文章编号:1001-6880(2015)6-0962-09

灯盏花及其药物灯盏花素中酚类成分的鉴定和含量测定

张人伟[1*],樊献俄[1],李来伟[1],林隆泽[2],James M. Harnly[2]

[1]昆明龙津药业股份有限公司,昆明 650091;

[2]美国农业部农业研究服务 Beltsville 人类营养研究中心食品成分和测定方法研究实验室,USA 20705

摘　要:本研究采用一个标准高效液相色谱质谱(HPLC-PDA-ESI/MS)分析方法和酚类成分定量方法确定了灯盏花中的33个肉桂酸衍生物和16个黄酮类化合物,灯盏花素中18个黄酮类化合物。使用干燥后的绿原酸(326 nm)作为标准品,根据326 nm处紫外吸收峰相对强度和克分子(mole)相对响应因子(MRRF)确定了每个肉桂酸衍生物的含量。以同样的方式,使用干燥后的芹菜素(336 nm)和芦丁(354 nm)确定了8个芹菜素,3个木犀草素,3个黄酮醇3-糖苷和7-葡萄糖醛酸苷的含量。这是首次报道十余微量黄酮类化合物存在于灯盏花素,以及灯盏花全植物中每个酚类化合物的含量。

关键词:灯盏花全草;灯盏花素;肉桂酸衍生物;黄酮苷;HPLC-PDA-ESI/MS;含量测定

中图分类号:R284.1;Q946.91　　**文献标识码**:A　　DOI:10.16333/j.1001-6880.2015.06.005

Identification and Quantification of Phenolic Components of *Erigeron breviscapus* and Its Derived Drug Breviscapine

ZHANG Ren-wei[1*], FAN Xian-er[1], LI Lai-wei[1], LIN Long-ze[2], HARNLY James M[2]

[1]*Longjin Pharmaceutical Co., Ltd., Kunming* 650091, *China*; [2]*Food Composition and Method Development Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, US Department of Agriculture, Maryland* 20705, *USA*

Abstract: A standardized HPLC-PDA-ESI/MS profiling method was used to identify the phenolic components of *Erigeron breviscapus* and breviscapine. A general phenolic quantification method was used to quantify the components. As a result, 33 hydroxycinnamates and 16 flavonoids were detected in *E. breviscapus*, a plant used in traditional Chinese medicine for treating cardiocerebral diseases. Eighteen flavonoids were detected in breviscapine, an *E. breviscapus* derived drug. The hydroxycinnamates were quantified using HPLC-PDA at the detection wavelength of 326 nm and the mole relative response factors (MRRF). Dried chlorogenic acid was used as calibration standard. Similarly, 8 apigenins, 4 luteolins, 3 flavonol 3-*O*-glycosides, and the 7-*O*-glucuronides of quercetin and isorhamnetin were quantified using apigenin (at 336 nm) and rutin (at 354 nm) as calibration standards and MRRF value for each compound. This was the first detection of over 10 flavonoids in breviscapine, and the quantification of each phenolic component in *E. breviscapus*.

Key words: *Erigeron breviscapus*; breviscapine; hydroxycinnamates; flavonoids; HPLC-PDA-ESI/MS; content quantification

Introduction

The whole plant of *Erigeron breviscapus* (Vant.) Hand-Mazz (Compositae) is an important traditional Chinese herbal medicine with anticoagulant activity and cerebrovascular and cardiovascular benefits. It has been used for treating various cardiocerebral diseases for years. It also has neuroprotective, antibacterial, antifungal and visual field protective activities [1-7]. Scutellarin (scutellarein 5,6,4′-trihydroxyflavone 7-glucuronide), one of its major components, has been developed into a therapeutic drug, breviscapine (dengzhanhuasu in Chinese), used for cardiocerebral diseases in China [8]. This drug contains more than 90% of scutellarin with apigenin 7-glucuronide, scutellarein and scutellarein 7-glucoside as its minor components. In addition, a therapeutic drug made from the hydroxycinnamates of this plant is in clinical trials [2].

Received: March 19, 2014 Accepted: July 3, 2014

* Corresponding authors Tel: 86-871-68520866-7488; E-mail: zrwcc3552@126.com

To date, approximately 50 hydroxycinnamates and 10 flavonoids were reported in this herb and most of them were identified using LC-DAD-MS^n[1-22]. Thirteen *E. breviscapus* polyphenols were confirmed to be free radical scavengers based on an on-line HPLC-ABTS/DPPH assay and MS detection [22]. In this study, 18 flavonoids were identified in breviscapine, and 33 hydroxycinnamates and 16 flavonoids were identified and quantified in *E. breviscapus* whole plant [27]. This was the first report of the detection of over 10 flavonoids in breviscapine, and the quantification of each phenolic component in *E. breviscapus*. The results of this study offered the possibility to understand the bioactivities of this herb more clearly and to use the herb more efficiently in the future.

Materials and Methods

Chemicals

HPLC grade solvents (methanol, acetonitrile), formic acid, HCl (37%) and dimethyl sulfoxide (DMSO) were purchased from VWR International, Inc. (Clarksburg, MD, USA). HPLC water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA, USA).

