Journal of Pharmaceutical and Biomedical Analysis 57 (2012) 133–142

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Chemical analysis of *Eriocaulon buergerianum* and adulterating species by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry

Xue Qiao[a], Guan Ye[b], Chun-Fang Liu[a], Zhi-Xiang Zhang[c], Qiang Tu[c], Jing Dong[d], Yue-Qi Li[d], De-An Guo[a], Min Ye[a,*]

[a] State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xueyuan Road, Beijing 100191, China
[b] Central Research Institute of Shanghai Pharmaceutical Group, Shanghai 201203, China
[c] College of Biological Science and Biotechnology, Beijing Forestry University, Beijing 100083, China
[d] Shimadzu International Trading (Shanghai) Co. Ltd., Beijing Office, Beijing 100020, China

ARTICLE INFO

Article history:
Received 16 May 2011
Received in revised form 9 July 2011
Accepted 22 August 2011
Available online 27 August 2011

Keywords:
Eriocaulon buergerianum
HPLC-DAD-ESI-MS^n
Flavonoids
Naphthopyranones
Chinese herbal medicine

ABSTRACT

Eriocaulon buergerianum Koern. is the botanical source for the Chinese herbal medicine Gu-Jing-Cao. Other *Eriocaulon* species are also used as the same herb in local areas and are difficult to be differentiated. In order to improve the quality control of Gu-Jing-Cao, chemical constituents of *E. buergerianum* and adulterating species were analyzed by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS^n). The 70% methanol extracts were separated on a Zorbax SB-C_{18} column and eluted with acetonitrile–water (each containing 0.1% formic acid). The compounds were identified by ion-trap mass spectrometry in both positive and negative ion modes. From *E. buergerianum*, *E. faberi*, *E. sexangulare* and *E. cinereum*, a total of 72 compounds were characterized, including 37 flavonols, 6 flavones, 4 isoflavones, 6 xanthones, 14 naphthopyranones, 3 phenolic acids, and 2 other flavonoids. Chemical variation of these four species were studied at three tiers, HPLC fingerprinting, quantitation of six major flavonoids, and semi-quantitative analysis of all characterized compounds, in combination with principal component analysis. *E. buergerianum* contained abundant flavonols and naphthopyranones, with minor flavones and xanthones; *E. cinereum* contained abundant isoflavones and flavones, together with few naphthopyranones; *E. sexangulare* was rich in flavones; and *E. faberi* contained abundant xanthones. Based on the above chemical analysis, *E. buergerianum* could be explicitly differentiated from the adulterating species, and the botanical species of 13 commercial batches of Gu-Jing-Cao were identified correctly. This is the first report on comprehensive chemical analysis of *Eriocaulon* species.

1. Introduction

Eriocaulon buergerianum Koern. (Eriocaulaceae Family) is a medicinal herb widely distributed in China. Its capitulae and scapes are used as the Chinese herbal medicine Gu-Jing-Cao (Eriocauli Flos), which is mainly used for the treatment of swelling eyes [1]. It contains flavonoids (flavonols, flavones, isoflavones, xanthones) and naphthopyranones as the major chemical constituents [2–4].

In the monograph of Chinese Pharmacopoeia, no effective analytical methods are available for its quality evaluation [1]. On the other side, the quality of Gu-Jing-Cao crude drug materials in Chinese herb market is bad [5]. A major reason is that multiple botanical species are used as the same herb. The genus of *Eriocaulon* has 435 species throughout the world and 34 in China [6,7]. Aside from the official *E. buergerianum*, at least four other *Eriocaulon* species are used as Gu-Jing-Cao in local areas [8]. Little is known about chemical constituents of these species, so far. Moreover, these adulterating species are very difficult to be differentiated according to their morphological characteristics. The capitulae of all *Eriocaulon* species are similar in size (3–8 mm in diameter), color (greenish yellow to dark brown), and shape (round, ovate, or cone). In order to guarantee therapeutic efficacy in Traditional Chinese Medicine clinical practice, fast and reliable analytical methods are demanded to improve the quality control of Gu-Jing-Cao.

In this study, chemical constituents of *E. buergerianum* and three adulterating species were analyzed by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS^n) and

* Corresponding author. Tel.: +86 10 82802024; fax: +86 10 82802024.
E-mail address: yemin@bjmu.edu.cn (M. Ye).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.08.033

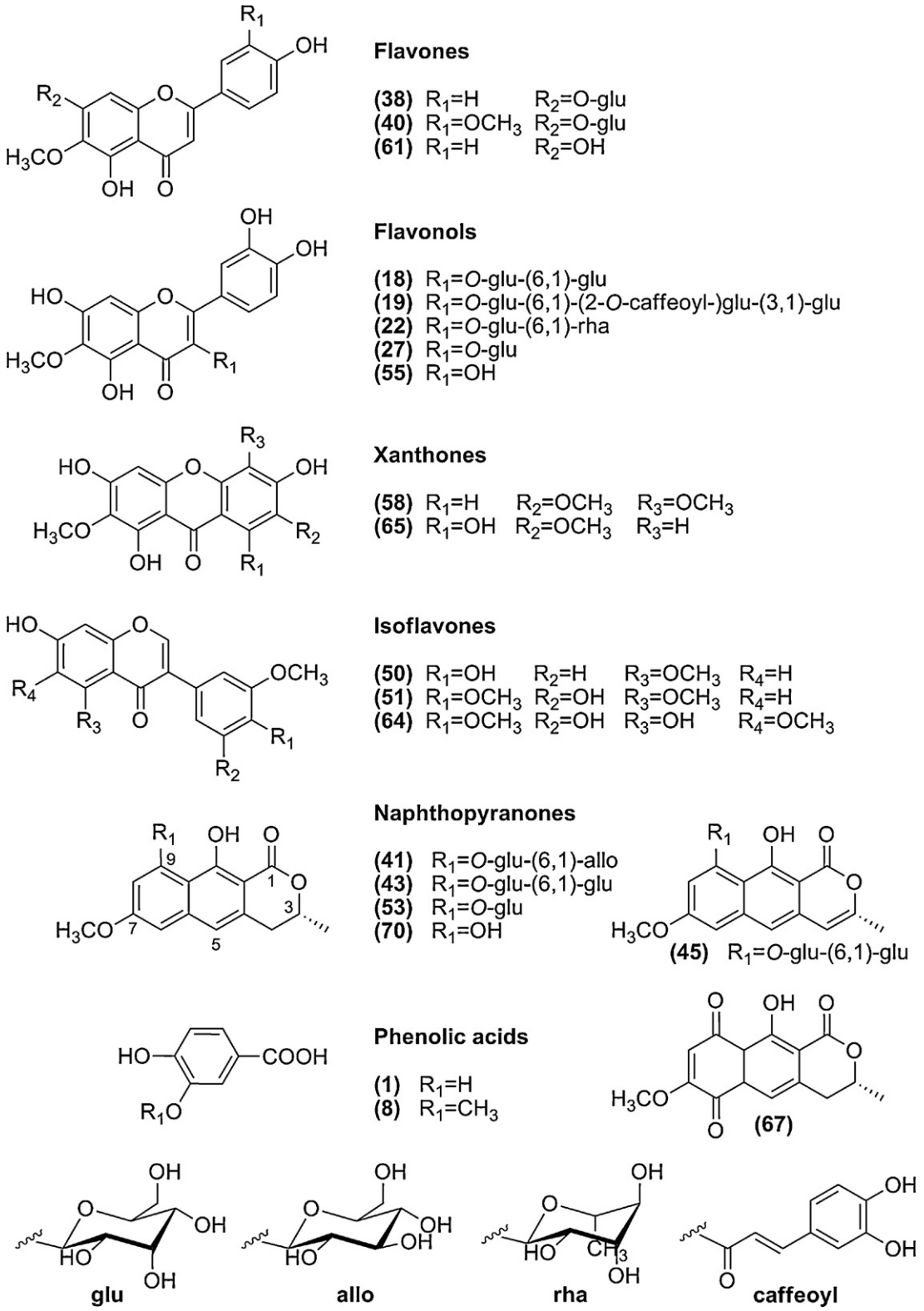

Fig. 1. Structures of reference standards isolated from *Eriocaulon buergerianum*.

liquid chromatography coupled with ion trap time-of-flight mass spectrometry (LC/IT-TOF-MS). The four species were then compared by HPLC fingerprinting, quantitative determination of six major flavonoids, and principal component analysis based on semi-quantification of all identified compounds. Differences among the species were summarized at a global level, rather than based on a few marker compounds [9,10]. This study could not only reveal the chemical composition of different *Eriocaulon* species, but also allowed rapid and correct species identification of Gu-Jing-Cao samples.

