Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 2674–2680
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Isolation of iridoid and secoiridoid glycosides and comparative study on *Radix gentianae* and related adulterants by HPLC analysis

Ren-Wang Jiang [a], Ka-Lok Wong [a], Yiu-Man Chan [a], Hong-Xi Xu [d], Paul Pui-Hay But [a,c,*], Pang-Chui Shaw [a,b,*]

[a] *Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*
[b] *Department of Biochemistry, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*
[c] *Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China*
[d] *Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong Science Park, Shatin, New Territories, Hong Kong, China*

Received 21 February 2005; received in revised form 3 August 2005

Abstract

HPLC profile guided study led to the isolation of an acylated secoiridoid glycoside, named gentiotrifloroside (**1**), together with six known compounds, i.e., loganic acid (**2**), 6-*O*-β-D-glucopyranosylgentiopicroside (**3**), swertiamarin (**4**), gentiopicroside (**5**), sweroside (**6**) and 2 -(*o*,*m*-dihydroxybenzyl)-sweroside (**7**) from *Gentiana triflora* and *Gentiana rigescens*. The structure of **1** was deduced from one- and two-dimensional NMR spectroscopic experiments. Compounds **1**–**7** were used successfully as chemical markers for the comparison of the four species of *Gentiana* used as *Radix gentianae*. Additionally, differentiation of *Gentiana* species mentioned and those used as adulterants was evaluated. The close similarity of chemical composition among the four genuine *Gentiana* species explain their popular usage as *R. gentianae* in Chinese medicine. We have also shown that the variation of chemical composition in *R. gentianae* and related adulterants agree well with their botanical phylogeny.

Keywords: Gentianaceae, *Gentiana manshurica*; *Gentiana scabra*; *Gentiana triflora*; *Gentiana rigescens*; Berberidaceae, *Podophyllum hexandrum*; HPLC analysis; Gentiotrifloroside

1. Introduction

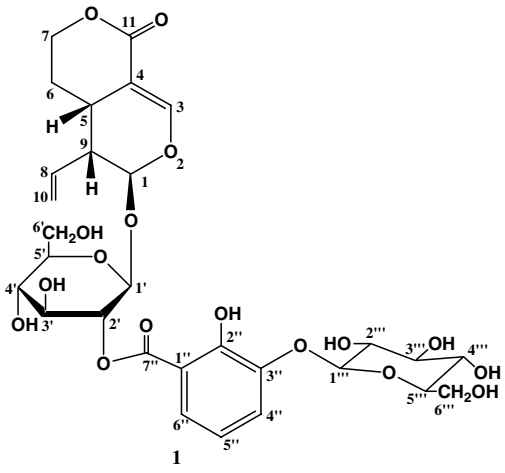
Gentiana, containing about 400 species, is the largest genus in the family Gentianaceae (Struwe et al., 2002). Plants belonging to this genus are best known for their bitter taste, and herbal extracts from various species have been included in many herbal formulations as remedies for poor appetite and digestive problems worldwide (Jensen and Schripsema, 2002). The dried root and rhizomes of four species, namely *Gentiana manshurica* Kitag, *Gentiana scabra* Bge, *Gentiana triflora* Pall and *Gentiana rigescens* Franch are officially listed in the Chinese Pharmacopoeia under the name of *Radix gentianae* (Longdan in Chinese). It is quite frequently used to eliminate damp-heat and quench the fire of the liver and gall bladder (Committee for the Pharmacopoeia of P.R. China, 2000). The first three species are distributed in northeastern China and reputed ''Guanlongdan'' while the latter one is located in southwestern part and called ''Jianlongdan''. A number of secoridoids have been isolated from *R. gentianae* to date (Rodriguez et al., 1998), for examples, gentiopicroside and sweroside were identified from *G. scabra* (Tang and Tan, 1997), and trifloroside was characterized in *G. triflora* (Inouye et al., 1974); however, detailed phytochemical comparison among different species and evidence for the equivalent application of these herbs are lacking. Furthermore, a related species *G. rhodantha* Franch is frequently found as a substitute in southwestern China due to the

* Corresponding authors. Tel.: +852 2609 6803; fax: +852 2603 5123.
E-mail addresses: paulbut@cuhk.edu.hk (P.Pui-Hay But), pcshaw@cuhk.edu.hk (P.-C. Shaw).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.09.028

demand for *R. gentianae*. Moreover, the neurotoxic *Podophyllum hexandrum* Royle in the family Berberidaceae had been found as an adulterant because of their similar appearance (Ng et al., 1991; But et al., 1996). Analytical methods for the comparative analysis of these herbs are thus necessary.

In this paper, we report the HPLC profile directed isolation and structure elucidation of a novel acylated secoiridoid glycoside, namely gentiotrifloroside (**1**) which was only found in *G. triflora* so far (Fig. 1), together with six known compounds, i.e., loganic acid (**2**), 6-*O*-β-D-glucopyranosylgentiopicroside (**3**), swertiamarin (**4**), gentiopicroside (**5**), sweroside (**6**) and 2-(*o*,*m*-dihydroxybenzyl)-sweroside (**7**), and comparative study on genuine *R. gentianae*, substitute and toxic adulterant by HPLC analysis.