Chlorogenic acid, apigenin, luteolin, quercetin dihydrate, rutin (quercetin 3-*O*-rutinoside) trihydrate, diosmetin and isorhamnetinnm were obtained from Chromadex, Inc. (Irvine, CA, USA).

Samples

E. breviscapus samples were collected from the planting field of Kunming Longjin Pharmaceutical Co., Ltd. for the determination of the phenolic components.

Breviscapine (scutellarin content 91%) was obtained from Yuxi Wanfang pharmaceutica Ltd (Yuxi, Yunnan Province, China). Scutellarin (99%) was derived from breviscapine by purification (repeat crystallization) in Kunming Longjin Pharmaceutical Co., Ltd., and the residue was used to identify the minor flavonoids in this therapeutic drug.

Instruments

An Agilent 1100 HPLC (Agilent, Palo Alto, CA, USA) coupled with photodiode array (PDA) detector and mass analyzer (MSD, model SL) was used in this study. A Symmetry C_{18} column (250 × 4.6 mm i.d., 5 μm, Waters Corp., Milford, MA, USA) with a Symmetry Sentry guard column (20 × 3.9 i.d., 5 μm) and SymmetryShield column (250 × 4.6 mm i.d., 5 μm) were used for the chromatographic separation.

Standard preparation

The standards of chlorogenic acid, apigenin, luteolin, and rutin were vacuum dried (National Appliance Co, Portland, OR, USA) at 110 ℃ until a constant weight was reached (around 24 hrs) and the dried standards were used for the polyphenol content quantification. Their water contents were determined as 6.4%, 5.4%, 8.6% and 10.0%, respectively [27].

Each standard was accurately weighed between 3.0-6.0 mg (the balance reading set for 0.01 mg) and put into a 10 mL volumetric flask (±0.2 mL). The standards were first dissolved in 2 mL of DMSO and then brought to volume with the aqueous methanol (60∶40, v/v). Their solutions were mixed in same molar concentration for each standard, and prepared at 3 different dilution factors to provide a range of signals suitable for the quantification of the major and minor phenolic compounds in the samples. Each solution was injected 3 times onto the column. The relative standard deviations (RSDs) for the peak areas were all <5% and the average integrated peak areas had a linear relationship with respect to concentration [23-27].

Sample preparation

Sample extraction

The dry materials were powdered and passed through 60 mesh sieves prior to extraction. The ground powder (200 mg) was put into a tube, and extracted with 10 mL methanol-water (60∶40, v/v) using an FS30 Ultrasonic sonicator (Fisher Scientific, Pittsburg, PA, USA) for 60 min, at room temperature. The slurry mixture was centrifuged at 2500 rpm for 15 minutes (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA), the supernatant was filtered through a 17mm (0.45 μ) PVDF syringe filter (VWR Scientific, Seattle, WA, USA), and 50 μL of the extract (or extract-1) was injected into the HPLC.

Acidic hydrolyzed extracts

The breviscapine residue solution (0.5 mg/0.40 mL)

and the whole plant extract (0.5 mL) were mixed with concentrated HCl (37%, 0.10 mL), and heated in a covered tube at 110 °C for 2 hrs, respectively. Then, 0.40 mL of methanol was added to each of the reaction mixture, and sonicated for 10 min. The solution was re-filtered prior to HPLC injection [23].

Identification of phenolic components

LC-PDA-ESI/MS condition

The LC-PDA-ESI/MS consisted of an Agilent 1100 HPLC (Agilent, Palo Alto, CA) coupled with photodiode array (PDA) detector and mass analyzer (MSD, model SL). A Symmetry C_{18} column was used at a flow rate of 1.0 mL/min for preliminary peak separation. SymmetryShield column was also used for the further separation of some overlapped peaks. The column oven temperature was set at 25 ℃. The mobile phase consisted of a combination of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was varied linearly from 10% to 26% B (v/v) in 40 min, to 65% B at 70 min. For the plant extracts, the PDA was set 326, 336, 348, 354 and 368 nm, recorded from 190-400 nm. Mass spectra were acquired using electrospray ionization in the positive and negative ionization (PI and NI) modes at low (100 V) and high (250 V) fragmentation voltages and recorded for the range of *m/z* 100-2000 [26].

Identification process

The preliminary putative compound identification was made based on its UV absorptions, retention time (elution order), protonated/deprotonated molecular ($[M+H]^+$/$[M-H]^-$) values, and the parent ions and main fragments. After a direct comparison of the obtained data with those of a standard or reference compound in in-house compound database, a reliable identification can be offered.

Quantification of phenolic components

HPLC-PDA conditions

The quantification of phenolic components, including the PDA settings, was carried out using HPLC-PDA. The HPLC-PDA method was the same as described in Section 2.6.1.