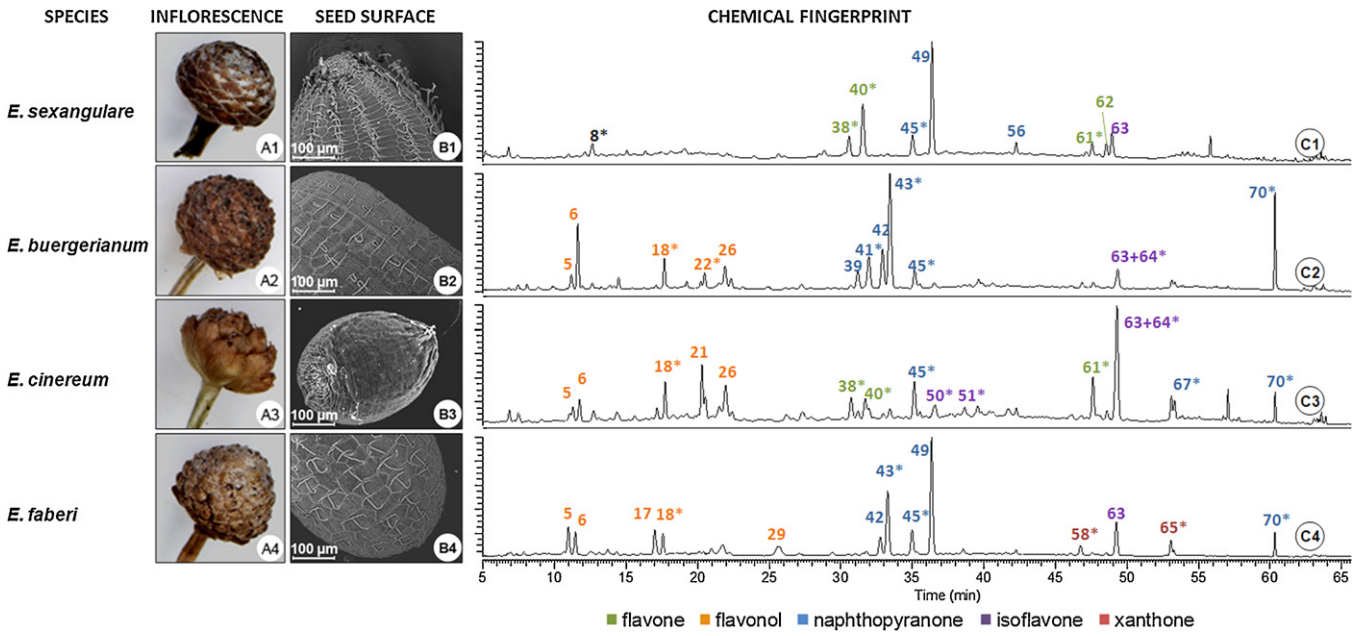

Fig. 2. Inflorescence by microscopy (A), seed surface by scanning electron microscopy (B) and chemical fingerprints by HPLC/UV (C) of four *Eriocaulon* species.

2. Experimental

2.1. Chemicals

Acetonitrile and formic acid were of HPLC grade (J.T. Baker, NJ, USA). De-ionized water was prepared by a Milli-Q system (Millipore, MA, USA). Solvents for sample extraction were of analytical grade. High purity nitrogen (99.9%) and helium (99.99%) were used for MS analysis. Reference compounds (Fig. 1) were isolated from *E. buergerianum* by the authors as previously reported [2]. These compounds were protocatechuic acid (**1**), vanillic acid (**8**), patuletin 3-*O*-*β*-D-gentiobioside (**18**), patuletin 3-*O*-[*β*-D-glucopyranosyl-(1→3)-2-*O*-*E*-caffeoyl-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside] (**19**), patuletin 3-*O*-*β*-D-rutinoside (**22**), patuletin 3-*O*-*β*-D-glucopyranoside (**27**), hispidulin 7-*O*-*β*-D-glucopyranoside (**38**), 5,4′-dihydroxy-6,3′-dimethoxyflavone 7-*O*-*β*-D-glucopyranoside (**40**), (*R*)-semivioxanthin 9-*O*-*β*-D-allopyranosyl-(1→6)-glucopyranoside (**41**), (*R*)-semivioxanthin 9-*O*-*β*-D-glucopyranosyl-(1→6)-glucopyranoside (**43**), toralactone 9-*O*-*β*-D-glucopyranosyl-(1→6)-glucopyranoside (**45**), gerontoisoflavone A (**50**), 7,3′-dihydroxy-5,4′,5′-trimethoxyisoflavone (**51**), (*R*)-semivioxanthin 9-*O*-*β*-D-glucopyranoside (**53**), patuletin (**55**), 1,3,6-trihydroxy-2,5,7-trimethoxyxanthone (**58**), hispidulin (**61**), 5,7,3′-trihydroxy-6,4′,5′-trimethoxyisoflavone (**64**), 1,3,6,8-tetrahydroxy-2,7-dimethoxyxanthone (**65**), (*R*)-semixanthomegnin (**67**), and (*R*)-semivioxanthin (**70**).

2.2. Crude drug materials

Thirteen commercial batches of Gu-Jing-Cao (Eriocauli Flos) were purchased from herb markets or pharmacies around China in 2009–2010 (Table 1S). Voucher specimens (GJC-Sx) were deposited at the authors' laboratory.

2.3. Morphological identification

The capitulae of Eriocauli Flos were cleaned and examined under an OLYMPUS SZX16 microscope (Olympus Corporation, Tokyo, Japan). The capitulae were then gently crushed, and the seeds were observed by scanning electron microscopy (SEM) under a HITACHI TM-1000 tabletop microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

2.4. Sample preparation

Crude materials of Eriocauli Flos were dried, finely powdered, sifted, and extracted in an ultrasonic bath for chemical analysis. For HPLC fingerprinting analysis and LC/MS (liquid chromatography coupled with mass spectrometry) analysis, 0.5 g of the dried powder was extracted in 5 mL of 70% methanol (v/v) for 30 min. For HPLC quantitative analysis, an amount of 0.5 g of the powder was extracted in 25 mL of 70% methanol (v/v) for 60 min. The sample was adjusted to the original mass with 70% methanol and centrifuged at 8000 rpm for 20 min. An aliquot of 20 mL of the supernatant was evaporated to dryness, and then reconstituted in 1 mL of 70% methanol. For both methods, the resulting solutions were filtered through a 0.22 μm membrane before use. A 10-μL aliquot was injected for analysis.

2.5. HPLC quantitative analysis

HPLC analysis was performed on an Agilent series 1100 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler, and a column compartment. Samples were separated on an Agilent Zorbax SB-C_{18} column (5 μm, ID 4.6 mm × 250 mm) protected with an Agilent Zorbax SB-C_{18} guard column (5 μm, ID 4.6 mm × 12.5 mm) (Agilent Technologies Inc., CA, USA). The mobile phase consisted of acetonitrile (A) and water (B), each containing 0.1% (v/v) formic acid. A gradient program was used for the elution: 0 min, 17% A; 12 min, 17% A; 12.5 min, 21% A; 20 min, 21% A; 32 min, 40% A; 40 min, 95% A. Flow rate, 1.0 mL/min; wavelength, 360 nm (scanned from 190 to 400 nm); column temperature, 40 °C.

2.6. LC/MS analysis

The HPLC conditions were the same as described under Section 2.5 except for a few modifications. The gradient elution program was 0 min, 10% A; 7 min, 13% A; 15 min, 18% A; 17–22 min, 19% A;

Table 1
Identification of chemical constituents in *Eriocaulon* species by HPLC-DAD-ESI-MS^n and LC/IT-TOF-MS.