1

Fig. 1. Structural formula of gentiotrifloroside (**1**).

2. Results and discussion

The crude methanol extracts of the powdered roots of *G. manshurica, G. scabra, G. triflora* and *G. rigescens* from different origins (Table 1) were screened by HPLC-UV. Typical chromatograms at a wavelength of 254 nm for the four herbs are shown in Fig. 2(a)–(d), which revealed five common peaks **2** ($t_R = 5.78$ min), **3**($t_R = 11.00$ min), **4** ($t_R = 11.55$ min), **5**($t_R = 16.68$ min) and **6** ($t_R = 18.53$ min) in the four herbs and two specific peaks **1** ($t_R = 28.87$ min) and **7** ($t_R = 33.15$ min) for *G. triflora* and *G. rigescens*, respectively. To identify the structures of **1**–**7**, the roots of *G. triflora* and *G. rigescens* were extracted for the purification of **1**–**6** and **7**, respectively, due to their relatively higher contents of the corresponding peaks. The extracts were separated by either precipitation or partition. Fractions were analyzed by HPLC-UV to check for the distribution of these peaks. Further purification of compounds **1**–**7** was achieved by semi-preparative HPLC.

The new compound **1** was isolated as a colorless powder. Assignment of its molecular formula $C_{29}H_{36}O_{17}$ was

Table 1
Sample list and contents of gentiopicroside (**5**) and sweroside (**6**)

No.	Species	Origins	Voucher number	**5** (%)[a]	**6** (%)[b]
1	*G. manshurica*	Yanbian, Jilin	2005-2701a	3.72	0.29
2	*G. manshurica*	Beian, Heilongjiang	2005-2701b	2.24	0.06
3	*G. manshurica*	Jilin city, Jilin	2005-2701c	4.19	0.05
4	*G. manshurica*	Dandong, Liaoling	2005-2701d	2.90	0.05
5	*G. scabra*	Beian, Heilongjiang	2005-2702a	1.08	2.90
6	*G. scabra*	Yinan, Heilongjiang	2005-2702b	2.31	1.93
7	*G. triflora*	Yinan, Heilongjiang	2005-2703a	6.47	0.03
8	*G. triflora*	Yinan, Heilongjiang	2005-2703b	4.45	0.03
9	*G. rigescens*	National Institute for the Control of Pharmaceutical and Biological Products	0988-200202	2.25	0.04
10	*G. rigescens*	Deqin, Yunnan	2005-2704a	6.28	0.06
11	*G. rigescens*	Weishan, Yunnan	2005-2704b	1.84	0.04
12	*G. rigescens*	Yiliang, Yunnan	2005-2704c	0.89	0.08
13	*G. rigescens*	Xilin, Guangxi	2005-2704d	1.02	0.04
14	*G. rigescens*	Dali, Yunnan	2005-2704e	3.01	0.03
15	*G. rigescens*	Lincang, Yunnan	2005-2704f	1.92	0.02
16	Commercial *Radix gentianae*	Wanzhen herbs shop, Hong Kong	2005-2705a	2.77	0.03
17	Commercial *Radix gentianae*	Guofeng herbs shop, Hong Kong	2005-2705b	2.16	0.02
18	Commercial *Radix gentianae*	Heilongjiang	2005-2705c	3.55	0.03
19	Commercial *Radix gentianae*	Wuhan, Hubei	2005-2705d	1.66	0.04
20	*G. rhodantha*	Xishui, Guizhou	2005-2706a	–	0.06
21	*G. rhodantha*	Shizhu, Chongqin	2005-2706b	–	0.04
22	*Podophyllum hexandrum*	Institute of Chinese Medicine, The Chinese University of Hong Kong (ICM-CUHK)	ICM 686	–	–
23	*Podophyllum hexandrum*	Chinese herbal museum, ICM-CUHK	ICM2148	–	–

"–", means too low to be determined.
[a] $y = 1.0058 \times 10^{-6}\chi + 0.557956$, $R^2 = 0.998$.
[b] $y = 7.12233 \times 10^{-7}\chi + 0.0723457$, $R^2 = 0.999$.