Quantification process

The relative UV absorption intensity of each separated compound to that of the standard was used for its content calculation. The concentration of the polyphenols were expressed as mg of the compound in 100 g of the dried plant materials, and were calculated using the following formula:

$$C(mg/100\ g) = [1000\ Ax \times MWx \times Ws \times Vs]/[As \times MWS \times Vx \times Wx \times MRRF]$$

Besides, each sample extract was prepared in triplicate, each preparation was analyzed in triplicate, and the relative standard deviations (RSDs) for each peak should be kept $< 5\%$. The efficiency of this extract preparation method for the main peaks was confirmed to be over 95%, thus, this method was able to be used for content quantification [23-27].

Results and Discussions

Identification of the flavonoids of breviscapine and phenolic compounds of *E. breviscapus*

Chemical structures of some phenolic compounds detected in *E. breviscapus* were shown in Fig. 1. HPLC-PDA chromatograms (326 nm) of breviscapine residue and the herb extract were shown in Fig. 2 and Fig. 3, respectively. The names, retention times (t_R), wavelengths of maximum absorbance (λ_{max}), molecular ion ($[M-H]^-$), and major fragment ions (the main and Breviscapine, prepared from *E. breviscapus*, contained

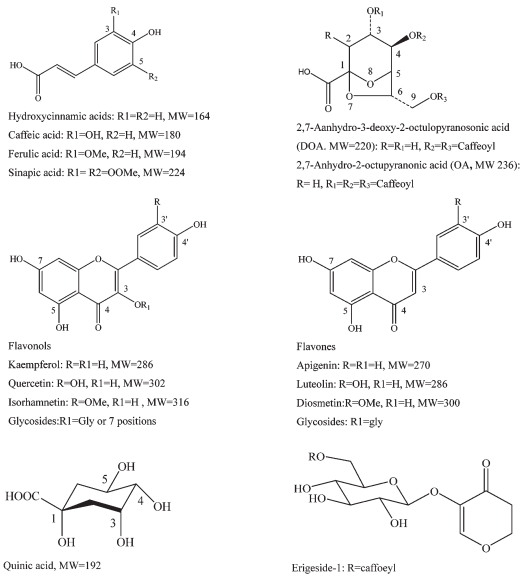

Fig. 1 Chemical structures of polyphenols of *E. breviscapus*

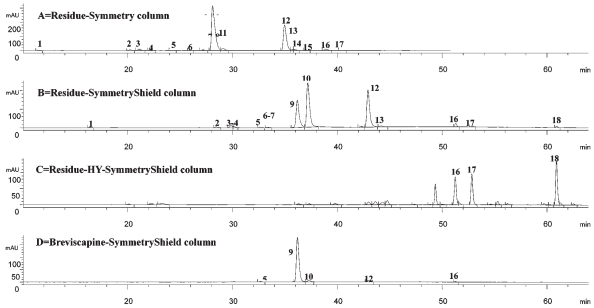

Fig. 2 HPLC chromatograms (326 nm) of the flavonoids of breviscapine residue (after removal of scutellarin) recorded with a Symmetry column (A) and a SymmetryShield column (B) and the flavonoid aglycones in the acidic hydrolyzed residue recorded with a SymmetryShield column (C) and bravilcupine recored with a SymmetryShield column (D)

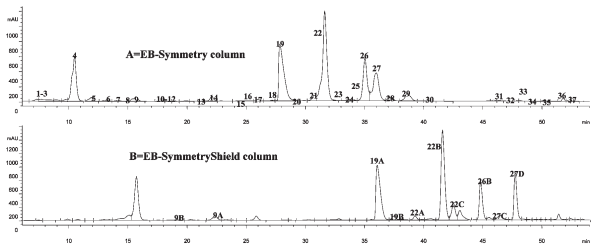

Fig. 3 HPLC chromatograms (326 nm) of the phenolic compounds of *E. breviscapus* recored with a Symmetry column (A) and a SymmetryShield column (B)

diagnostic ions) of the flavonoids of breviscapine residue were shown in Table 1 and 2. The concentration of each phenolic compound in the whole plant were listed in Table 2.

Table 1 Putative identification of the flavonoids in breviscapine and its residue