No.	t_R (min)	UV, λ_{max} (nm)	Identification	Formula	Meas. (m/z)	Pred. (m/z)	Diff (mDa)	Diff (ppm)
[a]1	6.83	230, 255, 295	Protocatechuic acid	$C_7H_6O_4$	–	–	–	–
2	8.12	w	Patuletin 3-*O*-hexosyl-hexosyl-hexoside	$C_{34}H_{42}O_{23}$	817.2027	817.2039	−1.20	−1.47
3	9.07	235, 255, 325	Quercetin 3-*O*-hexosyl-deoxyhexosyl-hexoside	$C_{33}H_{40}O_{21}$	771.1977	771.1984	−0.70	−0.91
4	10.47	235, 325	Patuletin 3-*O*-hexosyl-(*O*-deoxyhexosyl-)hexoside	$C_{34}H_{42}O_{22}$	801.2100	801.2089	1.10	1.37
5	10.97	255, 335	Quercetin 3-*O*-hexosyl-(*O*-caffeoyl-)hexosyl-hexoside	$C_{42}H_{46}O_{25}$	949.2244	949.2250	−0.60	−0.63
6	11.46	235, 255, 335	Patuletin 3-*O*-hexosyl-(*O*-caffeoyl-)hexosyl-hexoside	$C_{43}H_{48}O_{26}$	979.2352	979.2356	−0.40	−0.41
7	12.04	240, 255, 320	Demethylsemivioxanthin *C*-hexoside	$C_{20}H_{22}O_{10}$	421.1124	421.1135	−1.10	−2.61
[a]8	12.72	230, 260, 290	Vanillic acid	$C_8H_8O_4$	–	–	–	–
9	13.10	240, 325	Caffeic acid	$C_9H_8O_4$	–	–	–	–
10	13.73	235, 250, 335	Quercetin 3-*O*-hexosyl-methylhexosyl-(*O*-caffeoyl-)hexoside	$C_{43}H_{48}O_{25}$	963.2358	963.2406	−4.80	−4.98
11	14.06	235,255, 275, 345	Quercetagetin 3-*O*-deoxyhexosyl-hexoside	$C_{27}H_{30}O_{17}$	625.1429	625.1405	2.40	3.84
12	14.19	235, 270, 350	Patuletin 3-*O*-hexosyl-methylhexosyl-(*O*-caffeoyl-)hexoside	$C_{44}H_{50}O_{26}$	993.2477	993.2512	−3.50	−3.52
13	14.83	235, 270, 350	Quercetin *O*-pentosyl-*C*-deoxyhexoside	$C_{26}H_{28}O_{15}$	579.1341	579.1350	−0.90	−1.55
14	15.00	235, 255, 275, 345	Quercetagetin 3-*O*-hexoside	$C_{21}H_{20}O_{13}$	479.0835	479.0826	0.90	1.88
15	15.51	235, 270, 350	Quercetin *C*-deoxyhexosyl-*O*-pentoside	$C_{26}H_{28}O_{15}$	579.1333	579.1350	−1.70	−2.94
16	16.30	240, 270, 345	Quercetin *C*-deoxyhexoside	$C_{21}H_{20}O_{11}$	447.0916	447.0927	−1.10	−2.46
17	17.22	235, 275, 345	Quercetin 3-*O*-hexosyl-hexoside	$C_{27}H_{30}O_{17}$	625.1364	625.1405	−4.10	−6.56
[a]18	17.73	255, 345	Patuletin 3-*O*-*β*-D-gentiobioside	$C_{28}H_{32}O_{18}$	655.1499	655.1510	−1.10	−1.68
[a]19	19.14	250, 335	Patuletin 3-*O*-[*β*-D-Glucopyranosyl-(1→3)-2-*O*-*E*-caffeoyl-*β*-D-Glucopyranosyl-(1→6)-*β*-D-Glucopyranoside]	$C_{43}H_{48}O_{26}$	979.2311	979.2356	−4.50	−4.60
20	19.20	240, 270, 345	Patuletin 3-*O*-hexoside	$C_{22}H_{22}O_{13}$	493.0963	493.0982	−1.90	−3.85
21	20.36	255, 355	Quercetin 3-*O*-deoxyhexosyl-hexoside	$C_{27}H_{30}O_{16}$	609.1430	609.1456	−2.60	−4.27
[a]22	20.56	255, 355	Patuletin 3-*O*-*β*-D-rutinoside	$C_{28}H_{32}O_{17}$	639.1535	639.1561	−2.60	−4.07
23	21.10	235, 255, 350	Patuletin 3-*O*-deoxyhexosyl-hexoside	$C_{28}H_{32}O_{17}$	639.1536	639.1561	−2.50	−3.91
24	21.57	260, 350	Patuletin 3-*O*-pentosyl-(*O*-caffeoyl-)hexoside	$C_{36}H_{38}O_{21}$	805.1841	805.1827	1.40	1.74
25	21.85	w	Quercetin 3-*O*-hexosyl-hexoside	$C_{27}H_{30}O_{17}$	625.1403	625.1405	−0.20	−0.32
26	22.07	250, 335	Patuletin 3-*O*-caffeoyl-*O*-hexosyl-hexoside	$C_{37}H_{38}O_{21}$	817.1833	817.1827	0.60	0.73
[a]27	22.49	255, 350	Patuletin 3-*O*-*β*-D-glucopyranoside	$C_{22}H_{22}O_{13}$	493.0999	493.0982	1.70	3.45
28	23.95	250, 330	Quercetin 3-*O*-hexosyl-pentoside	$C_{26}H_{30}O_{17}$	613.1378	613.1405	−2.70	−4.40
29	25.68	255, 330	Patuletin 3-*O*-caffeoyl-*O*-hexosyl-hexoside	$C_{37}H_{38}O_{21}$	817.1826	817.1827	−0.10	−0.12
30	26.28	255	Methylquercetin 3-*O*-deoxyhexosyl-hexoside	$C_{28}H_{32}O_{16}$	623.1587	623.1612	−2.50	−4.01
31	26.28	255, 345	Methylpatuletin 3-*O*-deoxyhexosyl-hexoside	$C_{29}H_{34}O_{17}$	653.1687	653.1718	−3.10	−4.75
32	26.28	250, 320	Patuletin 3-*O*-deoxyhexosyl-(*O*-caffeoyl-)hexoside	$C_{37}H_{38}O_{20}$	801.1858	801.1878	−2.00	−2.50
33	27.44	250, 335	Quercetin 3-*O*-methylhexosyl-(*O*-caffeoyl-)hexoside	$C_{37}H_{38}O_{20}$	801.1840	801.1878	−3.80	−4.74
34	27.44	250, 335	Patuletin 3-*O*-methylhexosyl-(*O*-caffeoyl-)hexoside	$C_{38}H_{40}O_{21}$	831.1979	831.1984	−0.50	−0.60
35	28.53	265, 340	6-Methoxyluteolin 7-*O*-hexoside	$C_{22}H_{22}O_{12}$	477.1031	477.1033	−0.20	−0.42
36	28.88	240, 280, 335	6,4′-Dimethoxyquercetin 3-*O*-(trihydroxy-cinnamoyl-)glucoside	$C_{32}H_{30}O_{17}$	685.1367	685.1405	−3.80	−5.55
37	29.34	235, 270, 335	Methylquercetin 3-*O*-hexosyl-pentoside	$C_{27}H_{32}O_{17}$	627.1558	627.1561	−0.30	−0.48
[a]38	30.83	240, 270, 335	Hispidulin 7-*O*-*β*-D-glucopyranoside	$C_{22}H_{22}O_{11}$	461.1092	461.1084	0.80	1.73
39	31.30	260, 355	Semivioxanthin 9-*O*-hexosyl-hexoside	$C_{27}H_{34}O_{15}$	597.1790	597.1819	−2.90	−4.86

Table 1 (*Continued*)