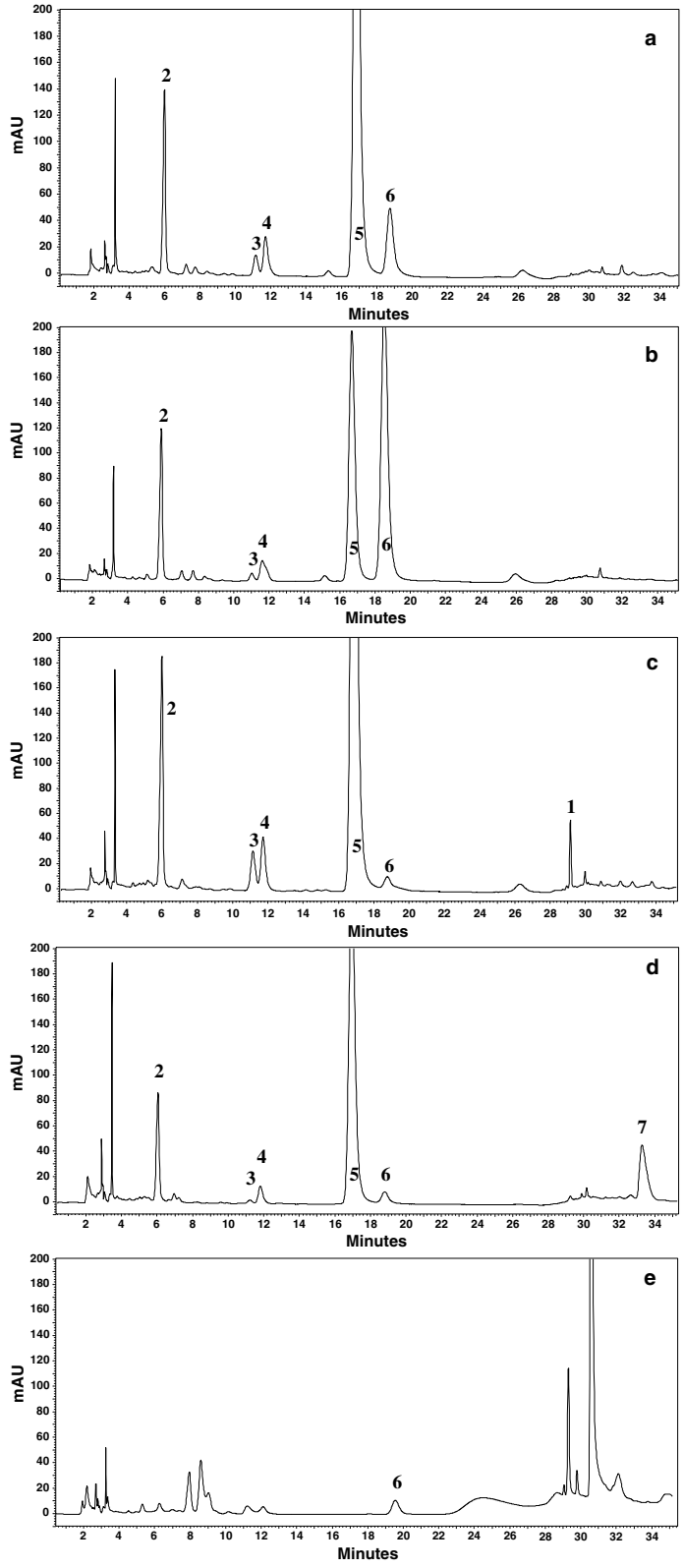


Fig. 2. Typical chromatograms at 254 nm for *Gentiana* species: *G. manshurica* (a, sample 1), *G. scabra* (b, sample 6) and *G. triflora* (c, sample 8), *G. rigescens* (d, sample 9) and *G. rhodantha* (e, sample 20).

based on negative HRFABMS (m/z $[M-H]^-$ 655.1886, calculated 655.1874).

The ^1H NMR spectrum (Table 2) showing a characteristic oxyolefinic proton at δ 7.133 (d, $J = 2.5$ Hz, H-3) suggested that **1** was a secoiridoid derivative of sweroside-type, which was further substantiated by liberation of sweroside from **1** with 5% NaOH.

Table 2
NMR spectroscopic data for gentiotrifloroside (**1**)[a]

Position	δ_H mult., J in Hz	δ_C mult.	HMBC
1	5.458, *d*, 1.5	97.71 *d*	C-3, C-5, C-8, C-1′
3	7.133, *d*, 2.5	153.38 *d*	C-1, C-4, C-5, C-11
4		105.68 *s*	
5	2.676, *m*	28.76 *d*	C-3, C-4, C-6, C-8, C-9
6	1.512, *m*, H-6α	25.71 *t*	C-5, C-7
	1.666, *m*, H-6β		C-5, C-4
7	4.093, *m*, H-7α	69.68 *t*	C-5, C-6
	4.304, *m*, H-7β		C-5, C-6, C-11
8	5.401, *dt*, 9.8, 17.1	132.72 *d*	C-1, C-5, C-9, C-10
9	2.620, *ddd*, 1.5, 5.5, 9.8	43.30 *d*	C-1, C-4, C-5, C-8, C-10
10	5.219, *dd*, 1.9, 9.8	121.04 *t*	C-1, C-8, C-9
	5.261, *dd*, 1.9, 17.1		C-1, C-8, C-9
11		167.11 *s*	
1′	4.996, *d*, 8.0	97.10 *d*	C-1, C-2′
2′	5.028, *dd*, 8.2, 8.9	75.68 *d*	C-1′, C-3′, C-7″
3′	3.523, *t*, 9.0	75.06 *d*	C-2′, C-4′, C-5′
4′	3.430, *t*, 8.2	71.43 *d*	C-3′, C-5′, C-6′
5′	3.470, *m*	78.33 *d*	C-3′, C-4′
6′	3.717, *dd*, 5.6, 10.2, H-6′α	62.59 *t*	C-4′, C-5′
	3.939, *dd*, 1.8, 11.9, H-6′β		C-4′
1″		114.35 *s*	
2″		153.12 *s*	
3″		147.35 *d*	
4″	7.420, *dd*, 1.5, 8.0	124.94 *d*	C-2″, C-3″, C-6″
5″	6.835, *t*, 8.0	120.46 *d*	C-1″, C-3″, C-4″, C-6″
6″	7.537, *dd*, 1.5, 8.0	124.68 *d*	C-2″, C-4″, C-7″
7″		170.68 *s*	
1‴	4.949, *d*, 7.4	103.44 *d*	C-3″, C-3‴
2‴	3.789, *dd*, 8.5, 8.7	75.06 *d*	C-4‴
3‴	3.496, *t*, 7.7	77.84 *d*	C-2‴, C-4‴, C-5‴
4‴	3.398, *t*, 9.2	71.78 *d*	C-3‴, C-5‴, C-6‴
5‴	3.451, *m*	78.74 *d*	C-3‴, C-4‴, C-6‴
6‴	3.694, *dd*, 5.6, 10.3, H-6′α	62.59 *t*	C-4‴, C-5‴
	3.896, *dd*, 2.1, 12.1, H-6′β		C-4‴