Peak No	t_R-1 (min)	t_R-2 (min)	$[M+H]^+$/ $[M-H]^-$ (m/z)	PI/NI main ions (m/z)	UV λ_{max} (nm)	Identification
1	11.5	16.5	641/nd	479,303	276,338	Pentahydroyflavone 7-*O*-glucuronid-4′-*O*-glucoside
2	20.2	28.5	447/445	271/269	268,328	trihydroxyflavone 7-*O*-glucuronide
3	21.1	29.8	517/515	319/317	260,274sh,354	quercetagetin 3-*O*-glucuronide
4	22.2	30.1	479/477	303/301	282,346	5,6,7,3′,4′-pentahydroxyflavone 7-*O*-glucuronide[a]
5	25.3	32.6	449/447	287/285	282,336	scutellarein 7-*O*-glucoside[a,b]
6	26.0	33.3	463/461	287/285	266,348	kaempferol 3-*O*- glucuronide[a,b]
7	27.1	33.9	463/461	287/285	256,264sh,368	kaempferol 7-*O*-glucuronide[a,b]
8	27.7	–	479/477	303/301	256,266sh,372	quercetin 7-*O*-glucuronide[a,b]
9	28.1	36.1	463/461	287/285	282,338	Scutellarin[a,b]
10	28.1	37.1	479/477	303/301	272,346	pentahydroxyflavone 7-*O*-glucuronide[b]
11	29.1	–	509/507	333/331	260,274sh,354	petuletin 3-*O*-glucuronide[a]
12	35.0	42.8	447/445	271/269	266,338	apigenin 7-*O*-glucuronide[a,b]
13	35.6	–	493/491	317/315	256,266sh,368	isorhamnetin 7-*O*-glucuronide[a]
14	35.9	42.1	477/475	301/299	254,268sh,350	diosmetin 7-*O*-glucuronide[a]
15	37.5	–	477/475	301/299	274,338	methoxytetrahydroxyflavone 7-*O*-glucuronide
16	38.9	51.3	287/285	–/–	282,338	Scutellarein[a,b]
17	40.4	52.9	303/301	–/–	274,350	Pentahydroxyflavone[b]
18	51.8	60.9	271/269	–/–	266,336	apigenin[a,b]

[a] identification was confirmed with a standard or positively identified compounds in reference plant materials.
[b] reported previously in this plant.

Table 2 The name, mass, UV absorbance, and content of each phenolic compounds of *E. breviscapus*

Peak	Compound name	$[M-H]^-$/ main ions	UV λ_{max}	Content (mg/100 g)
no	(retention time, min)	m/z	(nm)	± RSD

1	1-caffeoylquinic acid (6.3)[a,b]	353/191,179,135	328,300sh,240	1.70 ±0.07
2	3-caffeoylquinic acid (7.5)[a,b]	353/191,179,135	328,300sh,240	10.00 ±0.40
3	caffeoylquinic acid glucoside (7.7)[a,b]	515/353,179,135	328,300sh,240	9.75 ±0.39
4	5-caffeoylquinic acid (10.5)[a,b]	353/191,179	328,300sh,240	738.86 ±29.55
5	4-caffeoylquinic acid (11.9)[a,b]	353/191,179,135	328,300sh,240	10.50 ±0.42
6a	3-caffeoylDOA (13.3)[b]	381/201,179,161	328,300sh,240	12.50 ±0.50
6b	9-caffeoylOA (13.4)[b]	397/217,179,161	328,300sh,240	20.25 ±0.81
7	caffeic acid (14.1)[a,b]	179/135	328,300sh,240	9.35 ±0.37
9A	caffeoylOA (15.3)[b]	381/201,179	328,300sh,240	104.00 ±4.16
9B	caffeoylDOA (15.7)[b]	397/217,179	328,300sh,240	37.95 ±1.52
10	5-feruloylquinic acid (17.9)[a,b]	367/193,191	328,300sh,240	7.55 ±0.30
11	1,3-dicaffeoylquinic acid (18.1)[a,b]	515/353,191,179,135	nd	2.10 ±0.08
12a	4-feruloylquinic acid (18.3)[a,b]	367/193,191	328,300sh,240	4.10 ±0.17
12b	malonylcaffeoylquinic acid (18.3)[a,b]	439/191,179	328,300sh,240	103.40 ±4.14
14	erigeside-1 (22.1)[b]	435/323,179,161	328,300sh,240	103.40 ±4.14
15	dicaffeoylquinic acid glucoside (24.5)[a,b]	601/557,395,353,233	nd	3.20 ±0.13
16	dicaffeoylquinic acid glucoside (25.2)[a,b]	601/557,395,353,233	nd	2.70 ±0.11
21A	1,4-dicaffeoylquinic acid (30.7,39.8)[a,b]	515/353,191,179,161,135	328,300sh,240	9.50 ±0.38
22A	3,4-dicaffeoylquinic acid (31.4,39.3)[a,b]	515/353,191,179,161,135	328,300sh,240	53.60 ±2.14
22B	3,5-dicaffeoylquinic acid (31.6,41.6)[a,b]	515/353,191,179,161,135	328,300sh,240	1140.65 ±45.63
22C	1,5-dicaffeoylquinic acid (31.8,42.5)[a,b]	515/353,191,179	328,300sh,240	309.20 ±12.37
23	malonyldicaffeoylquinic acid (33.3)[a,b]	601/557,395,353,233	328,300sh,240	12.40 ±0.50
24	malonyldicaffeoylquinic acid (33.8)[a,b]	601/557,395,353,233	328,300sh,240	29.10 ±1.16
25	malonyldicaffeoylquinic acid (34.5)[a,b]	601/557,395,353,233	328,300sh,240	25.00 ±1.00
26B	4,5-caffeoylquinic acid (35.5)[a,b]	515/353,179	328,300sh,240	461.20 ±18.45
27C	erigoeter B isomer (4,9-diCDOA) (36.0,46.5)[b]	543/381,161	328,300sh,240	98.79 ±3.95
27D	erigoster B (3,9-diCDOA)(36.0,47.7)[b]	543/381,161	328,300sh,240	506.45 ±20.26
29	melonyldicaffeoylquinic acid (38.6)[a]	601/557,395,353,233	328,300sh,240	7.50 ±0.30
31	1,3,5-tricaffeoylquinic acid (40.2)[a,b]	677/515,497,353,191,179	328,300sh,240	3.45 ±0.14
33	erigoster A (3,9-diCOA) (47.9)[b]	557/393,353,233	328,300sh,240	24.35 ±0.97
34	3,4,5-tricaffeoylquinic acid (48.1)[a,b]	677/515,353,191,179	328,300sh,240	8.55 ±0.34
35A	erigoster A isomer (49.8)[b]	557/395,353	328,300sh,240	3.80 ±0.15
35B	3,4,9-triCDOA (49.9)[b]	705/491	328,300sh,240	2.10 ±0.08
	Total contents of 33 hydroxycinnamtes			3876.95 ±155.08
8	6,8-di-*C*-glucosylapigenin (14.7)[a,b]	593/475,355	nd	2.11 ±0.08
17	scutellarein 7-*O*-glucoside (25.8)[a,b]	447/285	336,282	24.52 ±0.98
19A	scutellarin (27.9)[a,b]	461/285	338,282	1411.52 ±56.46
21B	scutellarein 7-*O*-pentoside (30.8)[b]	417/285	336,282	7.15 ±0.29
26A	apigenin 7-*O*-glucuronide (35.0)[a,b]	445/269	336,266	52.12 ±2.08
28	methoxytrihydroxyflavone	475/299	338,266	94.34 ±3.78
30	scutellarein (38.8)[a,b]	285	338,282	76.45 ±3.06
36	apigenin (51.8)[a,b]	269	338,266	89.75 ±3.59

