.01	0.17 ± 0.00	0.10 ± 0.00	0.09 ± 0.00
12	Baisang, Kangding, Sichuan (3500 m)	0.03 ± 0.00	0.79 ± 0.00	0.50 ± 0.00	0.12 ± 0.00	0.13 ± 0.00
13	Bamei, Daofu, Sichuan (3500 m)	0.11 ± 0.00	1.27 ± 0.01	0.88 ± 0.01	0.19 ± 0.00	0.17 ± 0.00
14	Yinguanzai, Kangding, Sichuan (3500 m)	0.09 ± 0.00	1.20 ± 0.02	0.68 ± 0.01	0.18 ± 0.00	0.16 ± 0.00
15	Badan, Daofu, Sichuan (3950 m)	0.08 ± 0.00	0.83 ± 0.01	0.36 ± 0.00	0.13 ± 0.00	0.11 ± 0.00
16	Luoguoliangzi, Luhuo, Sichuan (4250 m)	0.05 ± 0.00	0.77 ± 0.00	0.52 ± 0.00	0.12 ± 0.00	0.11 ± 0.00
17	Zheduotang,Kangding, Sichuan (3000 m)	0.07 ± 0.00	1.01 ± 0.02	0.97 ± 0.01	0.15 ± 0.00	0.14 ± 0.00
18	Xinluhai, Dege, Sichuan (4000 m)	0.13 ± 0.01	1.65 ± 0.02	1.16 ± 0.02	0.25 ± 0.01	0.23 ± 0.01

Guangdong, China) were used for preparation of mobile phase. Analytical grade methanol (Huada, Guangdong, China) was used for sample preparation. The reference compounds 1–5 were extracted, isolated and purified from fresh *E. multiradiatus* (No. 12) in our laboratory. The purified compounds were identified by electrospray ionization (ESI)-MS and ^{1}H NMR, ^{13}C NMR spectrometric techniques and comparing with literatures. The purities were found to be greater than 98% based on the percentage of total peak area by HPLC analysis.

2.2. HPLC system and conditions

Experiments were performed on a Shimadzu HPLC N2010 system (Shimadzu Corp., Kyoto, Japan) consisting of a vacuum degasser, autosampler and DAD detector and a LCsolution workstation. For chromatographic analysis, a Shim-pack VP-ODS column (5 μm, 150 mm × 4.6 mm i.d.) with a guard column (5 μm, 7.5 mm × 4.6 mm i.d.) was used. The mobile phase consisted of acetonitrile (A) and 0.4% aqueous acetic acid (v/v, B) using a gradient program of 15–20% A in 0–10 min, 20–25.3% A in 10–30 min, 25.3–40% A in 30–50 min and 40–15% A in 50–60 min. The flow rate was 1.0 ml min^{-1} and column temperature was maintained at 35 °C. The detection wavelength was set at 335 nm for acquiring chromatograms.

2.3. LC–MS system and conditions

An Applied Biosystems/PESCIEX API 365 LC–MS system with an electrospray ionization source (Applied Biosystems, Foster City, CA, USA) was used for mass spectrometric measurements. The HPLC conditions for LC–MS analysis were the same as those used for HPLC-DAD analysis. The mass spectrometer conditions were optimized using flow injection analysis of the standards. For identification of the major constituents in *E. multiradiatus*, all data acquired were processed by Analyst 1.4.2 software. Ionization was achieved by using electrospray in the negative-ion mode with the spray voltage set at 4500 V. Nitrogen was used as nebulizer gas. Desolvation gas (nitrogen) was heated to 500 °C and delivered at a flow rate of 8 l min^{-1}.

2.4. Standard solution and sample preparation

For assay of the five analytes in *E. multiradiatus* herb, the standard stock solutions of 6′-*O*-cafferylerigeroside, scutellarin, apigenin-7-*O*-β-D-glucuronide, apigenin and kaempferol were prepared in 5.0 ml 60% methanol for each analyte and then diluted with methanol to appropriate concentrations for the establishment of calibration curves. All of the standard solutions were kept at 4 °C.

The dried powders of *E. multiradiatus* herb samples (0.5 g, 0.2 mm) were accurately weighed and extracted by ultrasonic with 25 ml 80% methanol for 40 min. Then the resultant mixture was adjusted to the original weight and the supernatant was filtered through a 0.45-μm membrane prior to HPLC injection. An aliquot of 10 μl of solution was injected in triplicate for HPLC analysis. All samples were prepared for analysis in triplicate.

