

Quality assessment and discrimination of the roots of *Cynanchum auriculatum* and *Cynanchum wilfordii* by HPLC–UV analysis

Ying Li · Donggen Piao · Haiyan Zhang · Mi-Hee Woo ·
Je-Hyun Lee · Dong-Cheul Moon · Seung-Ho Lee ·
Hyeun Wook Chang · Jong Keun Son

Received: 3 September 2012 / Accepted: 5 December 2012 / Published online: 26 February 2013
© The Pharmaceutical Society of Korea 2013

Abstract *Cynanchum auriculatum* and *Cynanchum wilfordii* are widely used as folk medicine in Eastern Asia. However, the indeterminacy in the authentic original plant material has resulted in the same appellative name being given to the two plants, and they are commonly misused. Therefore, it is necessary to establish an analytical method for discrimination as well as quality control of the two species. This study was to develop HPLC–UV methods for quality assessment of *C. auriculatum* and *C. wilfordii* and discrimination between the two species. Two HPLC methods to analyze eight marker compounds were established and validated. The first method analyzed seven marker compounds simultaneously on a reversed-phase column, while the second method analyzed a single marker compound, conduritol F, which exists only in *C. wilfordii*,

on a Si-column. Thirty-nine batches of *C. auriculatum* and nineteen batches of *C. wilfordii* that were collected from different geographical regions of South Korea were analyzed by these methods. The constructed data matrix was subjected to principal components analysis and hierarchical cluster analysis in order to classify the samples. The established methods offer a potential strategy for authentication and differentiation of the two species.

Keywords *Cynanchum auriculatum* · *Cynanchum wilfordii* · HPLC analysis

Introduction

The root tubers of *Cynanchum auriculatum* Royle ex Wight and *Cynanchum wilfordii* Maxim have been used as folk medicine for hundreds of years in Eastern Asia. Both of these species belong to the *Asclepiadaceae* family and appear morphologically similar. Of the two species, only *C. wilfordii* is registered in the Korean Herbal Pharmacopoeia. However, indeterminacy in the authentic original plant material has resulted in the same appellative name being given to both plants, and they have been commonly misused in the herbal drug market of Korea. Therefore, it is necessary to establish an analytical method not only to assess their quality but also to discriminate between the two species. While there are few studies comparing the chemical components between the two species by modern analytical methods, the extracts and constituents of *C. auriculatum* and *C. wilfordii* exhibit pharmacological actions such as multidrug-resistance-reversing activity (Kim et al. 1997; Hwang et al. 1999a), antioxidant effects (Lee et al. 1996, 1998, 2001), neuroprotective activity (Lee et al. 2000), inhibition of experimental gastric lesions

Ying Li and Donggen Piao contributed equally to this paper.

Electronic supplementary material The online version of this article (doi:10.1007/s12272-013-0060-3) contains supplementary material, which is available to authorized users.

Y. Li · D. Piao · H. Zhang · S.-H. Lee ·
H. W. Chang · J. K. Son (✉)
College of Pharmacy, Yeungnam University,
Gyeongsan 712-749, South Korea
e-mail: jkson@yu.ac.kr

M.-H. Woo
College of Pharmacy, Catholic University of Daegu,
Gyeongsan 712-702, South Korea

J.-H. Lee
College of Oriental Medicine, Dongguk University,
Gyeongju 780-714, South Korea

D.-C. Moon
College of Pharmacy, CBITRC, Chungbuk National University,
Cheongju 361-763, South Korea

(Shan et al. 2006) and anti-tumor activities (Shan et al. 2005; Zhang et al. 2007; Peng et al. 2008). Many different types of compounds have been isolated from the two species including acetophenones (Hwang et al. 1999b), cyclitol (Jiang et al. 2011), steroidal glycosides (Tsukamoto et al. 1985, 1986; Zhang et al. 2000; Gu et al. 2009; Xiang et al. 2009; Lu et al. 2011), alkaloids (Lee et al. 1998), and saccharides (Tsukamoto et al. 1989). HPLC–UV (Zhou et al. 2008) and LC–MS (Qi et al. 2009; Zhang et al. 2009) methods have previously been reported for component identification of *C. auriculatum*. The first method was a fingerprint analysis based on HPLC–UV separation to distinguish among *C. auriculatum*, *C. bungei* and *C. wilfordii*. However, it was still hard to discriminate between *C. auriculatum* and *C. wilfordii* based on the quantitation of three marker compounds without the help of the fingerprint analysis. Recently, we reported that conduritol F was isolated only from *C. wilfordii* but not from *C. auriculatum* and could be a marker compound to discriminate between the two species (Jiang et al. 2011). The purposes of this study were to develop a simultaneous determination method of eight marker compounds in order to evaluate their quality and discriminate between the two species and then to propose the developed method as an official analytical method for the two species in the Korean Herbal Pharmacopoeia.

Materials and methods

Plant materials and reagents

Different batches of the herbal samples were collected or purchased from different cultivation regions of Korea (supporting information), and identified by Professor Je-Hyun Lee, Dongguk University, Gyeongju, Korea. All the voucher specimens were deposited in the College of Pharmacy, Yeungnam University. Thirty-nine samples of *C. auriculatum* (A1–A39) and fifteen of *C. wilfordii* (W1–W15) were collected or purchased from four major regions of cultivation, Yeongju, Yeongcheon, Andong and Cheongsong, which were located on the Korean Peninsula. Four samples of *C. wilfordii* (W16–W19) were collected from Jeju Island, which was an island off the southern coast of the Korean Peninsula in the Korea Strait. Eight reference compounds, cynanonoside B (**1**), *p*-hydroxyacetophenone (**2**), 2,5-dihydroxyacetophenone (**3**), cynandione A (**4**), 2,4-dihydroxyacetophenone (**5**), wilfoside K1 N (**6**), wilfoside C1 N (**7**) and conduritol F (**8**), were isolated from *C. wilfordii* as reported previously by our research group (Fig. 1) and had a purity 95 % or more based on HPLC analysis (Jiang et al. 2011). An internal standard (IS) propyl paraben was obtained from Aldrich (St. Louis, MO, USA,

purity ≥ 99.0 %). HPLC-grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Water was prepared with an ELGA LabWater ultra-pure water purification systems (High Wycombe, Bucks, England). All solutions prepared for HPLC were filtered through 0.45- μm membrane filters before use.