	Total contents of 8 apigenin derivatives			1757.99 ± 70.32
19B	pentahydroxyflavone 7-*O*-glucuronide (28.0)[b]	477/301	346,272	116.58 ± 4.66
32	luteolin (46.2)[a,b]	285	348,266sh,256	20.90 ± 0.84
27B	diosmetin 7-*O*-glucuronide (36.1)[a]	475/299	350,268sh,254	56.45 ± 2.26
37	diosmetin (52.6)[a]	299	350,268sh,254	20.75 ± 0.83
	Total contents of 4 luteolin derivatives			214.68 ± 8.59
13	quercetagenin 3-*O*-glucuronide (21.1)[a]	493/317	354,274,254	21.90 ± 0.92
18	quercetin 7-*O*-glucuronide (27.4)[a]	477/301	372,266sh,254	64.10 ± 2.57
20	petuletin 3-*O*-glucuronide (29.1)[a]	507/331	354,266sh,254	100.00 ± 4.0
27A	isorhamnetin7-glucuronide (36.0)[a]	491/315	368,266sh,356	8.10 ± 0.32
	Total contents of 4 flavonols			194.10 ± 7.76
	Total contents of the 49 polyphenols			6043.72 ± 241.75

[a] the identification was confirmed with standard or positively identified compounds in reference plant materials
[b] reported previously in this plant;
[c] the content quantification was made on the related ratio of the UV band I absorption (the first one in the table) to that of the standard and their MRRF value.

91% scutellarin as its active component (Fig. 2D). The flavonoids of the breviscapine residue solution were firstly separated with Waters Symmetry column (Fig. 2A), and some un-separated compounds, for example, compounds **9** and **10**, were well separated with Waters SymmetryShield column (Fig. 2B). After the acidic hydrolysis, the glycosides were converted to their aglyocones (scutellarein peak 16, pentahydroxyflavone peak 17 and apigenin peak 18) and their peak intensities were increased, and appeared as the main peaks in Fig. 2C, while such aglyocones were minor peaks in Fig. 2B.

As mentioned in the section 2, the primary compound identification was made bade on the analysis of the elution order, UV and mass data of each compound. For example, based on loss of 176 Da, such as 463-287, 479-303, 493-317, 477-301, and showing same UV band I, peaks 8, 9, 13 and 14 were identified as the 7-glucuronide of kaempferol, quercetin, isorhamnetin and diosmetin, respectively. Peaks 3, 6 and 11 showed loss of 176 Da and a typical 12-17 nm shift of the UV band I movement to short wavelength to suggest them to be 3-*O*-glucuronide of quercetagetin, kaempferol and petuletin, respectively. After a direct comparison of the analytical data with those for the standards and the reference compounds from ten other Compositae plants in the database [24-26], 10 of the flavonoids were identified reliably as noted with superscript "a" in Table 1.