[a] Recorded in CD_3OD on a Bruker spectrometer at 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR). Carbon multiplicity was obtained from a DEPT experiment.

The appearance of a set of 1,2,3-trisubstituted benzene signals at δ 7.420 (*dd*, $J = 1.5$, 8.0 Hz, H-4″), 6.835 (*t*, $J = 8.0$ Hz, H-5″) and 7.537 (*dd*, $J = 1.5$, 8.0 Hz, H-6″) could be assigned to a 2″,3″-dioxygenated benzoyl group, which was reinforced by the typical carbon resonances at 114.35 (*s*, C-1″), 153.12 (*s*, C-2″), 147.35 (*s*, C-3″), 124.94 (*d*, C-4″), 120.46 (*d*, C-5″), 124.68 (*d*, C-6″) and 170.68 (*s*, C-7″ carbonyl). The positive reaction of **1** with $FeCl_3$ indicates the presence of a phenolic hydroxyl group and its location at 2-position was confirmed by the more downfield nature of C-2″ as compared with C-3″.

The existence of two glucosyl moieties was inferred from the two anomeric protons at δ 4.996 (*d*, $J = 8.0$ Hz, H-1′) and 4.949 (*d*, $J = 7.4$ Hz, H-1‴), and confirmed by the presence of two set of glucose carbon signals. The coupling constants of the anomeric protons prove the β-configuration for both sugar units. The more downfield position of one anomeric carbon atom C-l‴ (103.44, *d*) shows that this is the terminal glucose linked to the oxygenated benzoyl moiety; in contrast to that of the inner glucose C-1′ (97.10, *d*) which is linked to the secoiridoid nucleus. Accordingly, compound **1** was revealed to consist of one secoiridoid nucleus, one 2-hydroxyl-3-oxygenated benzoyl moiety and two glucosyl moieties.

The full assignments and connectivities were determined by ^1H–^1H COSY, HMQC and HMBC spectra. The ^1H–^1H COSY spectrum established spin systems involving H-1 → H-9 → H-8 → H-10, H-9 → H-5 → H_2-6 → H_2-7 and H-4″ → H-5″ → H-6″. The HMQC spectrum revealed that the protons at δ 5.458 (H-1) and 4.996 (H-1′) are attached to carbons at δ 97.71 (C-1) and 97.10 (C-1′), respectively, and the HMBC spectrum showed that H-1 is correlated to C-1′ and H-1′ is correlated to C-1, which established that the inner glucose is attached to the secoiridoid aglycone at C-1 via a glycosidic bond to form a sweroside skeleton. The HMBC correlation of H-2′ with the carbonyl group at C-7 indicates that the 2″-hydroxyl-3″-oxygenated benzoyl moiety is attached to C-2′ of the inner glucose via an ester bond. Similarly, the linkage between 2″,3″-dioxygenated benzoyl moiety and the terminal glucose was established by HMBC correlation of H-1‴ to C-3″. Accordingly, compound **1** was determined as 2-*O*-(2-hydroxyl-3-*O*-β-D-glucopyranosyl-benzoyl)-sweroside, which was named gentiotrifloroside.

A large number of acylated sweroside derivatives have been isolated from the Gentianaceae family. These kind of compounds vary primarily in the extent and pattern of acylation of the inner glucose. Close examination of the previously reported structures revealed three common acylation patterns: (i) single acylation with acetyl or hydroxylated aromatic acids, e.g., decentapicrin A (Kojić-Prodić et al., 1985) and amarogentin (Kawahara et al., 2001); (ii) multiple acylation with both acetyl and hydroxylated aromatic acids, e.g., 2-*O*-acetyl-4-*O*-*trans*-feruloylswertiamarin (Kikuzaki et al., 1996); (iii) multiple acylation with both acetyl group and glycosylated aromatic acid, e.g., trifloroside (Bergeron et al., 1997) and 2-gentisoyl gelidoside (Tan and Kong, 1997). To the best of our knowledge, compound **1** represents the first example of acylated sweroside derivative with a glycosylated aromatic acid served as the single acylation unit.