Sample preparation

The dried roots of *C. auriculatum* and *C. wilfordii* were comminuted and passed through a 50-mesh (0.30 mm) sieve. Each sample (0.2 g) was accurately weighed and extracted by sonication with 10 mL of 70 % aqueous methanol for 60 min at room temperature. After sonication, each solution was adjusted to the original volume and filtered through 0.2- μm membrane. A 10- μL aliquot of the filtrate was analyzed by HPLC.

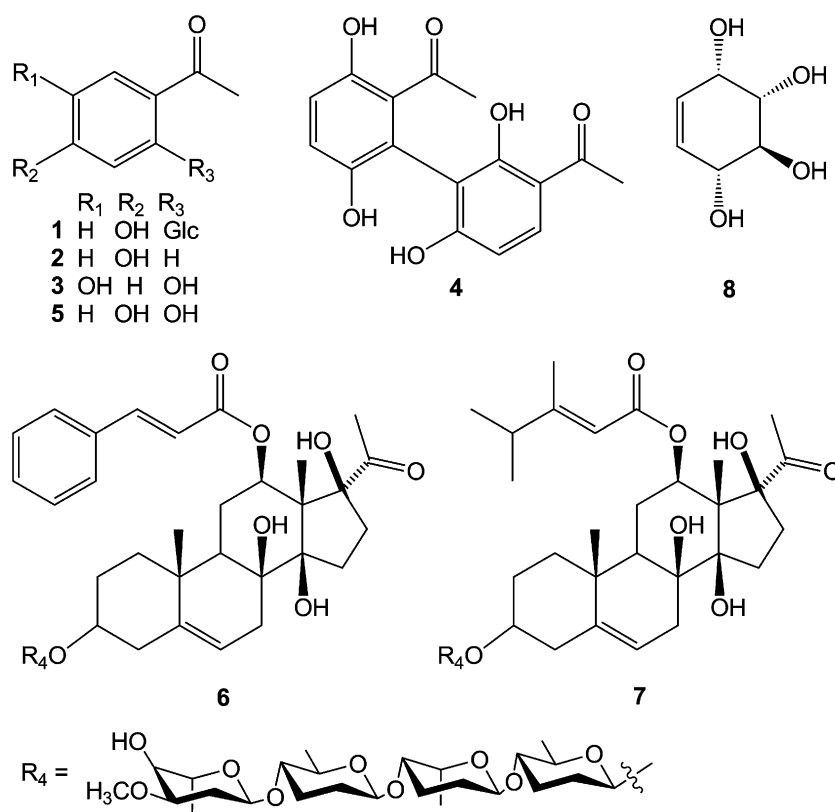
Chromatographic conditions

Two quantitative methods were developed on a Shimadzu HPLC system consisting of LC-20AD pump, SPD-M20A diode array detector (DAD), SIL-20A auto-sampler, CTO-20A column oven and DGU-20A3 solvent degasser. An internal standard method for separating marker compounds **1–7** with propyl paraben as an internal standard was performed on a Shiseido Capcell PAK C_{18} column (250 \times 4.6 mm, 5 μm) coupled with a guard cartridge C_{18} (4.0 \times 3.0 mm). The mobile phase consisted of 0.05 % (v/v) aqueous acetic acid (eluent A) and 0.05 % (v/v) acetic acid in acetonitrile (eluent B), which were applied in the gradient elution as follows: 0–30 min, linear gradient from A–B (90:10, v/v) to A–B (30:70, v/v); 30–40 min, isocratic elution of A–B (30:70, v/v). Prior to each run, the column was equilibrated to the starting conditions for 15 min. An external standard method for separating compound **8** was achieved on an Inertsil SIL column (250 \times 4.6 mm, 5 μm). The mobile phase consisted of 0.05 % (v/v) aqueous acetic acid (eluent A) and 0.05 % (v/v) acetic acid in acetonitrile (eluent B), which were applied in an isocratic elution of A–B (1:99, v/v) for 20 min. In both methods, the chromatogram was monitored at 210 nm, the column temperature set at 30 °C, the flow rate at 1.0 mL/min, and the injection volume 10 μL . Each sample was analyzed as three preparations from three parallel determinations. The data were collected and analyzed with Shimadzu LC solution software.

Sample extraction optimization

In order to find the optimal extraction efficiency for the marker compounds, extraction procedures including extraction solvent, extraction method, and extraction time

Fig. 1 Chemical structures of marker compounds: **1** cyanoneside B, **2** *p*-hydroxyacetophenone, **3** 2,5-dihydroxyacetophenone, **4** cynandione A, **5** 2,4-dihydroxyacetophenone, **6** wilfoside K1 N, **7** wilfoside C1 N, **8** conduritol F



were investigated. Different ratios of methanol/water (10:0, 7:3, 5:5, v/v), and ethanol/water (10:0, 7:3, 5:5, v/v) as extraction solvents, ultra-sonication, refluxing and shaking as extraction methods, and various extraction times (10, 30, 60, 90 min) were investigated.

Calibration curves and the limits of detection and quantification

For linear regression analysis, compounds **1–7** were assessed by an internal standard method, and **8** was assessed by an external standard method. The limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD and 10 for LOQ.

Precision

Precision of the chromatographic method was tested by examining repeatability and intermediate precision. The repeatability was assessed with six sample solutions according to previously described analytical methods on the same day. The intermediate precision of the method was evaluated by analyzing three individual extracts at three different concentration levels (low, middle and high). The experiment was repeated three times on the same day

for intra-day precision and on three consecutive days to determine inter-day precision.

Accuracy

Accuracy was determined by adding the mixed standard solutions at three different concentration levels (low, middle and high) to known amounts of the plant samples. Then the resultant samples were extracted and analyzed by the proposed method; triplicate experiments were performed at each level.

Stability of marker compounds in solution

Two sets of each marker compound (**1–8**) were dissolved in methanol (5–140 µg/mL) separately, stored at 4 and 25 °C, and analyzed by HPLC at 1, 2, 3, 4, 6, 11, 16 and 31 days.