Some of the remained flavonoids were previously reported in this herb (with superscript "b" in Table 1), and have the same identification except the aglycone for two flavonoids (i.e., peaks 1 and 10 in Table 1, and also peak 19A in Table 2, the herb extract) was previously identified as 3-glucuronide of 3,5,6,7,4′-flavanone (galetin) using LC-MS^n analysis [16]. However, the aglycone (i.e., peak 17 of Table 1, 40.4 min) of this flavonoid had a UV spectra (274, 350 nm) which was different from that of galetin (258sh, 276, 358 nm) [16,28]. Furthermore, this UV data were also different with those for 5,7,8,3′,4′-pentahydroxyflavone (266, 334 nm) [28], 5, 6, 7, 3′, 4′-pentahydroxyflavone (nepetin, 6-hydroxyluteolin) (282, 348 nm, 31.1 min), tricetin (5, 7, 3′, 4′, 5′-pentahydroxyflavone, 268, 352 nm, 37.4 min), herbacetin (3, 5, 7, 8, 4′-pentahydroxyflavone, 276, 329, 380 nm, 44.0 min), morin (3, 5, 7, 2′, 4′-pentahydroxyflavone, 252, 262sh, 352 nm, 41.22 min), or robinetin (3, 7, 3′, 4′, 5′-pentahydroxyflavone, 250, 318sh, 362 nm 25.33 min) too [23-26]. Thus, its positions for the 5 hydroxy functions is still not determined. Peak 1 might have the same aglycone with one additional gluosyl at 4′ position based on its UV band I λ_{max} at 338 nm, and retention times at 11.5 and 16.5 min, respectively. Peak 2 was identified as trihydroxyflavone 7-*O*-glucuronide, but the positions for the 3 hydroxy functione were not established. Peak 4 was identified as mostly 5, 6, 7, 3′, 4′-penta-

hydroxyflavone 7-*O*-glucuronide, based on its UV data. Several of the mentioned flavonoids were not reported in this herb previously [1-22].

Based on the relative peak area ratios, breviscapine contained scutellarin (peak 9, 90.9%), pentahydr oxyflavone-7-*O*-glucuronide (peak 10, 4.2%), apigenin-7-*O*-glucuronide (peak 12, 3.5%), scutellarein -7-*O*-glucoside (peak 5, 0.8%) and scutellarein (peak 16, 0.6%) (Fig. 2D). More than 10 other flavonoids were found in the breviscapine residue (Fig. 2A and 2B) with a combined concentration of less than 0.01%.

In the same way, 33 hydroxycinnamates and 16 flavonoids of *E. breviscapus* were separated and identified by two columns (Fig. 3). All of the flavonoids in the herb were the same flavonoids detected in breviscapine residue (Table 1). Twenty-nine of the polyphenols are caffeoylquinic acids (i. e., 6 mono-, 12 di- and 2 tri-caffeoylquinic acids and their malonyl or glucosyl derivatives), and were positively identified by direct comparison of their diagnostic UV (for caffeic acid conjugated system) and MS data ($[M-H]^-$, fragments, and the retention times recorded to in two different columns (Table 2) to those of the related standards[23-26]. Such compounds were always to present the quinic acid ion at m/z 191, with 1-3 caffoyls by loss of 162 amu in turn, *etc.* as their diagnostic mass fragments[23-26].

The remaining 10 hydroxycinnamates in the herb extract were easily identified based on reported structures, UV and mass spectra in the literature [10-14,16-18]. For example, peak 14 had molecular and major fragments at *m/z* 435, 323, 179 and 163, and was identified as erigeside-1. Similarly, peaks 6a and 9A ($[M-H]^-$ and major fragments at *m/z* 381, 201, 179, 161), peaks 6b and 9B ($[M-H]^-$ and major fragments at *m/z* 397, 217, 179 and 161, two isomers), peaks 27C and 27D ($[M-H]^-$ and major fragments at *m/z* 543, 381 and 161, two isomers), peaks 33 and 35A ($[M-H]^-$ and major fragments at *m/z* 557, 395, 353 and 233, two isomers), and peak 35 B ($[M-H]^-$ and major fragments at *m/z* 705, 543, 381 and 161) were identified as caffeoyl OA and its isomer, caffeoyl DOA and its isomer, erigoster B (4,9-Di-CDOA) and its isomer, erigoster A and its isomer, and 3, 4, 9-tri-CDOA, respectively. These compounds were among the caffeic acid derivatives of this herb that were previously identified using nuclear magnetic resonance (NMR) analysis [2,10-12] and also charaterized from LC-MS^n studies [16-18].

Quantification of phenolics in *E. breviscapus*

The phenolic concentrations, expressed as mg of the compound in 100 g of the dried plant materials, were listed in Table 2. Since the reference standard solution and plant extract were prepared in the same solvent volume (10.00 mL), the concentration (C in mg/100 g dry base of the plant material) of each polyphenol was calculated as follows:

$$C(mg/100g) = [1000\ Ax \times MWx \times Ws \times Vs]/[As \times MWS \times Vx \times Wx \times MRRF] \quad (1)$$

or the weight percent as:

$$C(\%, w/w) = [100Ax \times MWx \times Vs \times Ms \times Ws]/[As \times MWs \times Wx \times Vx \times MRRF] \quad (2)$$

Where, MWx, Wx, Vx and As, MWs, Ws, Vs are the peak area (μAU or mAU), molecular weight, and weight (mg) of the sample and the standard in same volume of the extract or solution, respectively. Depending on the calculation, the dried weight molar relative response factor ($MRRF_D$) or putative molar relative response factor ($MRRF_p$) were substituted for MRRF. This general quantification approach was shown to be suitable for the quantification of all the common plant polyphenols even for ones without their quantification standards [27].