The structures of other six known compounds were identified by comparison of their spectroscopic data with those of reported values and found to be loganic acid (**2**) (Bianco et al., 2003), 6-*O*-β-D-glucopyranosylgentiopicroside (**3**) (Kakuda et al., 2001), swertiamarin (**4**) (Luo and Nie, 1993), gentiopicroside (**5**) (Takeda et al., 1999), sweroside (**6**) (Beek et al., 1982) and 2-(*o*,*m*-dihydroxybenzyl)-sweroside (**7**) (Tan et al., 1996). Accordingly all seven peaks were characterized. Compounds **2** and **3** were isolated from *G. triflora* for the first time, and compound **7** was isolated from *G. rigescens* for the first time. A standard solution consisting of compounds **1**–**7** was analyzed under the same HPLC condition as the crude extracts. The resulting chromatogram is shown in Fig. 3.

The present HPLC-UV/PDA investigation reveals the close similarity of chemical compositions among the four genuine *Gentiana* species as the presence of five common

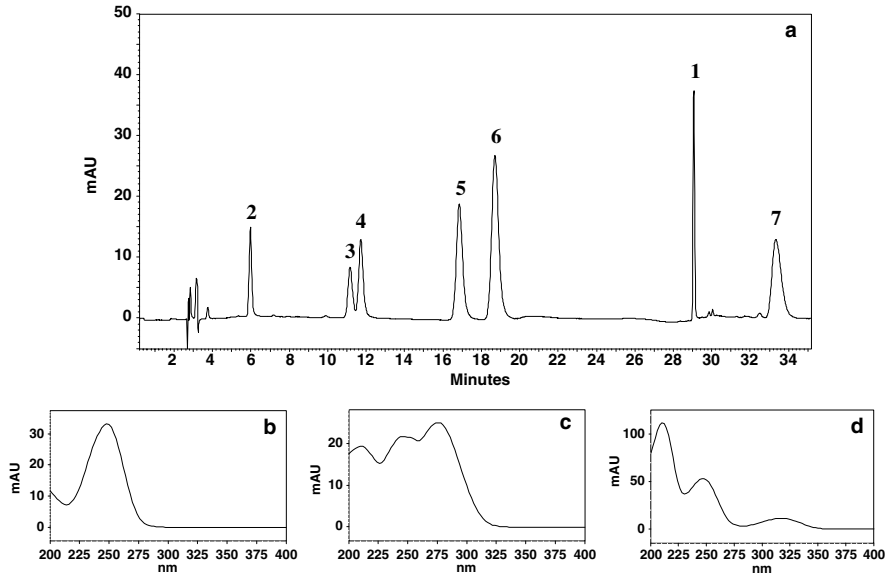


Fig. 3. HPLC chromatograms of chemical markers **1**–**7** at 254 nm (a), and online UV spectra of compounds **2**, **4** and **6** (b), compounds **3** and **5** (c), compounds **1** and **7** (d). Compounds **2**–**6**, **1** and **7** are eluted from the column successively.

compounds **2**–**6** in their methanol extracts (Fig. 2(a)–(d)) and therefore it is reasonable for their application under the same name of *R. gentianae* in Traditional Chinese Medicine.

Table 3
Relative abundance of compounds **1**–**7** in the underground part of *Gentiana* species and related adulterants

Sample No.	1	2	3	4	5	6	7
1		+	○	+	•	+	
2		○	○	+	•	○	
3		+	○	+	•+	○	
4		+	○	+	•	○	
5		+	○	○	•	•+	
6		+	○	+	•	•	
7	○	+	○	+	•+	○	
8	+	+	○	+	•+	○	
9		+	○	+	•	○	+
10		+	○	+	•+	○	+
11		○	○	○	•	○	+
12		○	○	○	+	+	+
13		○	○	○	•	○	+
14		+	○	○	•	○	+
15		○	○	○	•	○	+
16		+	+	+	•	○	
17		+	○	○	•	○	
18		+	○	+	•	○	
19		+	○	○	•	○	+
20						○	
21						○	
22							
23							

The symbols represent an estimate of the relative abundance on basis of peak area. "○" means a weak peak detected with peak area 2×10^4–4×10^5 units; "+" means a clear peak detected with peak area 4×10^5–4×10^6 units; "•" means a strong peak detected with peak area 4×10^6–2×10^7 units; and "•+" means a very strong peak detected with peak area larger than 2×10^7 units.