No.	t_R (min)	UV, λ_{max} (nm)	Identification	Formula	Meas. (*m*/*z*)	Pred. (*m*/*z*)	Diff (mDa)	Diff (ppm)
[a]40	31.79	255, 270, 345	5,4′-Dihydroxy-6,3′-dimethoxyflavone 7-*O*-*β*-D-glucopyranoside	$C_{23}H_{24}O_{12}$	491.1207	491.1190	1.70	3.46
[a]41	32.08	260, 355	(*R*)-semivioxanthin 9-*O*-*β*-D-allosyl-(1→6)-glucoside	$C_{27}H_{34}O_{15}$	597.1792	597.1819	−2.70	−4.52
42	33.02	260, 355	Semivioxanthin 9-*O*-hexosyl-hexoside	$C_{27}H_{34}O_{15}$	597.1801	597.1819	−1.80	−3.01
[a]43	33.52	260, 355	(*R*)-semivioxanthin 9-*O*-*β*-D-glucosyl-(1→6)-glucoside	$C_{27}H_{34}O_{15}$	597.1790	597.1819	−2.90	−4.86
44	34.08	255, 335	Patuletin 3-*O*-caffeoyl-*O*-deoxyhexosyl-hexoside	$C_{37}H_{38}O_{20}$	801.1827	801.1878	−5.10	−6.37
[a]45	35.12	280, 385	Toralactone 9-*O*-*β*-D-glucosyl-(1→6)-glucoside	$C_{27}H_{32}O_{15}$	595.1649	595.1663	−1.40	−2.35
46	35.55	255, 335	Quercetin 3-*O*-caffeoyl-*O*-deoxyhexosyl-hexoside	$C_{36}H_{36}O_{19}$	771.1751	771.1773	−2.20	−2.85
47	35.62	260, 310, 370	Semixanthomegnin *O*-hexoside	$C_{21}H_{22}O_{11}$	449.1098	449.1084	1.40	3.12
48	35.64	250, 335	Patuletin 3-*O*-caffeoyl-*O*-deoxyhexosyl-hexoside	$C_{37}H_{38}O_{20}$	801.1831	801.1878	−4.70	−5.87
49	36.36	280, 385	Toralactone 9-*O*-hexosyl-hexoside	$C_{27}H_{32}O_{15}$	595.1635	595.1663	−2.80	−4.70
[a]50	36.53	255	Gerontoisoflavone A	$C_{17}H_{14}O_6$	313.0702	313.0712	−1.00	−3.19
[a]51	38.64	255	7,3′-Dihydroxy-5,4′,5′-trimethoxyisoflavone	$C_{18}H_{16}O_7$	343.0802	343.0818	−1.60	−4.66
52	39.33	255, 325	1,3,6-Trihydroxy-5,7-dimethoxyxanthone	$C_{15}H_{12}O_7$	303.0495	303.0505	−1.00	−3.30
[a]53	39.37	260, 325	(*R*)-Semivioxanthin 9-*O*-*β*-D-glucopyranoside	$C_{21}H_{24}O_{10}$	435.1294	435.1291	0.30	0.69
54	40.07	245, 300, 325	7, 4′-Dihydroxyflavone *O*-hexoside	$C_{21}H_{20}O_9$	415.1043	415.1029	1.40	3.37
[a]55	41.58	255, 370	Patuletin	$C_{16}H_{12}O_8$	331.0438	331.0454	−1.60	−4.83
56	42.38	280, 380	Toralactone 9-*O*-glucoside	$C_{21}H_{22}O_{10}$	433.1139	433.1135	0.40	0.92
57	44.26	245, 280, 325	1,3,6,8-Tetrahydroxy-2-methoxyxanthone *C*-hexoside-(*O*-benzoyl-*O*-2-hydroxyphenol)	$C_{36}H_{30}O_{18}$	749.1331	749.1354	−2.30	−3.07
[a]58	46.09	244, 260, 326	1,3,6-Trihydroxy-2,5,7-trimethoxyxanthone	$C_{16}H_{14}O_8$	333.0607	333.0610	−0.30	−0.90
59	46.86	320, 365	1,3,6-Trihydroxy-2,5-dimethoxyxanthone	$C_{15}H_{12}O_7$	303.0520	303.0505	1.50	4.95
60	47.22	260	Emodin	$C_{15}H_{10}O_5$	269.0440	269.0450	−1.00	−3.72
[a]61	47.60	230, 275, 335	Hispidulin	$C_{16}H_{12}O_6$	299.0545	299.0556	−1.10	−3.68
62	48.63	250, 265, 345	4′,5,7-Trihydroxy-3′,6-dimethoxyflavone	$C_{17}H_{14}O_7$	329.0646	329.0661	−1.50	−4.56
63	49.34	265	Iristectorigenin A	$C_{17}H_{14}O_7$	329.0676	329.0661	1.50	4.56
[a]64	49.34	265	5,7,3′-Trihydroxy-6,4′,5′-trimethoxyisoflavone	$C_{18}H_{16}O_8$	359.0767	359.0767	0.00	0.00
[a]65	53.10	215, 260, 330	1,3,6,8-Tetrahydroxy-2,7-dimethoxyxanthone	$C_{15}H_{12}O_8$	319.0446	319.0454	−0.80	−2.51
66	53.33	250, 330	1,3,6,8-Tetrahydroxy-2-methoxyxanthone	$C_{14}H_{10}O_7$	289.0325	289.0348	−2.30	−7.96
[a]67	53.66	250, 330	(*R*)-Semixanthomegnin	$C_{15}H_{12}O_6$	287.0547	287.0556	−0.90	−3.14
68	54.09	250, 325	Semixanthomegnin isomer	$C_{15}H_{12}O_6$	287.0555	287.0556	−0.10	−0.35
69	59.04	w	3′,4′-Methylenedioxy-5,7-dimethoxyflavan	$C_{18}H_{18}O_5$	313.1058	313.1076	−1.80	−5.75
[a]70	60.37	260, 365	(*R*)-Semivioxanthin	$C_{15}H_{14}O_5$	273.0754	273.0763	−0.90	−3.30
71	60.65	w	Hesperetin 7-methyl ether	$C_{17}H_{16}O_6$	315.0855	315.0869	−1.40	−4.44
72	61.32	w	Patuletin isomer	$C_{16}H_{12}O_8$	331.0468	331.0454	1.40	4.23

w, signal too weak; –, not available.

[a] Identified by comparing with reference standards.

32 min, 24% A; 50 min, 40% A; 65 min, 95% A. Flow rate, 1.0 mL/min; wavelength, 262 nm (scanned from 190 to 400 nm); column temperature, 20 °C.

A Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFisher, CA, USA) was connected to the HPLC system via an ESI interface. Collision gas, high purity helium; nebulizing gas, high purity nitrogen; post-column splitting ratio to source, 4:1. Source-dependent parameters were as follows: sheath gas (N_2), 50 arb; auxiliary gas (N_2), 10 arb; spray voltage, 4.5 kV; capillary temperature, 330 °C; capillary voltage, 20 V/−10 V (positive/negative mode); tube lens offset voltage, 60 V/−45 V (positive/negative mode). MS full scan range, *m*/*z* 150–1200; Collision energy for collision induced dissociation (CID), 35%; Source-fragmentation voltage, 0 V/15 V (positive/negative mode); isolation width, 2.0 Th.

For high-accuracy mass determination, an LC/IT-TOF-MS system consisting of an LC-20AD pump, an SIL-20AC autosampler, a CTO-20A column oven, an ESI source, and an IT-TOF mass spectrometer (Shimadzu, Tokyo, Japan) was used. The HPLC conditions were the same as described above. The post-column splitting ratio to ESI source was 2:1. The IT-TOF-MS parameters were set as follows: collision and cooling gas, high purity argon (Ar); nebulizing gas, high purity nitrogen (N_2, 1.5 L/min); curved desolvation line temperature, 200 °C; electrospray ionization, negative mode; interface voltage, −3.5 kV; detector voltage, 1.7 kV; endcap acceleration,