The concentrations were calculated based on calibration with a standard solution containing chlorogenic acid, apigenin, and rutin and previously reported mole response factors ($MRRF_p$)[27]. As mentioned in this paper, the MRRFp values for the hydroxycinnamate containing mono-, di- and tri-caffeoyl or feruloyl function (s) were 1.0, 2.0 and 3.0, respectively [27]. Similarly, apigenins, luteolins, quercetin 3-glycosides, and two 7-glycosides of quercetin and isorhamnetin have same $MRRF_p$ of their group standard apigenin (at 336 nm), luteolin (at 348) and rutin (at 354), respectively. In general, the concentration for each component in each compound group are accurate within 10% since their UV band I λ_{max} values are close to that of the group

standard (Table 2) [27].

Some isomeric compounds, such as 3,4-, 3,5-, and 1,5-dicaffeoylquinic acid (peaks 22A, 22B and 22C) were overlapping on the Symmetry column, but were separated with the SymmetryShield column, which allowed quantification of each of the isomers. However, some compounds were not well separated with either column. When two or more compounds, having different molecular weights, appeared as an unresolved peak (peak 6a and 6b or 10a and 10b), selective ionization monitoring (SIM) detection or extracted ion chromatography (EIC) was used to determine the percent contribution of each compound to the overlapped UV peak. The total concentration obtained with UV detection was multiplied by the appropriate percentage to provide the concentration of each compound (Table 2) [28].

The total hydroxycinnamate content (3.7% dry weight) and scutellarin content (1.5%) were within the range reported previously [1,2,16,17,22]. The total polyphenol content was 5.9%. These phytochemical components are believed to contribute greatly to the various biological activities of this herb [1-5].

Conclusion

This study showed that the standardized methods developed in our lab were able to provide systematic identification and quantification of the major and minor phenolic components of *E. breviscapus*. The detailed identification and quantification of the phenolic components provided the data necessary to relate biological activities with the phytochemical content. This data will permit more efficient use of the herb and establish a basis for quality control.

Acknowledgements

This research is supported by the Agricultural Research Service of the U.S. Department of Agriculture and an Interagency Agreement with the Office of Dietary Supplements of the National Institutes of Health.

References

1 The Editorial Commission of Chinese Materia Medica. *Erigeron breviscapus* in Chinese Materia Medica. Shanghai: Shanghai Science and Technology Press, 1999. Vol. 7, 827-830.

2 Sun H, Zhao Q. A drug for treating cardio-cerebrovascular diseases-phenolic compounds of *Erigeron breviscapus*. *Prog Chem*, 2009, 21: 77-83.

3 Chen X, Cui L, Duan X, *et al*. Pharmacokinetics and metabolism of the flavonoid scutellarin in humans after a single oral administration. *J Pharmacol Exp Ther*, 2006, 34: 1345-1352.

4 Liu H, Yang XL, Ding J, *et al*. Antibacterialand antifungal activity of *Erigeron breviscapus*. *Fitoterapia*, 2003, 74, 387-389.

5 Liu H, Yang X, Tang R, *et al*. Effects of *Erigeron breviscapus* ethanol extract on neuronal oxidative injury induced bysuperoxide radical. *Fitoterapia*, 2005, 76: 666-670.

6 Gua H, Hu LM, Wang SX, *et al*. Neuroprotective effects of scutellarin against hypoxic-ischemic-induced cerebral injury via augmentation of antioxidant defense capacity. *Chin J Phys*, 2011, 54: 399-405.

7 Zhong Y, Xiang M, Ye W, *et al*. Visual field protective effect of *Erigeron breviscapus* (*vant.*) *Hand. Mazz.* extract on glaucoma with controlled intraocular pressure. A randomized, double-blind, clinical trial. *Drugs R D*, 2010, 10: 75-82.

8 Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China. Beijing: China Medical Science Press, 2010. 378-380.

9 Zhang RW, Yang SY, Lin YY. Studies on chemical constituents of dengzhanhua [*Erigeron breviscapus* (*Vant.*) *Hand-Mazz*]. I. The isolation and identification of pyromeconic acid and a new glucoside. *Acta Pharm Sin*, 1981, 16: 68-69.

10 Yue JM, Zhao QS, Lin ZW, *et al*. Phenolic compounds of *Erigeron breviscapus* (Compositae). *Acta Bot Sin*, 2000, 42: 311-315.

11 Zhang WD, Kong DY, Li HT, *et al*. A new glycoside form *Erigeron breviscapus*. *Chin Chem Lett*, 1999, 10: 125-126.

12 Yue JM, Lin ZW, Sun HD. A new caffeoyl conjugate from *Erigeron breviscapus*. *Chin Chem Lett*, 1997, 8: 225-228.

13 Li J, Yu DQ. Chemical constituents from herbs of *Erigeron breviscapus*. *Chin J Chin Mater Med*, 2011, 36: 1458-1462.