Results and discussion

Optimization of chromatographic conditions

Based on the absorption of the marker compounds on the UV spectrum, the detection wavelength was set at 210 nm. Two mobile phase systems, acetonitrile–water and methanol–water, were investigated. The acetonitrile–water system

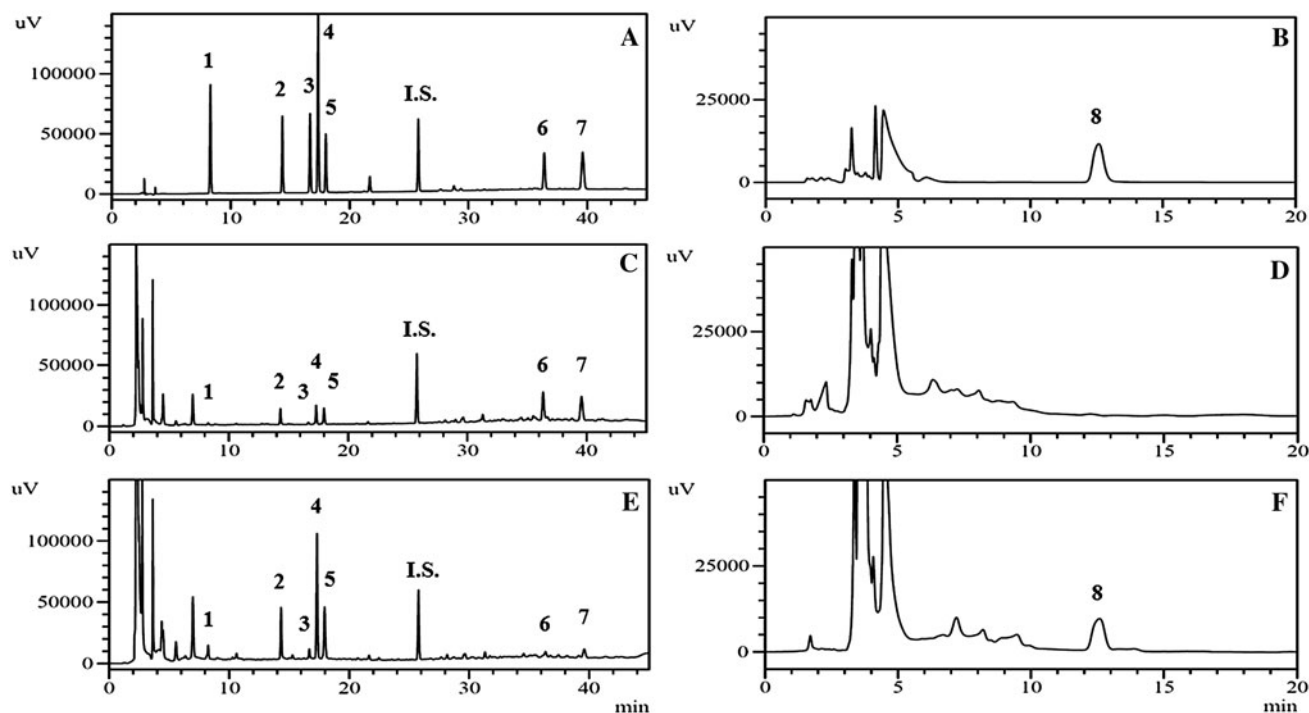


Fig. 2 Representative reverse phase (A, C, E) and normal phase (B, D, F) HPLC chromatograms of standard solution (A, B), extracts of *C. auriculatum* (C, D) and *C. wilfordii* (E, F). Peak number:

1 cyanoneside B, 2 *p*-hydroxyacetophenone, 3 2,5-dihydroxyacetophenone, 4 cynandione A, 5 2,4-dihydroxyacetophenone, 6 wilfoside K1 N, 7 wilfoside C1 N, 8 conduritol F, I.S.: propyl paraben

showed better separation ability for the marker compounds (1–7) than the methanol–water system, and the selected UV wavelength (210 nm) was further away from UV cutoff for acetonitrile (190 nm) than for methanol (205 nm). Addition of acetic acid to the mobile phase enhanced the resolution and reduced the peak tailing, but the effects of acetic acid concentration (0.01, 0.05, 0.1 %, v/v) in the mobile phase were not significant. From the above results, acetonitrile and water containing 0.05 % acetic acid were chosen as the eluting solvent. The optimum column temperature and flow rate were set as 30 °C and 1.0 mL/min, respectively. The chromatographic columns, Shiseido Capcell PAK C₁₈ (5 μm, 4.6 × 250 mm), Waters SunFire™ C₁₈ (5 μm, 4.6 × 250 mm) and Phenomenex Luna C₁₈ (5 μm, 4.6 × 250 mm),

were investigated for separation of seven marker compounds. The Shiseido Capcell PAK C₁₈ column (5 μm, 4.6 × 250 mm) proved to be better than the rest. Figure 2 shows the satisfactory separation and reasonable analytical time of a standard mixture (A), *C. auriculatum* extract (C) and *C. wilfordii* extract (E) obtained under the above optimized HPLC conditions. Two types of chromatographic columns were tested to separate 8 (conduritol F). On C₁₈ columns such as Shiseido Capcell PAK C₁₈ and Waters SunFire™ C₁₈, the peak of 8 was not retained longer than 5 min with poor resolution due to the polarity of 8. A Si-HPLC column (Inertsil SIL column) with an acetonitrile–water solvent system resulted in much better separation of 8, and these results are presented in Fig. 2B, F.

Table 1 Calibration curves of marker compounds

Compounds	Regression equation	r^2	Linear range (μg/mL)	LOD (ng/mL)	LOQ (ng/mL)
1	$y = 0.0546x + 0.0164$	0.9998	0.5–50	30	100
2	$y = 0.0909x + 0.0227$	0.9999	0.2–40	12	40
3	$y = 0.1067x + 0.0092$	0.9999	0.1–20	6	20
4	$y = 0.0983x + 0.1362$	0.9999	1.25–250	75	250
5	$y = 0.1486x + 0.0448$	0.9999	0.2–40	12	40
6	$y = 0.0183x + 0.0133$	0.9996	0.4–80	24	80
7	$y = 0.0172x + 0.0107$	0.9998	0.6–120	36	120
8	$y = 1,719.2x - 9,670.2$	0.9996	20.0–400	120	400

1 cyanoneside B, 2 *p*-hydroxyacetophenone, 3 2,5-dihydroxyacetophenone, 4 cynandione A, 5 2,4-dihydroxyacetophenone, 6 wilfoside K1 N, 7 wilfoside C1 N, 8 conduritol F

Table 2 Repeatability of eight marker compounds

Compounds	Repeatability ($n = 6$)			
	Observed conc. ($\mu\text{g/mL}$) ^a	RSD (%) ^b	Retention time (min)	RSD (%)
1	6.94 \pm 0.00	0.05	8.29 \pm 0.00	0.03
2	2.72 \pm 0.00	0.10	14.33 \pm 0.01	0.05
3	1.27 \pm 0.02	1.18	16.65 \pm 0.01	0.06
4	34.49 \pm 0.18	0.49	17.36 \pm 0.01	0.06
5	2.70 \pm 0.00	0.17	17.98 \pm 0.01	0.05
6	5.16 \pm 0.03	0.51	36.31 \pm 0.01	0.03
7	7.22 \pm 0.07	0.90	39.54 \pm 0.01	0.03
8	196.01 \pm 6.50	0.46	12.59 \pm 0.01	0.08