However, chemical variations were also observed in compounds **1**–**7** as shown in Table 3. Compounds **5** and **6** are the main active constituents which are mostly associated with the pharmacological activities of *R. gentianae*, e.g., liver-protecting (Liu et al., 2002, 1994) and smooth muscle relaxing (Rojas et al., 2000). Thus, the contents of compounds **5** and **6** were calibrated as listed in Table 1. The content of compound **5** ranged from 0.89% to 6.47% with an average of 2.97%. Sample 7 (*G. triflora*) possessed the highest content (6.47%) of compound **5** in all the samples, and samples 3, 6 and 10 had the highest content in *G. manshurica* (4.19%), *G. scabra* (2.31%) and *G. rigescens* (6.28%), respectively, whereas sample 12 was not qualified due to its lower content than 1% (Committee for the Pharmacopoeia of P.R. China, 2000). Compound **6** in most samples (from 1–4 to 7–15) appeared as a minor component with an average content of 0.06%; however, in the two localities of *G. scabra*, it is about fifty times higher than that in other samples. The average total contents of compounds **5** and **6** are 3.37%, 4.11%, 5.49% and 2.50% for *G. manshurica, G. scabra, G. triflora, G. rigescens*, respectively. Compounds **2**–**4** were observed in all four species as either a small or distinct peak; however, compounds **1** and **7** characterized in *G. triflora* and *G. rigescens*, respectively, were not detected in other samples.

Samples of four market commodities purchased in Hong Kong (samples 16 and 17) and mainland China (samples 18 and 19) were also studied. The average contents of compounds **5** and **6** in these samples are 2.53% and 0.03%, respectively, which are comparable to those of corresponding components in the collected samples. Samples 16–18 were inferred to be *G. manshurica* based on their similar HPLC pattern; while sample 19 was inferred to be *G. rigescens* due to the presence of a distinctive peak for compound **7** (Table 3).

The same HPLC conditions were also applied to differentiate *R. gentianae* from *G. rhodantha* and *P. hexandrum*. It was noted that the chromatogram of *G. rhodantha* is significantly different from the four species used as *R. gentianae* (Fig. 2(e)), and only a trace amount of compound **6** was detected. All seven markers were not observed in *P. hexandrum*. Thus, both *G. rhodantha* and *P. hexandrum* should be regarded as adulterants of *R. gentianae*.

The genus *Gentiana* is divided in 12 sections in China (19 sections in the world) (He, 1988). "Guanlongdan" (*G. manshurica, G. scabra* and *G. triflora*) and "Jianlongdan" (*G. rigescens*) belong to the adjacent sections of Pneumonanthe (section III) and Monopodiae (section IV), respectively, and also share a similar chemical pattern, while *G. rhodantha* belongs to the section of Senogyne (section IX) and large variation in HPLC chromatogram was observed. *P. hexandrum* belongs to another family and shows completely different chemical profile. Hence, the HPLC profiles agree well with their botanical phylogeny. In conclusion, current investigation provides a detailed comparison of the chemical composition of four species used as *R. gentianae* and differentiation from related adulterants. Results of this research thus now explain the popular usage of four species in their clinical applications.

3. Experimental

3.1. General

The UV spectra were obtained on an online Beckman 168 DAD spectrophotometer. ESIMS were recorded on a Finnigan MAT TSQ 7000 instrument. HRFABMS measurements were made on an API QSTAR Pulsar i system Q-TOF mass spectrometer. NMR spectra were obtained (^{1}H, ^{13}C, DEPT, NOESY, ^{1}H–^{1}H COSY, HMQC and HMBC) on a Bruker spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts are reported in ppm with reference to the solvent signals CD_3OD (δ_H 4.87 and δ_C 49.2), and coupling constants are in Hz. Column chromatography was performed with reversed phase silica gel (Merck, Germany), and macroporous resin D101 (Tianjin Agricultural Chemical Co. Ltd., China). TLC were performed on precoated Si gel 60 F_{254} plates (0.2 mm thick, Merck) with $CHCl_3$–CH_3OH (4:1 and 3:1 v/v), and spots were detected by UV illumination and by spraying with vanillin–H_2SO_4 (1%) and 1% $FeCl_3$ reagent.

3.2. Plant material

The herbal samples of *G. manshurica, G. scabra* and *G. triflora* were collected in northeastern China (Jilin, Liaoning and Heilongjiang provinces) and *G. rigescens* and *G. rhodantha* were collected in southwestern China (Yunnan, Guangxi, Guizhou and Chongqing provinces). All of the plant materials were collected from February to April of 2004. The botanical origins of all the collected samples were identified by Dr. Cao Hui during the field collection. The specimens of all these materials were kept in the Institute of Chinese Medicine, The Chinese University of Hong Kong. The voucher numbers are shown in Table 1.

3.3. Extraction and isolation of compounds 1–6 from G. triflora

The dried and pulverized roots and rhizomes of *G. triflora* (120 g) were extracted with MeOH (300 mL) in an ultrasonic water bath for 1 h. This extraction was repeated one more time. The extracted portions were combined and concentrated by evaporation under reduced pressure to give a crude extract (18.5 g), which was dissolved in MeOH (100 mL). $CHCl_3$ was slowly added to the MeOH solution until its concentration reached 65% to afford two fractions, i.e., precipitate fraction and a 65% chloroform soluble fraction.