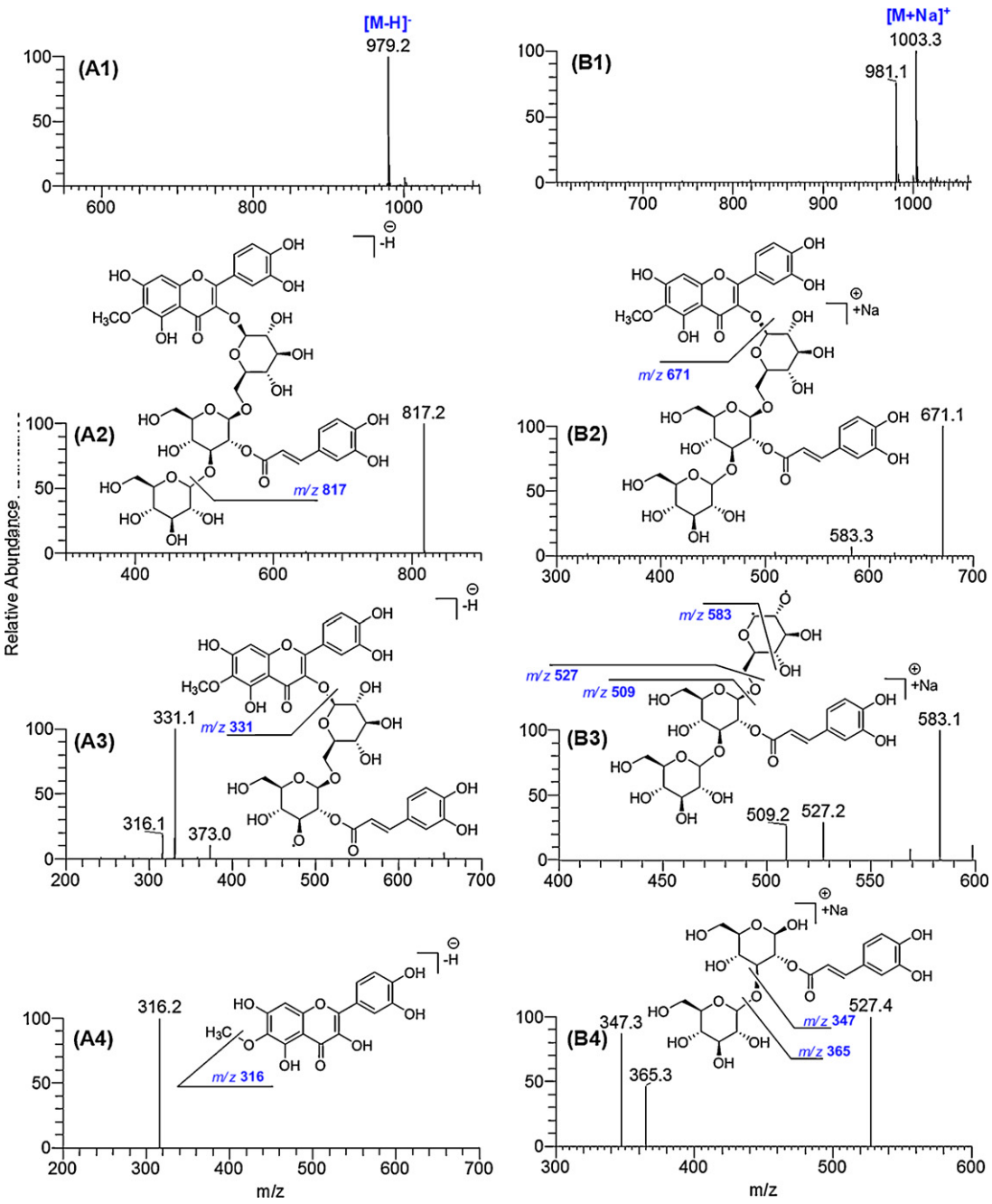

Fig. 3. ESI-MS^n spectra of patuletin 3-*O*-glucosyl-(1→6)-(2-*O*-caffeoyl-)glucosyl-(1→6)-glucoside (**19**) in negative and positive ion modes. (A1) full scan mass spectrum in the negative mode; (A2) MS/MS spectrum of *m*/*z* 979; (A3) MS^3 spectrum of *m*/*z* 817; (A4) MS^4 spectrum of *m*/*z* 331; (B1) full scan mass spectrum in the positive mode; (B2) MS/MS spectrum of *m*/*z* 1003; (B3) MS^3 spectrum of *m*/*z* 671 and (B4) MS^4 spectrum of *m*/*z* 583.

3.5 V; flight tube voltage, +7.0 kV; CID energy, 50%; CID gas, 50%. MS full scan range, *m*/*z* 220–2000; MS^n range, *m*/*z* 220–1200; precursor ion isolation width, 3.0 Th. The mass analyzer was calibrated by external standard method with a 0.05 mg/mL methanol solution of patuletin 3-*O*-glucosyl-(1→6)-glucoside (**18**, $C_{28}H_{32}O_{18}$, $[M-H]^-$ *m*/*z* 655.1510).

2.7. Data processing and statistical analysis

HPLC and LC/MS data were processed with Agilent ChemStation (Agilent, Waldbronn, Germany) and Xcalibur™ 2.0.7 software (ThermoFinnigan, San Jose, CA, USA), respectively. Accurate mass data were recorded and processed by LC/MS solution V3.41 software including a formula predictor (Shimadzu, Tokyo, Japan). Principal component analysis was conducted with SPSS Statistics 17.0 (SPSS Inc., IL, USA), using peak areas in extracted ion chromatograms (EIC) for all identified compounds.

3. Results and discussion

3.1. Morphological identification by SEM

A total of 25 batches of Gu-Jing-Cao (Eriocauli Flos) were collected from herb markets or pharmacies around China. Although the inflorescence (capitulae) and scapes were slightly different in shape, size and color, the botanical species were difficult to be identified (Fig. 2A). Currently, scanning electron microscopy is the most authentic method to identify *Eriocaulon* species [7,11].

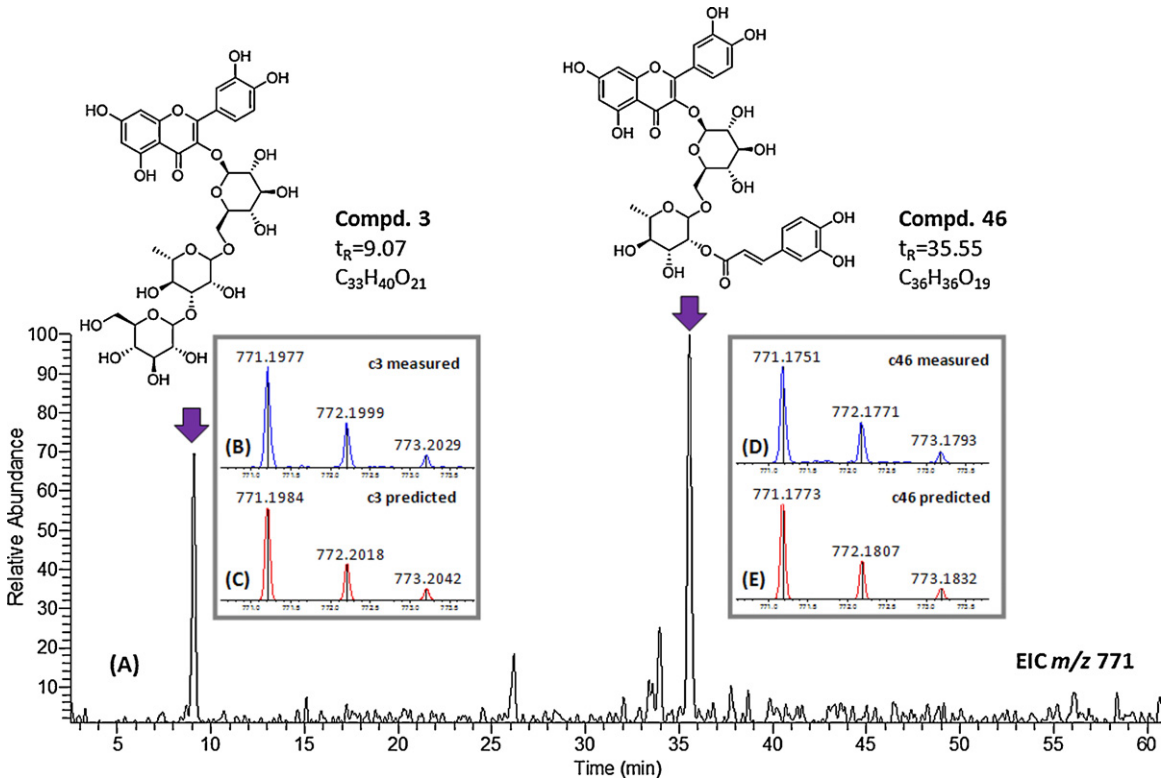

Fig. 4. Differentiation of compounds **3** and **46** by time-of-flight mass spectrometry. (A) Extracted ion chromatogram of *m*/*z* 771; (B, D) measured mass; (C, E) predicted mass.