2.5. Linearity

An aliquot of 10 μl of solution for each calibration standard solution was injected in triplicate for HPLC analysis. The calibration curve was constructed by plotting the peak areas versus the concentration for each analyte.

2.6. Limits of detection and quantification

The stock solutions mentioned above were diluted to a series of appropriate concentrations with methanol, and an aliquot of the diluted solutions were injected for HPLC analysis. The limits of detection (LOD) and limits of quantification (LOQ) for each analyte were determined under the present chromatographic conditions at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

2.7. Precision

The intra- and inter-day variations were chosen to deduce the precision of the developed method. For intra-day variability test, the assays were carried out on six replicate samples (No. 12) that were extracted and analyzed during the same day. The inter-day variations were determined for 3 consecutive days. The quantity of each ingredient presented in this herbal sample was determined from corresponding calibration curve. Variations were expressed by the relative standard deviation (R.S.D.).

2.8. Accuracy

Recovery was used to further evaluate the accuracy of the method. The spiking known quantities of the mixed standard solution were added to known amounts of *E. multiradiatus* samples. The resultant samples were extracted and then analyzed with the HPLC procedure described above. The added standard solutions were prepared in three different concentration levels (low, medium and high) within the concentration range of calibration curve and three replicates were performed for the test.

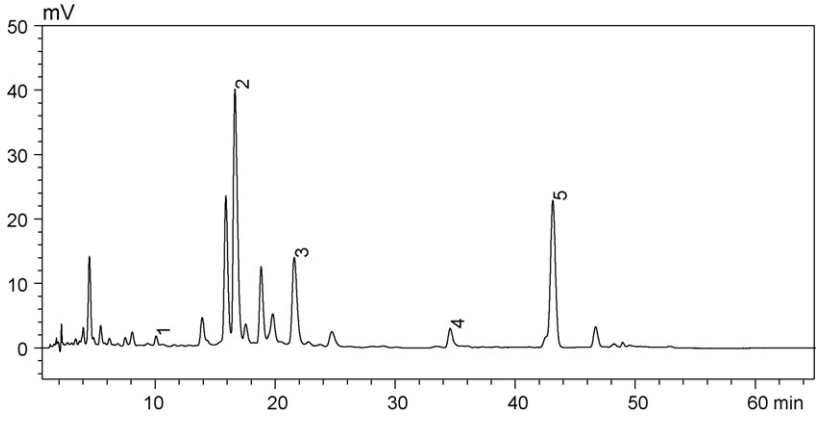

Fig. 2. Typical HPLC chromatogram of *E. multiradiatus* samples at 335 nm.

3. Results and discussion

3.1. Optimization of extraction procedure

Extraction method, extraction solvent and extraction time were investigated in an effort to optimize the extraction procedure. First, heat-refluxing (4 h, 80 °C) was compared with ultrasonic extraction (30 min, 490 W). The results showed that ultrasonic extraction was more effective. Second, different solvents including 20% methanol, 50% methanol, 80% methanol and 100% methanol were used with ultrasonic extraction to evaluate the efficiency of the solvent extraction. Eighty percent methanol was the most suitable extraction solvent; it contributed to extraction of all the constituents in high yields. Finally, the influence of the extraction time on the efficiency of extraction was also optimized. Powdered samples were extracted by ultrasonic extraction with 80% methanol for 10, 20, 40 and 60 min, respectively. Ultrasonic extraction for 40 min obtained optimal results, and there was no obvious difference between 40 and 60 min. In summary, ultrasonic extraction of the samples in 80% methanol for 40 min gave the best extraction efficiency.

3.2. Choice of detection wavelength for HPLC-DAD analysis

By comparing the HPLC chromatograms of *E. multiradiatus* acquired at different wavelengths in the range of 210–400 nm and the corresponding UV absorption maximum for each standard compound, it was found that 335 nm best represents the profile of the major constituents. A representative HPLC chromatogram is shown in Fig. 2.