The precipitate (16 g) was dissolved in distilled water and subjected to column chromatography on macro-porous resin (2.5×30 cm) which was eluted with H_2O (600 mL) followed by EtOH–H_2O (4:6) (600 mL) to give two fractions: water eluate and EtOH–H_2O (4:6) eluate. The water eluate mainly contained polysaccharides, with no secoiridoid detected by HPLC analysis. The EtOH–H_2O (4:6) eluate was subjected to semi-preparative HPLC with a mobile phase of CH_3OH:H_2O = 25:75. The peaks at 10.4, 12.8, 14.6 and 19.2 min were collected and condensed by rotary evaporator to afford compounds **2** (25 mg), **3** (20 mg), **4** (16 mg) and **5** (120 mg).

The 65% chloroform solubles were submitted to an isolation procedure as described for the precipitate fraction, except that the EtOH concentration for the column chromatography was changed to 60% and the mobile phase for the semi-preparative HPLC was changed to methanol: H_2O = 30:70. The peaks at 15.2 and 17.4 min were collected to afford compounds **6** (15 mg) and **1** (14.8 mg).

*Gentiotrifloroside (**1**)*: Colorless powder, formula $C_{29}H_{36}O_{17}$, $[\alpha]_D^{20}$ -109.5° (*c* 0.5, CH_3OH); online UV (CH_3CN–H_2O) λ_{max} 212, 247 and 318 nm, ESMS *m/z*: 655 $[M-H]^-$, 493 $[M-H\text{-glucosyl}]^-$, negative HRFABMS *m/z* $[M-H]^-$ 655.1886 (calculated 655.1874). For ^{1}H and ^{13}C NMR spectroscopic data, see Table 2.

*Liberation of sweroside from compound **1***. To confirm the structure deduced from spectroscopic analyses, **1** was deacylated with 0.5 M NaOH followed by an adjustment of pH to 4. Sweroside was identified by HPLC based on the comparison of retention time and online UV spectrum with the reference compound.

3.4. Extraction and isolation of compound 7 from G. rigescens

The dried and pulverized roots and rhizomes of *G. rigescens* (200 g) were extracted with the same methods as *G triflora* to give a crude extract (20 g), which was suspended

in distilled water and extracted with Et_2O and EtOAc successively to afford Et_2O (1.0 g), EtOAc (1.0 g) and H_2O soluble fractions (14.2 g). Peak **7** was present in the EtOAc subfraction by analytical HPLC, which was then subjected to semi-preparative HPLC with the mobile phase changed to 35:65. The peak at 12.5 min was collected to afford compound **7** (20 mg).

3.5. HPLC analysis

The powdered roots (0.5 g) of *G. manshurica*, *G. scabra*, *G. triflora* and *G. rigescens* from different origins (Table 1) were extracted with MeOH (10 mL) under ultrasonic condition. The extracted solutions were filtered through a 0.22 μm PTFE syringe filter, with an aliquot of each filtrate (10 μl) was injected in the HPLC instrument for analysis. Analytical HPLC was performed on a Beckman System Gold instrument equipped with a 125 solvent module, a 168 photo diode-array detector and a 508 autosampler. Chromatographic separation was carried out on a C18 column (250 × 4.6 mm, 5 μm; Beckman, USA), using a gradient solvent system comprised of H_2O (A) and CH_3CN (B). Gradient profile: 0–22.5 min: isocratic 10% B; 22.5–25 min: linear 10–20% of B; 25–32.5 min, isocratic 20% B; 32.5–35 min, linear 20–10% of B; On-line UV spectra were recorded with a diode-array detector from 200 to 400 nm.

3.6. Calibration of compounds **5** and **6**

Calibration curves for compounds **5** and **6** were prepared for the present study. Triplicate injections were made at six concentrations. The linearity of each standard curve was made by plotting the peak area versus concentration. The equations and correlation coefficients obtained from the linearity studies are shown as footnotes in Table 1.

3.7. Semi-preparative HPLC

Semi-preparative HPLC was also performed on a Beckman System Gold instrument but equipped with an Alltima C-8 semi-preparative column (250 × 10 mm, 5 μm) using a flow rate of 3 mL/min and a detection wavelength at 254 nm.

Acknowledgements

The research is funded by the Hong Kong Jockey Club Charities Trust. We also thank Dr. Hui Cao for his assistance in collecting samples of *R. gentianae*.

References

Beek, T.A., van Lankhorst, P.P., Verpoorte, R., Svendsen, A.B., 1982. Isolation of the secoiridoid-glucoside sweroside from *Tabernaemontana psorocarpa*. Planta Med. 44, 30–31.

Bergeron, C., Marston, A., Gauthier, R., Hostettmann, K., 1997. Iridoids and secoiridoids from *Gentiana linearis*. Phytochemistry 44, 633–637.

Bianco, A., Ramunno, A., Melchioni, C., 2003. Iridoids from seeds of *Gentiana lutea*. Nat. Prod. Res. 17, 221–224.

