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Short communication

Three guaianolides from *Saussurea involucrata* and their contents determination by HPLC

Yan Li, Chunlan Wang, Shunxing Guo *, Junshan Yang, Peigen Xiao

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, PR China

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Abstract

Saussurea involucrata (Kar. et Kir.) Sch. Bip. (family Asteraceae) is a rare traditional Chinese medicinal herb. A new guaianolide, 11β,13-dihydrodehydrocostuslactone-8-*O*-[6′-*O*-acetyl-β-D-glucoside] (**1**), together with two known guaianolides, namely 11β,13-dihydrodehydrocostuslactone-8-*O*-β-D-glucoside (**2**) and 3α-hydroxyl-11β,13-dihydrodehydrocostuslactone-8-*O*-β-D-glucoside (**3**), was isolated from the 95% ethanol extract of the whole plants. Their structures were elucidated on the basis of spectral analysis. Their contents in the *S. involucrata* were determined by HPLC for the first time. Chromatographic separation was achieved on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at 25 °C and the mobile phase was a mixture of acetonitrile and water in a mode of gradient elution detected at 209.8 nm at a flow rate of 1.0 ml/min. This method was validated in terms of selectivity, linearity, precision and accuracy. The linearity ranges were 0.066–0.44 μg for **1**, 0.23–2.30 μg for **2**, and 0.258–2.58 μg for **3**, respectively.

Keywords: Guaianolides; 11β,13-Dihydrodehydrocostuslactone-8-*O*-[6′-*O*-acetyl-β-D-glucoside]; Structural elucidation; High performance liquid chromatography; *Saussurea involucrata*

1. Introduction

Saussurea involucrata (Kar. et Kir.) Sch. Bip. (family Asteraceae) is a rare traditional Chinese medicinal herb on the verge of extinction. According to the theories of traditional Chinese medicine, it has the efficacy of warming kidney and activating 'yang', expelling wind and eliminating dampness, inducing menstruation and promoting blood circulation. Therefore, it has been used as a principal drug for the cure of rheumatic arthritis and gynecological disorders [1]. Modern pharmacological studies have demonstrated that it possesses activities such as free radical scavenging, anti-fatigue [2], anti-inflammation [3], anti-cancer [4], and immunomodulation [5].

It is well known that a change in the chemical constituents of a botanical drug may induce different levels of effectiveness. Therefore, it is necessary to evaluate the quality of the botanical drugs. For the purpose of quality control for *S. involucrata*, its chemical constituents rutin and chlorogenic acid are listed in the Chinese Pharmacopoeia [1]. However, previous chemical investigations of this herb have revealed that the flavonoids [6,7] and the guaianolides [8] are the main constituents in *S. involucrata*, and therefore would be superior indicators of quality assessment. While the flavonoids are ubiquitous in the plant kingdom, most of the guaianolides isolated from this herb are found exclusively in *S. involucrata* [8]. Thus, the content of guaianolides demonstrated to be an important index to evaluate the quality of this botanical drug. The purpose of this study is to improve the standard of quality control for the characteristic chemical constituents of *S. involucrata*. Furthermore, a new guaianolide named 11β,13-dihydrodehydrocostuslactone-8-*O*-[6′-*O*-acetyl-β-D-glucoside] (**1**), along with two other known guaianolides, was isolated from the 95% ethanol extract of the whole herbs. The new guaianolide's structure was elucidated by the spectral analysis.

Currently, there are several reports comprised of analytical methods available for the determination of sesquiterpene lactones by HPLC [9–18]. In these reports, several combined techniques with HPLC, such as HPLC-NMR [14,15] and HPLC-MS [16–18] are adopted in the qualitative and quantitative analyses of sesquiterpene lactones. A review of the literature

* Corresponding author. Tel.: +86 10 62829619; fax: +86 10 62895120.
E-mail address: sxguo2006@yahoo.com.cn (S. Guo).