By using seed surface SEM, we found that 13 batches (Table 1S) were derived from four *Eriocaulon* species, i.e. *E. buergerianum* (7 batches), *E. faberi* (1 batch), *E. sexangulare* (3 batches), and *E. cinereum* (2 batches) according to seed shape and girds on seed surfaces (Fig. 2B, Table 2S). The other 12 batches contained mixed species. Therefore, the 13 single-species batches were further studied in later chemical analysis. SEM ensured all analyzed samples as genuine species which improved the reliability of chemical variation. Subsequently, the three-tier chemical analysis was performed to identify *Eriocaulon* species.

3.2. HPLC fingerprinting analysis

Sample preparation and HPLC conditions were optimized. For sample preparation, various solvents (methanol, aqueous methanol, chloroform, cyclohexane) and extraction methods (maceration, heat-reflux, ultrasonic bath, Soxhlet extraction) were tested. Peak areas of ten major constituents were compared. The most efficient method was ultrasonic extraction with 70% methanol. For HPLC analysis, several C_{18} columns (Zorbax SB-C_{18}, Agilent, Agilent Technologies Inc., CA, USA; Extend-C_{18}, Agilent, Agilent Technologies Inc., CA, USA; Atlantis T3, Waters, Waters Corporation, MA, USA) were tested. Atlantis T3 and Zorbax SB-C_{18} performed similarly in chromatographic separation, and the latter was selected because the analysis time was shorter. Moreover, column temperatures of 20 °C, 30 °C and 40 °C were compared. The 20 °C condition provided desirable separation for hydrophilic constituents, and was hence used. In addition, the ultraviolet (UV) detection wavelength was optimized (230 nm, 262 nm, 300 nm, 360 nm, and photo-diode array detection). Flavonoids quantitation was monitored at 360 nm, which was the absorption maximum for flavonoids. HPLC fingerprints were monitored at 262 nm, which could show more peaks other than flavonoids. Detailed data are given in the supplementary material (Fig. 1S–5S).

HPLC/UV fingerprints for 13 batches of Gu-Jing-Cao were established (Fig. 6S) as the first step of the three-tier solution. Representative fingerprints of the four species are shown in Fig. 2C. The chemo-types of major peaks were labeled in different colors, and their identification will be discussed later. Although differences in HPLC/UV fingerprints of these species were observable, identities of the peaks were unknown, so far. Therefore, HPLC-DAD-ESI-MS^n was employed to characterize the HPLC peaks as a complement.

3.3. Identification of Eriocaulon compounds by HPLC-DAD-ESI-MS^n and LC/IT-TOF-MS

Previous to the identification of unknown *Eriocaulon* compounds, tandem mass spectrometry of 21 reference compounds were studied to understand the fragmentation pathways. Each compound (0.1 mg/mL in methanol) was individually injected into the ESI source by continuous infusion. The $[M-H]^-$, $[M+H]^+$ or $[M+Na]^+$ ions were selected to produce MS/MS and MS^n fragments (collision energy 30–40%).

The tandem mass spectrometry fragmentation pathways of flavonoids and their glycosides were consistent with literature reports [12–14]. Both positive and negative ion modes were used because they provided complementary structural information. Here we discuss the fragmentation of patuletin 3-*O*-glucosyl-(1→6)-(2-*O*-caffeoyl-)glucosyl-(1→3)-glucoside (**19**, MW 980) as an example. In the negative mode, the $[M-H]^-$ ion at *m*/*z* 979 yielded *m*/*z* 817 by losing 162 u, which could be attributed to either the glucosyl- or the caffeoyl-group at the terminal position. This neutral loss was finally confirmed to be glucosyl group by high-resolution mass spectrometry. The parent ion *m*/*z* 979.2311 ($C_{43}H_{48}O_{26}$, Δ = −4.50 ppm) yielded *m*/*z* 817.1847 ($C_{37}H_{38}O_{21}$, Δ = 2.45 ppm) by losing $C_6H_{10}O_5$ (Fig. 3A). In the MS^3 spectra, the *m*/*z* 817 ion could lose the sugar moiety to yield the aglycone ion at *m*/*z* 331, which could further lose a methyl group to

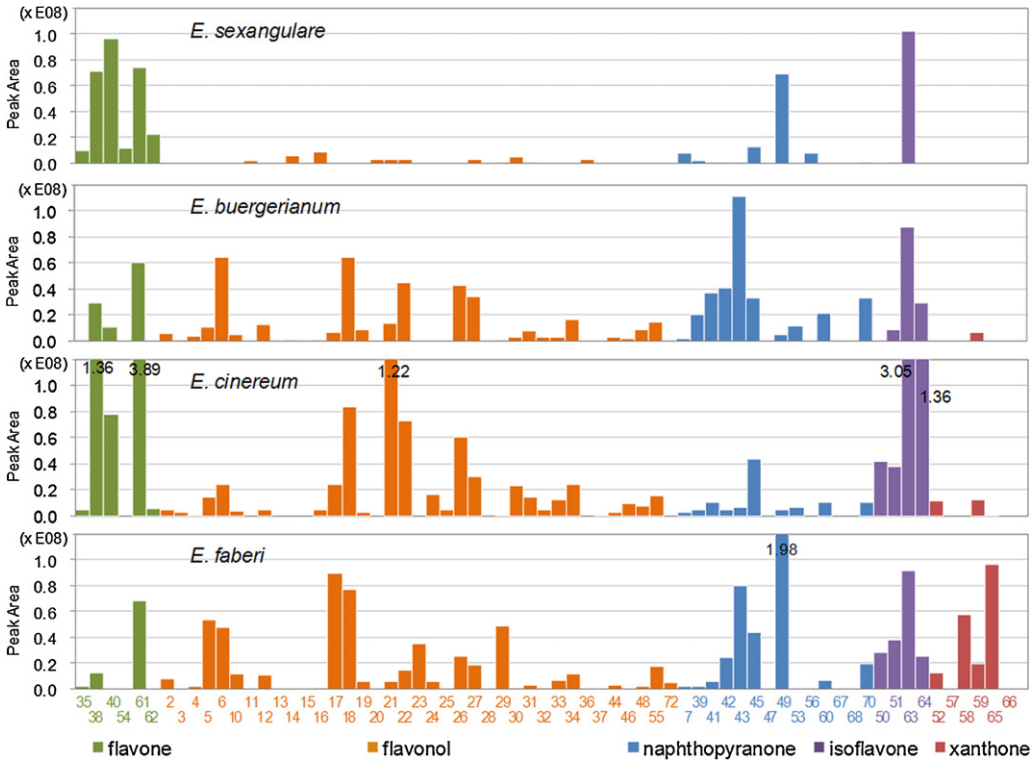

Fig. 5. Peak areas of 67 compounds in extracted ion chromatograms (EIC) of four *Eriocaulon* species.

produce *m*/*z* 316. The loss of 15 Da was common for flavonoids with methoxyl groups [15]. In the positive mode, the $[M+Na]^+$ ion at *m*/*z* 1003 was the base peak (Fig. 3B). It could lose the aglycone part to yield the sugar moiety ion at *m*/*z* 671, which could further fragment into *m*/*z* 583 possibly due to $^{1,4}X$ cleavage of the glucose ring. Similar fragmentation (neutral loss of 88 u) was also observed in compound **43**. Further fragmentations of the sugar moiety took place at the glycosidic bonds to produce *m*/*z* 527, 365, and 347. The combination of positive and negative modes was powerful for flavonoid glycosides and allowed their identification. For naphthopyranones, (*R*)-semivioxanthin 9-*O*-*β*-D-glucosyl-(1→6)-glucoside (**43**) was taken as an example and the fragmentation pathways are illustrated in Fig. 7S.