But, P.H., Tomlinson, B., Cheung, K.O., Yong, S.P., Szeto, M.L., Lee, C.K., 1996. Adulterants of herbal products can cause poisoning. Brit. Med. J. 313, 117.

Committee for the Pharmacopoeia of P.R. China, 2000. Pharmacopoeia of P.R. China, Part I. Guangdong Science and Technology Publishing House and Chemical Industry Publishing House, P.R. China, pp. 72–73.

He, T.N., 1988. GentianaceaeDictionary of Chinese Botany, vol. 62. Science Press, Beijing, P.R. China, pp. 23–24.

Inouye, H., Ueda, S., Nakamura, Y., Inoue, K., Hayano, T., Matsumura, H., 1974. Monoterpene glucosides and related natural products. XXIV. Trifloroside, a new secoiridoid glucoside from *Gentiana triflora*. Tetrahedron 30, 571–577.

Jensen, S.R., Schripsema, J., 2002. Chemotaxonomy and pharmacology of Gentianaceae. In: Struwe, L., Albert, V.A. (Eds.), Gentianaceae – Systematics and Natural History. Cambridge University Press, Cambridge, New York, pp. 573–631.

Kakuda, R., Iijima, T., Yaoita, Y., Machida, K., Kikuchi, M., 2001. Secoiridoid glycosides from *Gentiana scabra*. J. Nat. Prod. 64, 1574–1575.

Kawahara, N., Masuda, K., Sekita, S., Satake, M., 2001. A new secoiridoid glucoside, amaronitidin, from the Peruvian folk medicine "Hercampuri" (*Gentianella nitida*). Chem. Pharm. Bull. 49, 771–772.

Kikuzaki, H., Kawasaki, Y., Kitamura, S., Nakatani, N., 1996. Secoiridoid glucosides from *Swertia mileensis*. Planta Med. 62, 35–38.

Kojić-Prodić, B., Spek, A.L., Van der Sluis, W.G., Labadie, R.P., 1985. Structure of decentapicrin A: (−)-(4aS)-6-[3′-*O*-(3-hydroxybenzoyl)-β-D-glucopyranosyloxy]-5-vinyl-4,4aα,5β,6α-tetrahydro-1*H*,3*H*-pyrano[3,4-*c*]pyran-l-one, $C_{23}H_{26}O_{11}$. Acta Cryst. C41, 379–383.

Liu, J., Liu, Y., Klaassen, C.D., 1994. The effect of Chinese hepatoprotective medicines on experimental liver injury in mice. J. Ethnopharmacol. 42, 183–191.

Liu, Z.W., Chen, C.X., Jin, R.M., Shi, G.Q., Song, C.Q., Hu, Z.B., 2002. Studies on liver-protecting and bile secretion-promoting effects of gentiopicroside. Zhongcaoyao 33, 47–50.

Luo, Y.H., Nie, R.L., 1993. The secoiridoids isolated from *Swertia nervosa* (G. don) Wall. Ex C.B. Clarke. Acta Bot. Sin. 35, 307–310.

Ng, T.H.K., Chan, Y.W., Yu, Y.L., Chang, C.M., Ho, H.C., Leung, S.Y., But, P.P.H., 1991. Encephalopathy and neuropathy following ingestion of a Chinese herbal broth containing podophyllin. J. Neurol. Sci. 101, 107–113.

Rodriguez, S., Marston, A., Wolfender, J.L., Hostettmann, K., 1998. Iridoids and secoiridoids in the Gentianaceae. Curr. Org. Chem. 2, 627–648.

Rojas, A., Bah, M., Rojas, J.I., Gutierrez, D.M., 2000. Smooth muscle relaxing activity of gentiopicroside isolated from *Gentiana spathacea*. Planta Med. 66, 765–767.

Struwe, L., Kadereit, J.W., Klackenberg, J., Nilsson, S., Thiv, M., Von Hagen, K.B., Albert, V.A., 2002. Systematics, character evolution, and biogeography of Gentianaceae, including a new tribal and subtribal classification. In: Struwe, L., Albert, V.A. (Eds.), Gentianaceae – Systematics and Natural History. Cambridge University Press, Cambridge, New York, pp. 26–27.

Takeda, Y., Masuda, T., Honda, G., Takaishi, Y., Ito, M., Ashurmetov, O.A., Khodzhimatov, O.K., Otsuka, H., 1999. Secoiridoid glucosides from *Gentiana olivieri*. Chem. Pharm. Bull. 47, 1338–1340.

Tan, R.X., Kong, L.D., 1997. Secoiridoids from *Gentiana siphonantha*. Phytochemistry 46, 1035–1038.

Tan, R.X., Wolfender, J.L., Ma, W.G., Zhang, L.X., Hostettmann, K., 1996. Secoiridoids and antifungal aromatic acid from *Gentiana algida*. Phytochemistry 41, 111–116.

Tang, H.Q., Tan, R.X., 1997. Glycosides from *Gentiana scabra*. Planta Med. 63, 388.