^a Observed conc. = mean \pm SD ($n = 3$)^b RSD (%) = (SD/mean) \times 100**Table 3** Intra- and inter-day precision data of eight marker compounds

Compounds	Intra-day precision ($n = 3$, mean)						Inter-day precision ($n = 9$, mean)	
	Day 1		Day 2		Day 3		Observed conc. ($\mu\text{g/mL}$)	RSD (%)
	Observed conc. ($\mu\text{g/mL}$)	RSD (%)	Observed conc. ($\mu\text{g/mL}$)	RSD (%)	Observed conc. ($\mu\text{g/mL}$)	RSD (%)		
1	4.66 \pm 0.01	0.25	4.62 \pm 0.03	0.66	4.77 \pm 0.04	0.83	4.68 \pm 0.07	1.59
	7.69 \pm 0.02	0.30	7.54 \pm 0.05	0.60	7.73 \pm 0.01	0.07	7.65 \pm 0.10	1.28
	23.73 \pm 0.05	0.21	23.95 \pm 0.14	0.57	23.70 \pm 0.17	0.73	23.79 \pm 0.14	0.58
2	7.31 \pm 0.01	0.16	7.36 \pm 0.02	0.32	7.53 \pm 0.06	0.74	7.40 \pm 0.12	1.55
	10.07 \pm 0.01	0.08	10.10 \pm 0.01	0.13	10.26 \pm 0.06	0.56	10.14 \pm 0.11	1.01
	24.45 \pm 0.04	0.18	24.68 \pm 0.13	0.51	24.55 \pm 0.06	0.22	24.56 \pm 0.12	0.47
3	3.44 \pm 0.01	0.10	3.45 \pm 0.01	0.36	3.56 \pm 0.01	0.27	3.48 \pm 0.07	1.87
	4.92 \pm 0.01	0.18	4.92 \pm 0.01	0.06	4.99 \pm 0.03	0.59	4.94 \pm 0.04	0.78
	12.71 \pm 0.02	0.17	12.83 \pm 0.12	0.94	12.61 \pm 0.03	0.27	12.72 \pm 0.11	0.90
4	6.16 \pm 0.07	1.20	6.13 \pm 0.01	0.22	6.28 \pm 0.04	0.68	6.19 \pm 0.08	1.35
	8.87 \pm 0.07	0.75	8.88 \pm 0.01	0.16	8.92 \pm 0.06	0.63	8.89 \pm 0.03	0.33
	23.06 \pm 0.03	0.14	23.29 \pm 0.19	0.80	23.06 \pm 0.06	0.28	23.14 \pm 0.13	0.58
5	33.23 \pm 0.01	0.04	33.66 \pm 0.44	1.31	33.71 \pm 0.06	0.17	33.53 \pm 0.27	0.79
	38.48 \pm 0.16	0.41	38.92 \pm 0.27	0.69	39.51 \pm 0.16	0.41	38.97 \pm 0.52	1.32
	64.47 \pm 0.16	0.25	65.21 \pm 0.32	0.50	63.92 \pm 0.27	0.42	64.53 \pm 0.65	1.00
6	47.47 \pm 0.20	0.43	47.09 \pm 0.09	0.19	49.22 \pm 0.14	0.29	47.93 \pm 1.14	2.38
	53.94 \pm 0.10	0.18	53.53 \pm 0.15	0.28	55.12 \pm 0.16	0.29	54.20 \pm 0.83	1.52
	86.97 \pm 0.17	0.20	85.70 \pm 0.74	0.87	87.41 \pm 0.13	0.15	86.69 \pm 0.88	1.02
7	28.09 \pm 0.03	0.12	27.79 \pm 0.02	0.06	28.02 \pm 0.06	0.21	27.96 \pm 0.16	0.56
	35.92 \pm 0.10	0.27	35.43 \pm 0.06	0.17	35.27 \pm 0.24	0.69	35.54 \pm 0.34	0.95
	76.11 \pm 0.14	0.18	75.30 \pm 0.48	0.64	74.20 \pm 0.14	0.18	75.21 \pm 0.96	1.27
8	117.54 \pm 0.23	0.20	116.8 \pm 0.09	0.08	115.61 \pm 0.45	0.39	116.65 \pm 0.18	0.15
	143.29 \pm 0.52	0.36	141.45 \pm 0.47	0.34	142.03 \pm 0.22	0.16	142.25 \pm 0.16	0.11
	194.90 \pm 0.82	0.42	192.33 \pm 0.68	0.36	190.32 \pm 1.60	0.84	192.51 \pm 0.49	0.26

Sample extraction optimization

Extraction by methanol/water (7:3, v/v) gave a higher yield of marker compounds than other solvents. In terms of extraction

methods, the ultrasonic method was better than the others. An extraction time of 60 min was sufficient to extract all eight marker compounds. Finally, the optimized extraction conditions were 70 % methanol under ultra-sonication for 60 min.

Table 4 Recoveries of marker compounds ($n = 3$)

Compounds	Fortified conc. ($\mu\text{g/mL}$)	Observed conc. ($\mu\text{g/mL}$)	RSD (%)	Recovery ^a (%)
1	0	1.53 ± 0.00	–	–
	2.5	4.06 ± 0.01	0.39	101.25
	5.0	6.62 ± 0.04	0.80	101.71
	20.0	21.69 ± 0.08	0.42	100.77
2	0	4.52 ± 0.01	–	–
	2.5	7.04 ± 0.01	0.48	100.54
	5.0	9.80 ± 0.03	0.65	105.50
	20.0	24.31 ± 0.05	0.25	98.92
3	0	2.05 ± 0.01	–	–
	1.25	3.30 ± 0.01	0.61	100.19
	2.5	4.65 ± 0.01	0.46	104.05
	10.0	12.21 ± 0.02	0.20	101.60
4	0	21.18 ± 0.04	–	–
	7.5	28.86 ± 0.03	0.43	102.33
	15.0	36.66 ± 0.06	0.39	103.19
	60.0	77.64 ± 0.29	0.52	94.09
5	0	3.70 ± 0.02	–	–
	2.5	6.33 ± 0.05	1.72	105.47
	5.0	8.98 ± 0.08	1.44	105.68
	20.0	23.45 ± 0.21	1.04	98.76
6	0	27.92 ± 0.04	–	–
	5.0	33.24 ± 0.05	1.01	106.49
	10.0	38.42 ± 0.04	0.41	105.06
	40.0	64.85 ± 0.26	0.69	92.33
7	0	41.8 ± 0.17	–	–
	5.0	46.85 ± 0.12	2.29	101.03
	10.0	51.98 ± 0.23	2.25	101.83
	40.0	82.64 ± 0.89	2.17	102.11
8	0	92.42 ± 0.93	–	–
	25.0	117.54 ± 0.23	0.36	97.43
	50.0	143.30 ± 0.52	0.95	98.01
	100.0	194.90 ± 0.82	0.68	99.89