Based on UV spectra and ESI-MS^n fragmentation pathways of reference compounds, chemical constituents of the four *Eriocaulon* species were characterized. The same procedure as those in our previous reports was used for structural elucidation [16–18]. Compounds of different chemotypes could be explicitly differentiated by UV (Fig. 8S, 1) and MS (Fig. 8S, 2–5) spectral data. Isomeric compounds were identified on the basis of: (a) consistency in chromatography, UV and MS spectrometry with reference compounds; (b) fragmentation pathway and chromatographic behavior. A total of 72 compounds (**1**–**72**) were identified from four species, including 37 flavonols, 6 flavones, 4 isoflavones, 6 xanthones, 14 naphthopyranones, 3 phenolic acids, and 2 other flavonoids (Table 1). Except for 21 compounds with reference standards, the other compounds were tentatively characterized (Table 3S) referring to literature reports [19–26].

The proposed chemical formulae were further confirmed by high-accuracy mass spectrometry using an LC/IT-TOF-MS instrument. The measured accurate masses and isotopic mass distribution patterns matched the predicted values well (Table 1). For most of the compounds, the mass error values were below 5 ppm. For instance, a pair of isobaric compounds (**3** and **46**) were successfully differentiated, as shown in Fig. 4. Their high-accuracy $[M-H]^-$ ions were *m*/*z* 771.1977 and 771.1751, corresponding to the elemental composition of $C_{33}H_{40}O_{21}$ and $C_{36}H_{36}O_{19}$, respectively. In combination with tandem mass spectrometry data, **3** was identified as quercetin *O*-trisaccharide, and **46** was quercetin *O*-caffeoyl-disaccharide.

According to the first tier of chemical analysis, structural features of the *Eriocaulon* compounds were revealed. Flavonols were mostly di- or tri-glycosides of patuletin and quercetin, and the sugar chain was sometimes substituted by a caffeoyl group. Naphthopyranones were mostly diglycosides of (*R*)-semivioxanthin. Xanthones and isoflavones were mainly present in free form.

3.4. Quantitative analysis of selected flavonoids

The second level of the three-tier strategy is to understand the contents of *Eriocaulon* compounds. A fast HPLC/UV method was established to simultaneously determine six representative flavonoids, **18**, **22**, **27**, **38**, **55**, and **61**. The analytes were selected on the basis of previous phytochemical studies. 6-Methoxylated flavonols form *E. buergerianum* were reported for antibacterial activity [2,27], and was relatively abundant than other active compounds (phenolic acids and xanthones). Moreover, 6-methoxylated flavonols and flavones were characteristic for Eriocaulaceae family [28]. Therefore, six 6-methoxylated flavonoids were chosen for quantitative analysis. The method was fully validated. All the six analytes showed good linearity ($r^2 > 0.9998$), and the limits of detection ranged from 0.95 to 12.8 ng/mL (Table 4S). The intra-day ($n = 5$) and inter-day ($n = 5$) precision, as well as reproducibility ($n = 6$) of the method was good with a relative standard deviation (RSD) lower than 5% (Tables 5S and 6S). The recoveries were 93.12–100.83% (Table 7S). All the six flavonoids were stable within 24 h after sample preparation (RSD 0.58–3.86%) (Table 8S). Detailed

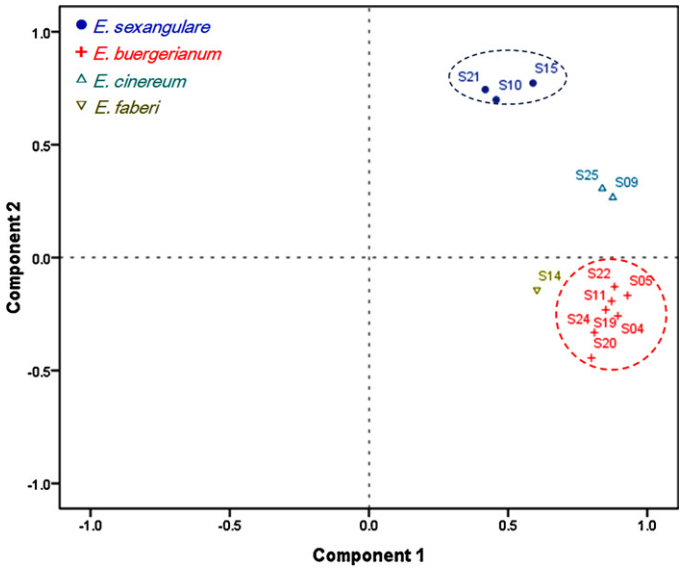

Fig. 6. PC1-PC2 projection plot of different Gu-Jing-Cao samples.

experimental procedures were provided in the supplementary material.

Contents of six flavonoids varied significantly among different species (Table 9S). Total amounts of these flavonoids in *Eriocaulon* species were not higher than 1.2 mg/g, and *E. sexangulare* was the lowest (lower than 0.3 mg/g for all three batches). The flavonoid patterns were also different. Among the six determined flavonoids, **18**, **22** and **27** are glycosides of **55** (patuletin), and **38** is a glycoside of **61** (hispidulin). It was noteworthy that *E. sexangulare* contained relatively high amounts (>75% relative abundance) of hispidulin group and low amounts of patuletin group flavonoids, which was vice versa for the other three species (Fig. 9S). *E. buergerianum* and *E. faberi* contained less than 25%, and *E. cinereum* 40–50% of hispidulin group flavonoids. In addition, flavonoids content also varied intra-species. For instance, flavonoid content of S19 was 3-fold higher than S11, which were both derived from *E. buergerianum*.

3.5. Semi-quantification and principal component analysis

To better reflect the complicated chemical composition of *Eriocaulon* plants, a global semi-quantitative analysis as the third level of the three-tier manner was conducted using LC/MS. Chemical composition of the four *Eriocaulon* species was compared based on the abundances of all detected compounds. The abundance was obtained from the extracted ion chromatogram (EIC) for each identified compound (listed in Table 1). To ensure the accuracy and reproducibility of the relative abundance, mass ranges for all EICs were 3 mass units, centered at $[M-H]^-$ of the compound. It should be noted that three phenolic compounds, protocatechuic acid (**1**), vanillic acid (**8**) and caffeic acid (**9**) were not counted due to their poor MS response. Also, one flavan (**69**) and one flavanone (**71**) were not counted due to their fairly low contents. The remaining 67 compounds could be classified into five groups, flavones, flavonols, isoflavones, xanthones, and naphthopyranones. Their EIC peak areas were reconstructed in Fig. 5, from which the chemical patterns of different *Eriocaulon* species could be summarized:

- *E. sexangulare* mainly contains flavones, and its major characteristic compounds are 3′-methoxyhispidulin 7-*O*-*β*-D-glucopyranoside (**40**) and toralactone hexosyl-hexoside (**49**).
- *E. cinereum* contains abundant flavones and isoflavones, as both glycosides and aglycones. The amount of naphthopyranones in *E. cinereum* is relatively low. Its major characteristic compounds are hispidulin (**61**), iristectorigenin A (**63**), and irigenin (**64**).
- *E. buergerianum* contains abundant isoflavones and naphthopyranones, mainly as glycosides. Amounts of flavones and xanthones are much lower. The major characteristic compound of *E. buergerianum* is semivioxanthin 9-*O*-*β*-D-glucosyl-glucoside (**43**).
- *E. faberi* contains remarkably more xanthones than other species. Its major characteristic compounds are 1,3,6,8-tetrahydroxy-2,7-dimethoxyxanthone (**65**), quercetin 3-*O*-hexosyl-hexoside (**17**), and patuletin 3-*O*-*β*-D-gentiobioside (**18**).

Subsequently, relative abundance values of same structural group were added, averaged within species, and the mean values were compared among the inter-species. Chemical profile of each *Eriocaulon* species could be summarized in Fig. 10S.

The results of semi-quantification were then used for principal component analysis (PCA). Calculation was preformed with the standard-projection plot of 1–2 principal component sized correlation matrix (extraction, univariate initial PC; method, correlation; fixed number of factors, 2; maximum iterations for convergence, 25; rotation, none; missing listwise). The principal factorial plane summarized 77.82% of the whole variability, and two PCA axes explained 59.88% and 17.94% of the variance, respectively. As shown in the distribution plot, the 13 batches could be explicitly clustered into four groups (Fig. 6). Interestingly, these four groups were consistent with the four species very well. These results indicated that chemical differences between the *Eriocaulon* species are greater than those within species. Thus these *Eriocaulon* plants could be distinguished by PCA analysis as well. In addition, we found that all the five classes of *Eriocaulon* compounds were statistically significant in chemotaxonomy, according to the correlation circle plot (Fig. 11S).