14 Qu J, Wang Y, Luo G, *et al*. Identification and determination of glucuronides and their aglycones in *Erigeron breviscapus* by liquid chromatography-tandem mass spectrometry. *J Chromatogr A*, 2001, 928: 155-162.

15 Chu QC, Wu T, Fu L, *et al*. Simultaneous determination of active ingredients in *Erigeron breviscapus* (*Vant.*) *Hand-Mazz.* by capillary electrophoresis with electrochemical detection. *J Pharm Biomed Anal*, 2005, 37: 535-541.

16 Zhang Y, Shi P, Qu H, *et al*. Characterization of phenolic compounds in *Erigeron breviscapus* by liquid chromatography

coupled to electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom*, 2007, 21: 2971-2984.

17 Wang LW, Li HJ, Li P, *et al*. Simultaneous quantification of two major classes of constituents in *Erigeron breviscapus* and its extract Injection by RP-HPLC. *Chromatographia*, 2007, 66: 395-399.

18 Liao SG, Zhang LJ, Li CB, *et al*. Rapid screening and identification of caffeic acid and its esters in *Erigeron breviscapus* by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*, 2010, 24: 2533-2541.

19 Wang YF, Hu LM, Liu YN, *et al*. A rapid method for qualitative and quantitative analysis of major constituents in dengzhanxixin injection by LC-DAD-ESI-MSn. *Chromatographia*, 2010, 71: 845-853.

20 Li F, Zhang LD, Li BC, *et al*. Screening of free radical scavengers from *Erigeron breviscapus* using on-line HPLC-ABTS/DPPH based assay and mass spectrometer detection. *Free Rad Res*, 2012, 46: 286-294.

21 Zhang Y, Zhao Q, Ma J, *et al*. Chemical characterization of phenolic compounds in *Erigeron* Injection by rapid-resolution LC coupled with multi-stage and quadrupole-TOF-MS. *Chromatographia*, 2010, 72: 651-658.

22 Liu Q, Shi Y, Wang Y, *et al*. Metabolism profile of scutellarin in urine following oral administration to rats by ultra performance liquid chromatography coupled to time-of-flight mass spectrometry. *Talanta*, 2009, 80: 84-91.

23 Lin LZ, Harnly JM. A screening method for the identification of glycosylated flavonoids and other phenolic compounds using a standard analytical approach for all plant materials. *J Agric Food Chem*, 2007, 55: 1084-1096.

24 Lin LZ, Harnly JM. Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC-DAD-ESI/MS profiling method. *J Agric Food Chem*, 2008, 56: 10105-10114.

25 Lin LZ, Harnly JM. Identification of the phenolic components of Chrysanthemum flowers (*Chrysanthemin morifolium* Ramat). *Food Chem*, 2010, 120: 319-326.

26 Lin LZ, Harnly JM. LC-MS profiling and quantification of food phenolic components using a standard analytical approach for all plants. *Nova Publishers*, 2008: 1-103.

27 Lin LZ, Harnly JM, Zhang RW, *et al*. Quantitation of the hydroxycinnamic acid derivatives and the glycosides of flavonols and flavones by UV absorbance after identification by LC-MS. *J Agric Food Chem*, 2012, 60: 544-553.

28 Greenham J, Harborne JB, Williams CA. Identification of lipophilic flavones and flavonols by comparative HPLC, TLC and UV spectral analysis. *Phytochem Anal*, 2003, 14: 100-118.

(上接第 1031 页)

9 Huang SL(黄少兰), Dong WJ(董文娟), Xue YH(薛艳红), *et al*. Protective effect of *Solanum cathayanum* alkaloid on H_2O_2 induced SH-damage in SY5Y cells. *Jiangsu J Tradit Chin Med*(江苏中医药), 2011, 9: 90-93.

10 Su XP(苏香萍), Hu GH(胡格华), Wu J(吴军), *et al*. Isolation and antimicrobial activities of endophytic fungi in *Solanum cathayanum*. *Lishizhen Med Mater Med Res*(时珍国医国药), 2012, 6: 337-339.

11 Guo XL(郭夏丽), Luo LP(罗丽萍), Leng TT(冷婷婷), *et al*. Chemical compositions and antioxidant activities of seven honeys from different floral sources. *Nat Prod Res Dev*(天然产物研究与开发), 2010, 22: 665-670.

12 Nie SP(聂少平), Xie MY(谢明勇), Luo Z(罗珍). Studies on antioxidant activity of tea polysaccharide. *Nat Prod Res Dev*(天然产物研究与开发), 2005, 17: 549-552.

13 Deng J(邓靖), Mo ZC(莫正昌), Ji GQ(汲广全), *et al*. Antioxidant activity of extract from *Balanophora spicata* Hayata *in vitro*. Food Scince(食品科学), 2010, 31(5): 23-25.