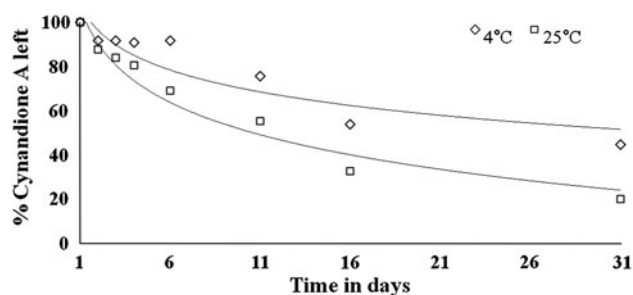
^a Recovery (%) = [(measured concentration – original concentration) / spiked concentration] \times 100

Validation

For linear regression analysis for each of the eight compounds, all the calibration curves showed good linearity ($r^2 \geq 0.9996$) over the concentration ranges tested. The LODs and LOQs of the eight marker compounds ranged from 6 to 120 and 20 to 400 ng/mL, respectively (Table 1). Six samples from the same plant origin were extracted and analyzed by the proposed method, and the relative standard deviation (RSD) value of content and retention time for

Table 5 Stability of marker compounds in methanol

Compounds	Stability (30 day, $n = 3$)			RSD (%)
	4 °C Observed conc. ($\mu\text{g/mL}$)	RSD (%)	25 °C Observed conc. ($\mu\text{g/mL}$)	
1	26.23 ± 0.14	0.54	26.35 ± 0.10	0.36
2	10.39 ± 0.05	0.47	10.41 ± 0.02	0.17
3	9.88 ± 0.21	2.08	9.92 ± 0.19	1.95
4	20.85 ± 5.28	25.34	17.22 ± 7.32	42.50
5	5.04 ± 0.03	0.62	5.05 ± 0.04	0.79
6	45.64 ± 0.79	1.74	45.69 ± 0.22	0.48
7	58.74 ± 0.30	0.51	59.25 ± 0.29	0.49
8	131.60 ± 1.90	1.44	131.30 ± 1.65	1.26

**Fig. 3** Degradation plots of cynandione A in methanol

each standard compound was calculated as a measurement of method repeatability. The RSD values of the eight compounds were less than 1.18 %, which showed high repeatability of the method in Table 2. Three individual sample solutions were made daily on three consecutive days. The RSD values of content for the eight marker compounds were calculated to assess precision. The RSDs ranged from 0.04 to 1.31 % for intra-day variation and from 0.11 to 2.38 % for inter-day variation (Table 3). The low RSD values indicated the high precision in the method. Accuracy was evaluated by a recovery experiment. The percentage recoveries were calculated according to the following equation: [(determined amount – original amount) / spiked amount] \times 100. As shown in Table 4, the developed analytical method was reproducible with good accuracy in the range of 92.33–106.49 % (RSD < 2.29 %). Specificity of the method was proved not only by measuring the resolution factor between each standard peak and the nearest resolving peak on the chromatogram from the plant extract in addition to consistency of the retention times of marker compounds with those from plant extract but also by comparison between the UV spectra of the marker compounds and those of obtained from HPLC–DAD of plant extract (Figs. 2, 3, 4; Table 2 in the supporting information).