The above studies compared the chemical constituents at a global level rather than based on a few marker compounds, and thus led to more reliable results. This is the first systematic chemical analysis for *Eriocaulon* plants, in which the chemical profile of *Eriocaulon* plants was depicted. Importantly, chemical difference among species was elucidated for the first time. Moreover, each level of this method, i.e. HPLC fingerprints, quantitative analysis, and semi-quantification with PCA analysis, could be used for quality control and species authentification.

4. Conclusions

A three-tier chemical analysis, including HPLC fingerprints, quantitative analysis, and global semi-quantification in combination with PCA analysis, was established to differentiate official *E. buergerianum* with its adulterating species, *E. faberi*, *E. sexangulare*, and *E. cinereum*. From these four species, a total of 72 compounds were characterized by HPLC-DAD-ESI-MS^n. This is the first report on comprehensive chemical analysis of *Eriocaulon* species. The HPLC fingerprinting and quantification methods could be used for the quality control of Gu-Jing-Cao.

Acknowledgement

This work was supported by the 985 Project of Peking University Health Science Center (No. 985-2-119-121).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.08.033.

References

[1] Chinese Pharmacopoeia Commission, Pharmacopoeia of the People's Republic of China, vol. 1, 2010 ed., Chinese Medical Science and Technology Press, Beijing, 2010, pp. 168.

[2] J.J. Fang, G. Ye, W.L. Chen, W.M. Zhao, Antibacterial phenolic components from *Eriocaulon buergerianum*, Phytochemistry 69 (2008) 1279–1286.

[3] J.C. Ho, C.M. Chen, Flavonoids from the aquatic plant *Eriocaulon buergerianum*, Phytochemistry 61 (2002) 405–408.

[4] E.C. Bate-Smith, J.B. Harborne, Quercetagetin and patuletin in *Eriocaulon*, Phytochemistry 8 (1969) 1035–1037.

[5] Z.L. Liao, C.Y. Tian, L. Chen, X.J. Zhou, K. Sun, Textual criticism on the original plant of Flos Eriocauli in great compendium of Chinese Materia Medica, Shanghai Zhongyiyao Zazhi 38 (2004) 60–62.

[6] Flora of China Editorial, Committee, Flora of China, vol. 133, Science Press, Beijing, 1979, pp. 20–63.

[7] Z.X. Zhang, T. Stützel, SEM studies on morphology of seed surface structure of *Eriocaulon* from eastern and southeastern Asia, J. Beijing Forestry Univ. 24 (2002) 81–83.

[8] W.C. Yang, H. Liu, S.F. Ni, R.F. Luo, Z.X. Li, Q.L. Chen, Recent progress on *Eriocaulon* species: a review, Acta Chin. Med. Pharmcol. 37 (2009) 92–93.

[9] C. Tistaert, B. Dejaegher, Y.V. Heyden, Chromatographic separation techniques and data handling methods for herbal fingerprints: a review, Anal. Chim. Acta 690 (2011) 148–161.

[10] J.L. Zhou, L.W. Qi, P. Li, Herbal medicine analysis by liquid chromatography/time-of-flight mass spectrometry, J. Chromatogr. A 1216 (2009) 7582–7594.

[11] V.L. Scatena, A.M. Giulietti, E.L. Borba, C. van den Berg, Anatomy of Brazilian Eriocaulaceae: correlation with taxonomy and habitat using multivariate analyses, Plant Syst. Evol. 253 (2005) 1–22.

[12] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, J. Mass Spectrom. 39 (2004) 1–15.

[13] V. Vukics, A. Guttman, Structural characterization of flavonoid glycosides by multi-stage mass spectrometry, Mass Spectrom. Rev. 29 (2010) 1–16.

[14] N. Fabre, I. Rustan, E. Hoffmann, J. Quetin-Leclercq, Determination of flavone, flavonol, and flavanone aglycones by negative Ion liquid chromatography electrospray ion trap mass spectrometry, J. Am. Soc. Mass Spectrom. 12 (2001) 707–715.

[15] U. Justesen, Collision-induced fragmentation of deprotonated methoxylated flavonoids, obtained by electrospray ionization mass spectrometry, J. Mass Spectrom. 36 (2001) 169–178.

[16] X. Qiao, X. Zhang, M. Ye, Y.F. Su, J. Dong, J. Han, J. Yin, D.A. Guo, Rapid characterization of triterpene saponins from *Conyza blinii* by liquid chromatography coupled with mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 3340–3350.

[17] M. Ye, Y.N. Yan, D.A. Guo, Characterization of phenolic compounds in the Chinese herbal drug Tu-Si-Zi by liquid chromatography coupled to electrospray ionization mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 1469–1484.

[18] M. Ye, J. Han, H.B. Chen, J.H. Zheng, D.A. Guo, Analysis of phenolic compounds in rhubarbs using liquid chromatography coupled with electrospray ionization mass spectrometry, J. Am. Soc. Mass Spectrom. 18 (2007) 82–91.

[19] M.A. da Silva, A.P.S. Oliveira, M. Sannomiya, P.T. Sano, E.A. Varanda, W. Vilegas, L.C. dos Santos, Flavonoids and a naphthopyranone from *Eriocaulon ligulatum* and their mutagenic activity, Chem. Pharm. Bull. 55 (2007) 1635–1639.

[20] L.C. dos Santos, C.M. Rodrigues, M.A. da Silva, R.G. Coelho, M. Sannomiya, W. Vilegas, Chemical profile of *Eriocaulon ligulatum* (Vell.) L.B. Smith (Eriocaulaceae), Biochem. Syst. Ecol. 33 (2005) 1159–1166.

[21] M. Iinuma, H. Tosa, N. Toriyama, T. Tanaka, T. Ito, V. Chelladurai, Six xanthones from *Calophyllum austroindicum*, Phytochemistry 43 (1996) 681–685.

[22] J.H. Lee, I.Y. Baek, N.S. Kang, J.M. Ko, W.Y. Han, H.T. Kim, K.W. Oh, D.Y. Suh, T.J. Ha, K.H. Park, Isolation and characterization of phytochemical constituents from soybean (*Glycine max* L. Merr.), Food Sci. Biotechnol. 15 (2006) 392–398.

[23] A. Rehman, A. Malik, N. Riaz, H.R. Nawaz, H. Ahmad, S.A. Nawaz, M.I. Choudhary, Lipoxygenase inhibitory constituents from *Periploca aphylla*, J. Nat. Prod. 67 (2004) 1450–1454.

[24] S.W. Kang, M.C. Kim, C.Y. Kim, S.H. Jung, B.H. Um, The rapid identification of isoflavonoids from *Belamcanda chinensis* by LC-NMR and LC–MS, Chem. Pharm. Bull. 56 (2008) 1452–1454.

[25] S.W. Mina, N.J. Kimb, N.I. Baekc, D.H. Kima, Inhibitory effect of eupatilin and jaceosidin isolated from *Artemisia princeps* on carrageenan-induced inflammation in mice, J. Ethnopharmacol. 125 (2009) 497–500.

[26] M. Miyazawa, Y. Okuno, S. Nakamura, H. Kosaka, Antimutagenic activity of flavonoids from *Pogostemon cablin*, J. Agric. Food Chem. 48 (2000) 642–647.

[27] S. Faizi, H. Siddiqi, A. Naz, S. Bano, Lubna, Specific deuteration in patuletin and related flavonoids via keto-enol tautomerism: solvent- and temperature-dependent ^{1}H-NMR studies, Helv. Chim. Acta 93 (2010) 466–481.

[28] A. Salatino, M.L.F. Salatino, D.Y.A.C. dos Santos, M.C.B. Patricio, Distribution and evolution of secondary metabolites in Eriocaulaceae, Lythraceae and Velloziaceae from "campos rupestres", Genet. Mol. Biol. 23 (2000) 931–940.