3.3. Optimization of separation conditions

In order to obtain chromatograms with better resolution of adjacent peaks within shorter time, the chromatographic conditions were optimized. The resolutions of these compounds were tested and compared with different reversed phase conditions using a variety of analytical columns such as Kromasil C_{18} (5 μm, 250 mm × 4.6 mm i.d.), Diamonsil TM C_{18} (5 μm, 250 mm × 4.6 mm i.d.) and Shim-pack VP-ODS (5 μm, 150 mm × 4.6 mm i.d.). Different mobile phase compositions were also optimized (Table 2). Various mixtures of water and methanol were used as mobile phase, but separation was not satisfactory. The resolution was greatly improved and shorter duration of analysis was achieved when methanol was replaced by acetonitrile. A low concentration acid was added to improve the resolution and eliminate the tailing of the target peak. We compared three types of acid: 0.3% trifluoroacetic acid, 0.4% acetic acid and 0.01% phosphoric acid. As a result, a Shim-pack VP-ODS (5 μm, 150 mm × 4.6 mm i.d.) column with acetonitrile and 0.4% aqueous acetic acid (v/v) as the mobile phase was chosen as the preferred chromatographic conditions, and gradient elution was applied. It was also suggested that separation was better when column temperature was kept at 35 °C rather than 20, 25 and 40 °C.

Table 2
The tried mobile phases in selection of solution systems

Systems	Gradient programs
Methanol (A) and water (B)	5–90% A and 95–10% B in 60 min 15–70% A and 85–30% B in 60 min
Methanol (A) and 0.3% trifluoroacetic acid (B)	15–60% A and 85–40% B in 60 min
Methanol (A) and 0.01% phosphoric acid (B)	15–60% A and 85–40% B in 60 min
Methanol (A) and 0.4% acetic acid (B)	5–90% A and 95–10% B in 60 min 15–70% A and 85–30% B in 60 min 15–60% A and 85–40% B in 60 min
Acetonitrile (A) and 0.4% acetic acid (B)	5–60% A and 95–40% B in 60 min 15–65% A and 85–35% B in 60 min 15–60% A and 85–40% B in 60 min 15–60% A and 85–40% B in 90 min

3.4. HPLC–MS identification of major constituents in E. multiradiatus

In this study, the mass spectral conditions were optimized in both positive- and negative-ion modes, and the negative-ion mode

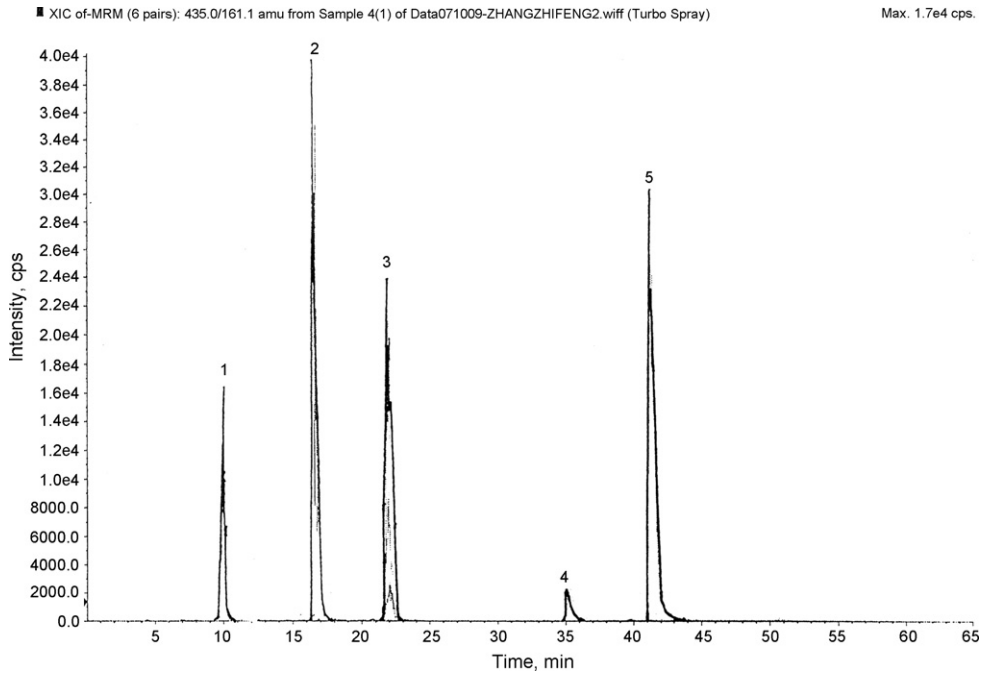

Fig. 3. The TIC chromatogram of *E. multiradiatus* sample in negative-ion mode.