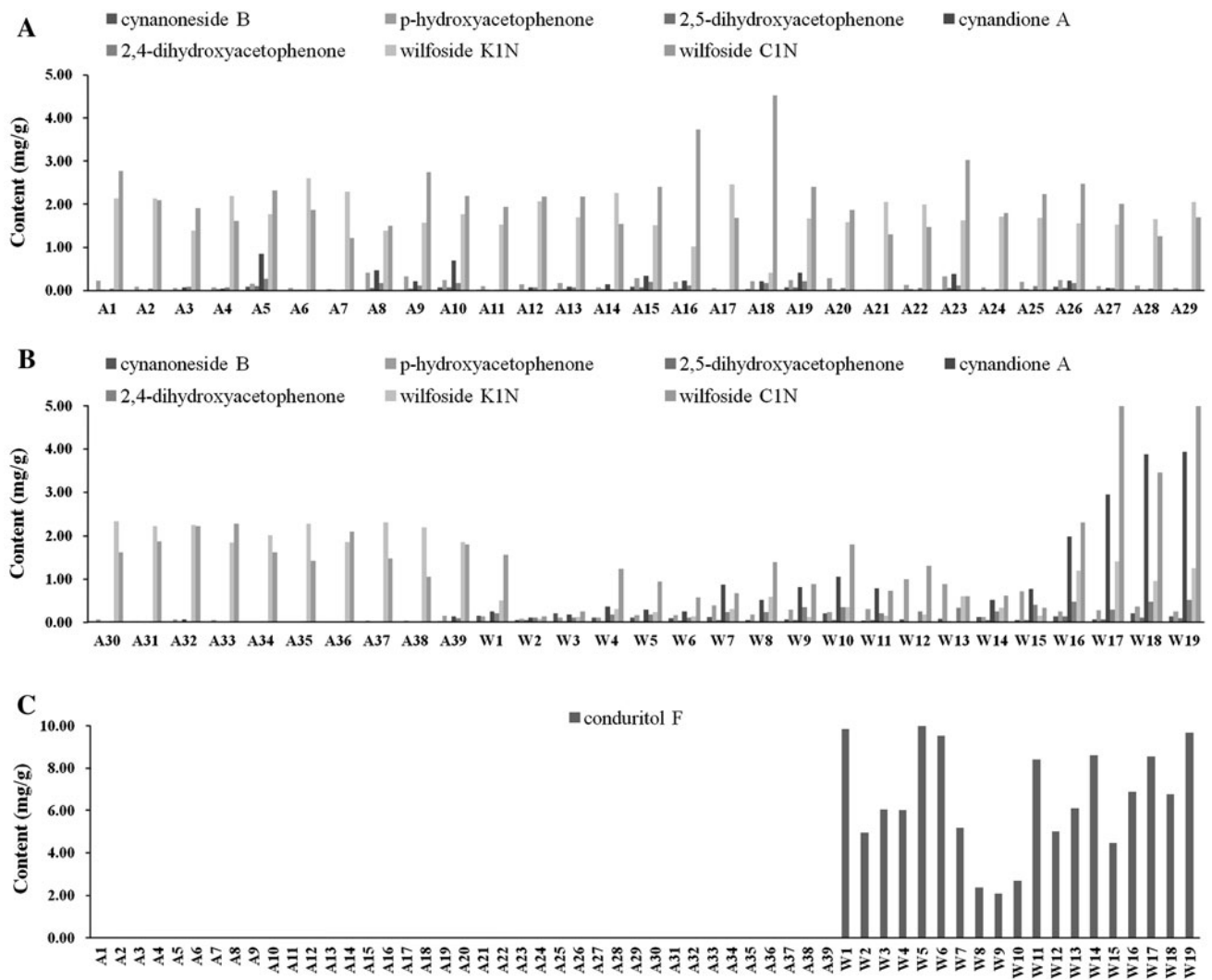


Fig. 4 Contents of the seven marker compounds (A and B) and conduritol F (C) for *C. auriculatum* (A1–A39) and *C. wilfordii* (W1–W19)

Table 6 Analysis of *C. auriculatum* and *C. wilfordii* samples

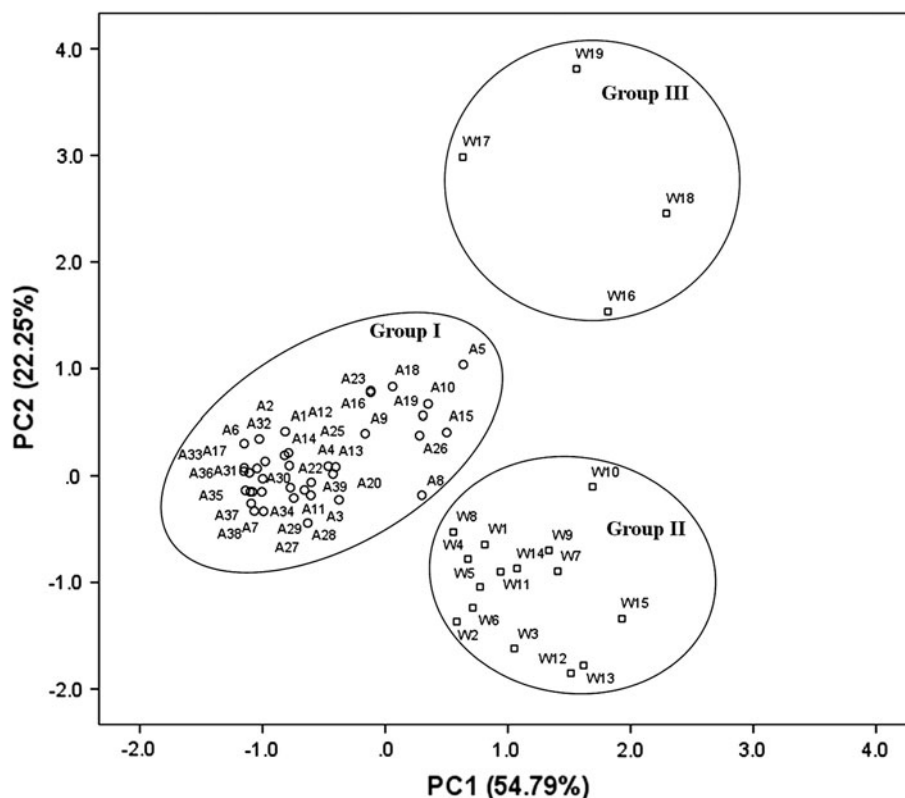
Compounds	Content (mean ± SD) (mg/g)		
	<i>C. auriculatum</i> Sample no.: A1–A39	<i>C. wilfordii</i> (Inland Korea) W1–W15	<i>C. wilfordii</i> (Jeju Island) W16–W19
1	0.02 ± 0.03	0.11 ± 0.05	0.14 ± 0.06
2	0.14 ± 0.11	0.33 ± 0.30	0.29 ± 0.05
3	0.03 ± 0.03	0.04 ± 0.02	0.10 ± 0.03
4	0.12 ± 0.21	0.45 ± 0.34	3.19 ± 0.92
5	0.08 ± 0.07	0.24 ± 0.09	0.44 ± 0.10
6	1.86 ± 0.43	0.28 ± 0.17	1.21 ± 0.19
7	2.04 ± 0.69	0.87 ± 0.50	4.20 ± 1.60
8	0	6.12 ± 2.72	7.98 ± 1.41

Stability

The stability of two sets of the eight marker compounds in methanol was tested by analyzing the solutions at various

time intervals within 1 month at 4 and 25 °C, respectively. The RSD values of the content of each sample were taken as a measurement of stability. Seven compounds except 4 showed good stability for 1 month at both temperatures,

Fig. 5 The scatter plot obtained by PCA of 58 samples based on the contents of marker compounds: *C. auriculatum* (Group I); *C. wilfordii* from inland on the Korean Peninsula (Group II); *C. wilfordii* from Jeju Island (Group III)



and the RSD values were all lower than 2.08 % (Table 5). Compound **4** was quite unstable in both conditions; >50 % of **4** appeared to be decomposed after 30 days under either condition (Fig. 3). However, **4** was stable within 18 h at both 4 and 25 °C (RSD < 2.55 %).

Sample analysis

The developed analytical methods were applied for quantitation of the eight marker compounds from 58 herbal samples as shown in Fig. 2. The contents of each marker compound in the samples are shown in Fig. 4 and summarized in Table 6 as an average value. As shown in Fig. 4C, **8** (conduritol F) was detected from *C. wilfordii* (W1–W19) in a range of 2.07–10.38 mg/g, but not from *C. auriculatum* (A1–A39). Samples of *C. auriculatum* (A1–A39) exhibited similar patterns in content ratios among seven marker compounds, and **6** and **7** were detected as major marker compounds in samples (Fig. 4A, B). However, samples of *C. wilfordii* (W1–W19) showed two different patterns in the content ratios of the seven marker compounds according to their origin of cultivation (Fig. 4B). While samples W1–W15 were cultivated inland on the Korean Peninsula, samples W16–W19 were collected from Jeju Island off the southern coast of the Korean Peninsula in the Korea Strait. The samples of W16–W19 contained four times higher contents of both **6** and **7** than other samples of *C. wilfordii* (W1–W15).

Principal components analysis (PCA)

PCA efficiently summarizes multivariate variation into a few principle components that represent maximum possible variability (Da et al. 2012). PCA of the contents of seven marker compounds (1–7) were calculated simultaneously because compound **8** had already been identified as a unique marker to distinguish between the two species, as it was only detected in *C. wilfordii*. A 58 object \times 7 variable data matrix containing the contents of the seven components was submitted to PCA. Two components (PC1 and PC2) were extracted on the basis of the eigenvalues-greater-than-one rule. Those two components together accounted for 77.04 % data variance (PC1 = 54.79 % and PC2 = 22.25 %). As shown in Fig. 5, the whole sample dots could be classified into three groups. The data of *C. auriculatum* (Group I) were effectively separated from those of *C. wilfordii* (Group II, III), but there was no definite confine such as all of the *C. wilfordii* were distributed with positive scores on PC1. Furthermore, data of the *C. wilfordii* was separated into two groups on PC2 with positive and negative scores, and such results might emerge from actual geologic and climatic differences that are reflected in the contents of the components. The lower group (Group II) contained four inland producers and the upper group (Group III) included outlying Jeju Island producers.

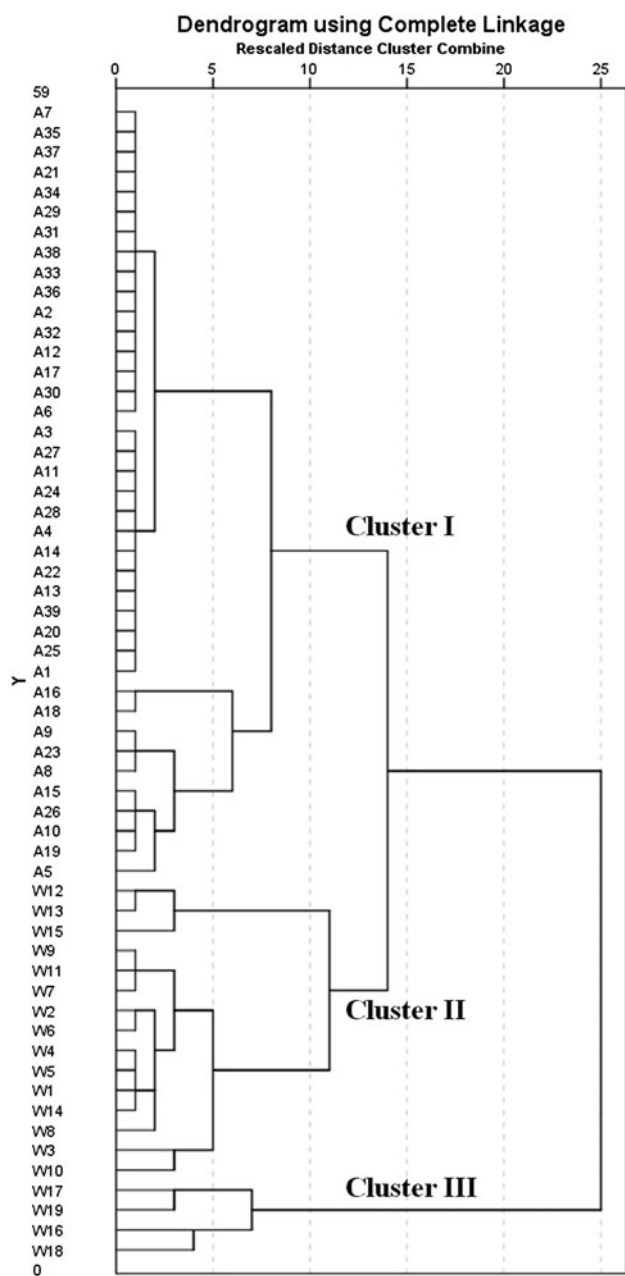


Fig. 6 Hierarchical clustering dendrogram of 58 samples based on the contents of marker compounds: *C. auriculatum* (Cluster I); *C. wilfordii* from inland on the Korean Peninsula (Cluster II); *C. wilfordii* from Jeju Island (Cluster III)

Hierarchical cluster analysis (HCA)

HCA was also applied to evaluate the reliability of the categorization. HCA is different from PCA, which reveals patterns across variables, in that it reveals patterns across whole cases. The hierarchical clustering was conducted by using Ward's Linkage as a clustering method and applying squared Euclidean Distance as a way of measuring distances between the cases and clusters. The presentation of

HCA resulted in a dendrogram (Fig. 6) that intuitively depicted the relationship characteristics. All samples could be classified into three clusters, and the results were similar to that of the PCA. A1–A39 were compactly grouped into Cluster I. W1–W19 comprised two subclusters; W16–W19 in Cluster III were obtained from Jeju Island, while others in Cluster II were from inland areas. The HCA result was consistent with the PCA result, and both methods could successfully distinguish the two species based on contents.

Conclusions

For quality assessment of *C. auriculatum* and *C. wilfordii* and discrimination between the two species, two HPLC methods were established to analyze eight marker compounds. Thirty-nine batches of *C. auriculatum* and nineteen batches of *C. wilfordii* that were obtained from different geographical regions of South Korea were analyzed by these methods. The method of simultaneous RP–HPLC analysis of seven marker compounds was validated to identify the chemical content differences between the two species. The constructed data matrix was subjected to principal components analysis (PCA) and hierarchical cluster analysis (HCA) in order to classify the samples. As a result, not only samples of *C. auriculatum* and *C. wilfordii* but also samples of two groups of *C. wilfordii* that were harvested in different areas could be discriminated. The other single marker compound, conduritol F, which exists only in *C. wilfordii*, was analyzed by Si–HPLC and proved to be a unique marker compound that could be used to discriminate between *C. wilfordii* and *C. auriculatum* based on analysis of multiple samples and our previous report (Jiang et al. 2011). The established methods offer a potential strategy for authentication and differentiation of the two species.

Acknowledgments This research was supported by a Yeungnam University research grant in 2010.