Table 3
The detected mass spectral and spectrometric data

Peak no.	Identified	Major ions m/z for $[M-H]^-$	λ_{max} (nm)	t_R (min)
1	6′-*O*-Cafferylerigeroside	435	213, 252	10.39
2	Scutellarin	461	280, 335	17.02
3	Apigenin-7-O-β-D-glucuronide	445	269, 354	22.01
4	Apigenin	269	267, 330	35.12
5	Kaempferol	285	255, 368	43.38

Table 4
Linear relation between peak area and concentration ($n = 6$)

Compound	Regression equation	r	Linear range (μg/ml)	LOD (ng)	LOQ (ng)
1	$y = 763286x + 1424$	0.9994	5.32–104.84	2.2	8.7
2	$y = 9E+07x - 198043$	0.9998	24.72–492.56	1.7	5.9
3	$y = 2E+08x - 257462$	0.9995	26.53–532.48	1.8	6.2
4	$y = 1E+08x + 62991$	0.9991	12.64–253.74	2.3	2.5
5	$y = 2E+08x - 13572$	0.9993	5.61–112.82	1.5	6.4

Table 5
Intra- and inter-day precision of the five major constituents in *Erigeron multiradiatus*

Compound	Intra-day ($n = 6$)						Inter-day ($n = 3$)	
	Day 1		Day 2		Day 3			
	Calculated content (%)[a]	R.S.D. (%)	Calculated content (%)[a]	R.S.D. (%)	Calculated content (%)[a]	R.S.D. (%)	Calculated content (%)[a]	R.S.D. (%)
1	0.06 ± 0.00	1.2	0.06 ± 0.00	0.8	0.06 ± 0.00	1.4	0.06 ± 0.00	0.6
2	0.79 ± 0.02	2.4	0.77 ± 0.01	2.7	0.78 ± 0.02	2.1	0.79 ± 0.02	2.3
3	0.52 ± 0.01	1.8	0.51 ± 0.01	1.5	0.50 ± 0.01	1.9	0.51 ± 0.01	2.0
4	0.12 ± 0.00	0.7	0.12 ± 0.00	2.4	0.11 ± 0.00	0.3	0.12 ± 0.00	1.1
5	0.11 ± 0.00	1.9	0.11 ± 0.00	0.9	0.11 ± 0.00	0.7	0.10 ± 0.00	0.8

[a] Data were gram constituents per 100 g drug herb.

was found to be more sensitive (Fig. 3). The constituents exhibited quasi-molecular ions $[M-H]^-$ in this mode.

Based on comparisons with standard compounds, five peaks were unambiguously identified as 6′-*O*-cafferylerigeroside (1), scutellarin (2), apigenin-7-*O*-β-D-glucuronide (3), apigenin (4) and kaempferol (5), by comparing their m/z values and UV spectra with the data reported in the literature [5,6]. The results are listed in Table 3.

3.5. Method validation

Suitability of the developed method for its intended use can be concluded from several analytical parameters. All the compounds showed good linearity ($r > 0.999$) within a particular concentration range. Typical equations for the calibration were shown in Table 4. The data of LOD (based on a 10-μl injection) and LOQ for each investigated compounds were found to be below 2.3 and 8.7 ng, indicating that method is sensitive for the quantitative evaluation of major constituents in *E. multiradiatus*. Table 5 indicated that the intra- and inter-day R.S.D. values of the five compounds were all less than 2.7%, showing good precision of the developed method. Accuracy was determined in recovery experiments. As seen in Table 6, all results were within the usually required recovery range of 100 ± 5%, with maximum deviation reached for compound 2 (recovery at low spike: 95.3%) and compound 3 (recovery at high spike: 102.4%), respectively. Relative standard deviations below 2.3% indicated the method accuracy. Considering the results, the method was considered to have good precision and accuracy.

3.6. Application

The developed analytical method was also utilized to simultaneously analyze the 5 constituents in 18 natural samples of *E. multiradiatus*. The contents were calculated with external standard method and the data were summarized in Table 1 with the mean values of three parallel determination. Peaks in the obtained chromatograms were identified by comparing the retention times and UV spectra with those of the standards. Retention time parameters for 1–5 were 10.39, 17.02, 22.01, 35.12 and 43.38 min, respectively. There were remarkable differences among the content of the five constituents from different places. For example, scutellarin was the most dominant constituent in all samples. Its contents varied from 0.48 to 1.65%. The same variation could also be found in other constituents. These results suggested that each procedure involved

Table 6
Recoveries of the five major constituents in *Erigeron multiradiatus* ($n = 3$)

Compound	Added (mg/ml)	Detected (mg/ml)[a]	Recovery (%)[b]	R.S.D. (%)
1	0.004	0.0038 ± 0.0001	95.8	2.3
	0.008	0.0770 ± 0.0006	96.2	0.8
	0.016	0.0156 ± 0.0001	97.5	0.8
2	0.042	0.0400 ± 0.0005	95.3	1.2
	0.084	0.0829 ± 0.0007	98.7	0.8
	0.168	0.1705 ± 0.0020	101.5	1.2
3	0.025	0.0242 ± 0.0002	96.8	0.7
	0.050	0.0496 ± 0.0003	99.2	0.6
	0.010	0.0100 ± 0.0002	102.4	1.5
4	0.005	0.0048 ± 0.0001	96.5	2.3
	0.010	0.0099 ± 0.0002	99.4	1.7
	0.020	0.0196 ± 0.0002	98.1	0.8
5	0.004	0.0038 ± 0.0001	95.7	1.5
	0.008	0.0078 ± 0.0001	97.2	1.3
	0.016	0.0156 ± 0.0001	97.8	0.9

[a] Calculated by subtracting the total amount after spiking from the amount in the herb before spiking. Data were expressed as means of three experiments.

[b] Calculated as detected amount/added amount × 100%. Data were expressed as means of three experiments.

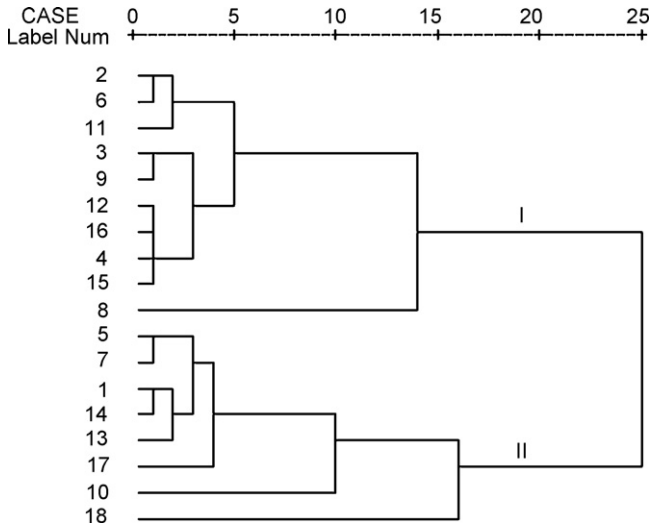

Fig. 4. Dendrograms of hierarchical cluster analysis for the 18 tested samples of *E. multiradiatus*. The hierarchical clustering was done by SPSS 11.5 software. Dendrogram resulting from the five investigated compounds peaks' area derived from HPLC profiles of the tested samples. The 18 samples are the same as in Table 1.

should be standardized to assure the quality, clinic efficacy and safety of *E. multiradiatus*.

3.7. Quality assessment by HCA

In order to evaluate the variation of *E. multiradiatus*, hierarchical cluster analysis (HCA) was performed based on five investigated constituents from HPLC profiles. Fig. 4 shows the result of the 18 tested samples of *E. multiradiatus;* they were divided into two main clusters (I and II) according to their contents (low to high). The chemical variation was obvious among the different locations of *E. multiradiatus.* In fact, the differences in the level of active ingredients among different samples may attribute to the following factors: genetic variation, plant origin, altitude, drying process and storage conditions. However, definite differentiation of these samples may require development of HPLC fingerprint of a large sample size analysis.

4. Conclusion

The present paper described a method for the simultaneous identification and quantification of 5 major components in 18 samples of *E. multiradiatus*. The samples were divided into two clusters based on their contents. The developed method exhibited good precision and accuracy. The five major bioactive constituents in *E. multiradiatus* are generally regarded as a quality index for this herb. The developed method was simple, sensitive and reproducible, and could be used for the comprehensive evaluation of *E. multiradiatus*.

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