References

- Da, J., W.Y. Wu, J.J. Hou, H.L. Long, S. Yao, Z. Yang, L.Y. Cai, M. Yang, B.H. Jiang, X. Liu, C.R. Cheng, Y.F. Li, and D.A. Guo. 2012. Comparison of two officinal Chinese pharmacopoeia species of *Ganoderma* based on chemical research with multiple technologies and chemometrics analysis. *Journal of Chromatography A* 1222: 59–70.
- Gu, X.J., N. Yao, S.H. Qian, Y.B. Li, and P. Li. 2009. Four new C₂₁ steroidal glycosides from the roots of *Cynanchum auriculatum*. *Helvetica Chimica Acta* 92: 88–97.
- Hwang, B.Y., S.E. Kim, Y.H. Kim, Y.S. Hong, J.S. Ro, K.S. Lee, and J.J. Lee. 1999a. Pregnane glycoside multidrug-resistance modulators from *Cynanchum wilfordii*. *Journal of Natural Products* 62: 640–643.

- Hwang, B.Y., Y.H. Kim, J.S. Ro, K.S. Lee, and J.J. Lee. 1999b. Acetophenones from the roots of *Cynanchum wilfordii* HEMSLEY. *Archives of Pharmacal Research* 22: 72–74.
- Jiang, Y.F., H.G. Choi, Y. Li, Y.M. Park, J.H. Lee, D.H. Kim, J.H. Lee, J.K. Son, M.K. Na, and S.H. Lee. 2011. Chemical constituents of *Cynanchum wilfordii* and the chemotaxonomy of two species of the family Asclepiadaceae, *C. wilfordii* and *C. auriculatum*. *Archives of Pharmacal Research* 34: 2021–2027.
- Kim, S.E., B.Y. Hwang, Y.H. Kim, Y.C. Kim, K.S. Lee, and J.J. Lee. 1997. Multidrug-resistance reversing activity of medicinal plants. *Korean Journal of Pharmacognosy* 28: 174–178.
- Lee, D.U., U.S. Shin, and K. Huh. 1996. Inhibitory effects of gagaminine, a steroidal alkaloid from *Cynanchum wilfordii*, on lipid peroxidation and aldehyde oxidase activity. *Planta Medica* 62: 485–487.
- Lee, D.U., U.S. Shin, and K. Huh. 1998. Structure-activity relationships of gagaminine and its derivatives on the inhibition of hepatic aldehyde oxidase activity and lipid peroxidation. *Archives of Pharmacal Research* 21: 273–277.
- Lee, D.W., C. Kim, and D.U. Lee. 2001. Effect of culture conditions on the biosynthesis of gagaminine, a potent antioxidant from the roots of *Cynanchum wilfordii*. *Biological &/and Pharmaceutical Bulletin* 24: 1451–1453.
- Lee, M.K., H. Yeo, J. Kim, G.J. Markelonis, T.H. Oh, and Y.C. Kim. 2000. Cynandione A from *Cynanchum wilfordii* protects cultured cortical neurons from toxicity induced by H₂O₂, L-glutamate, and kainite. *Journal of Neuroscience Research* 59: 259–264.
- Lu, Y., H.L. Teng, G.Z. Yang, and Z.N. Mei. 2011. Three new steroidal glycosides from the roots of *Cynanchum auriculatum*. *Molecules* 16: 1901–1909.
- Peng, Y.R., Y.B. Li, X.D. Liu, J.F. Zhang, and J.A. Duan. 2008. Antitumor activity of C-21 steroidal glycosides from *Cynanchum auriculatum* Royle ex Wight. *Phytomedicine* 15: 1016–1020.
- Qi, L.W., X.J. Gu, P. Li, Y. Liang, H. Hao, and G. Wang. 2009. Structural characterization of pregnane glycosides from *Cynanchum auriculatum* by liquid chromatography on a hybrid ion trap time-of-flight mass spectrometer. *Rapid Communications in Mass Spectrometry* 23: 2151–2160.
- Shan, L., R.H. Liu, Y.H. Shen, W.D. Zhang, C. Zhang, D.Z. Wu, L. Min, J. Su, and X.K. Xu. 2006. Gastroprotective effect of a traditional Chinese herbal drug “Baishouwu” on experimental gastric lesions in rats. *Journal of Ethnopharmacology* 107: 389–394.
- Shan, L., W.D. Zhang, C. Zhang, R.H. Liu, J. Su, and Y. Zhou. 2005. Antitumor activity of crude extract and fractions from root tuber of *Cynanchum auriculatum* Royle ex Wight. *Phytotherapy Research* 19: 259–261.
- Tsukamoto, S., K. Hayashi, K. Kaneko, H. Mitsuhashi, and M. Shiro. 1986. Revised structure of a novel disaccharide, wilforibiose, obtained from the hydrolysate of *Cynanchum wilfordii* HEMSLEY glycosides. *Chemical & Pharmaceutical Bulletin* 34: 1067–1074.
- Tsukamoto, S., K. Hayashi, and H. Mitsuhashi. 1985. Studies on the constituents of Asclepiadaceae plants. LX. Further studies on glycosides with a novel sugar chain containing a pair of optically isomeric sugars, D- and L-cymarose, from *Cynanchum wilfordii*. *Chemical & Pharmaceutical Bulletin* 33: 2294–2304.
- Tsukamoto, S., K. Kaneko, and K. Hayashi. 1989. A method to identify the absolute configuration of rhamnose lyxose and 2,6-dideoxy sugars cymarose, oleandrose, diginose, and digitoxose, using a chiral HPLC column. *Chemical & Pharmaceutical Bulletin* 37: 637–641.
- Xiang, W.J., L. Ma, and L.H. Hu. 2009. C₂₁ steroidal glycosides from *Cynanchum wilfordii*. *Helvetica Chimica Acta* 92: 2659–2674.
- Zhang, R., Y. Liu, Y. Wang, Y. Ye, and X. Li. 2007. Cytotoxic and apoptosis-inducing properties of auriculolide A in tumor cells. *Chemistry & Biodiversity* 4: 887–892.
- Zhang, R.S., Y.P. Ye, Y.M. Shen, and H.L. Liang. 2000. Two new cytotoxic C-21 steroidal glycosides from the root of *Cynanchum auriculatum*. *Tetrahedron* 56: 3875–3879.
- Zhang, X., L. Shan, H. Huang, X. Yang, X. Liang, A. Xing, H. Huang, X. Liu, J. Su, and W. Zhang. 2009. Rapid identification of acetophenones in two *Cynanchum* species using liquid chromatography–electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 49: 715–725.
- Zhou, J., T. Zhang, Q. Wang, and J. Chen. 2008. Chromatographic fingerprint analysis of varietal differences among three species of Baishouwu and simultaneous analysis of three bioactive constituents by use of LC-DAD. *Chromatographia* 68: 213–218.