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revealed all of the methods utilized within the last two decades for sesquiterpenes including their lactones [19]. However, there have not been any reports on the determination of the levels of sesquiterpene lactones in the *S. involucrata*, until now. Therefore, the aim of this study is to develop a HPLC method which is able to quantify the three markers simultaneously in *S. involucrata*. Consequently, a simple, accurate and reliable analytical method for the simultaneous determination of three guaianolides contained in *S. involucrata* was developed using HPLC coupled with DAD-UV detector.

2. Experimental

2.1. Materials and reagents

S. involucrata was collected from Xinjiang province and identified by Prof. Shunxing Guo, Institute of Medicinal Plant Development, Peking Union Medical College and Chinese Academy of Medical Sciences. Compounds **1**, **2** and **3** were isolated and their structures were identified in our laboratories. All of their purities confirmed by HPLC were over 99%. HPLC grade acetonitrile was purchased from Honeywell Burdick & Jackson Company. Other reagents were all of analytical grade and the water used was double distilled.

Column chromatography was performed on glass column using silica gel (300–400 mesh; Qingdao Marine Chemical Factory, China) as adsorbent and a Sephadex LH-20 column (35 cm × 3.5 cm i.d., Amersham Pharmacia Biotech, Sweden).

2.2. Apparatus and chromatographic conditions

Melting points were measured on an X-4 micromelting point apparatus (uncorrected). The UV spectra were recorded on a Shimadzu UV-2100s spectrophotometer. Optical rotations were taken on a Perkin-Elmer 341 digital Polarimeter. The IR (KBr) spectra were obtained on a Perkin-Elmer 983G infrared spectrophotometer. The MS spectra were recorded on an Autospec-Ultima ETOF instrument (FAB+; BpM:415; BpI:12070; TIC:24526; Flags: NORM). The ^{1}H, ^{13}C and 2D NMR spectra were recorded on Bruker AM-500 instrument (in DMSO-d_6 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane as the internal standard.

All analyses were performed on a Waters HPLC system, equipped with a Waters^{TM} 600 pump, a Waters^{TM} 600 system controller and a 2996 DAD-UV detector. All separations were carried out on a column Agilent Zorbax SB-C_{18} (250 mm × 4.6 mm i.d., 5 μm). The chromatographic data were recorded and processed with a Waters empower workstation. The elution program was well optimized as follows, the elution composition of mobile phase was 10:90 (acetonitrile:water, v/v) during the first 20 min; subsequently, the composition of the mobile phase was changed from 10:90 to 57:43 in the next 15 min, and then the mobile phase composition was kept at the 57:43 in the following 10 min; finally, the column was eluted with acetonitrile for another 15 min. The detected wavelength was 209.8 nm. The loop volume was 20 μl and the column temperature was maintained at 25 °C.

2.3. Extraction and isolation

The air-dried whole plants (5.0 kg) of *S. involucrata* were chipped and extracted with 95% ethanol for three times (75 l per time), for 2 h each time at 78 °C. The resulting extract was concentrated to give a gummy mass (950 g). The gum was suspended in water and partitioned successively with petroleum ether, $CHCl_3$, EtOAc and *n*-BuOH. The $CHCl_3$ extract (10 g) was fractioned in a glass column using silica gel (40 cm × 3.5 cm i.d.) as adsorbent and eluted with gradients $CHCl_3$–MeOH (10:0 to 8:2, v/v) to afford four fractions. Fraction 4 was purified repeatedly over a Sephadex LH-20 column with a mixture of $CHCl_3$–MeOH (1:1, v/v) to give compound **1** (50 mg). The EtOAc extract (55 g) was chromatographed on a glass column using silica gel (70 cm × 8.0 cm i.d.) as adsorbent and eluted successively with gradients $CHCl_3$–MeOH (9:1 to 1:1, v/v) to afford seven fractions. Fraction 5 was separated by a glass column using silica gel (40 cm × 3.5 cm i.d.) as adsorbent to yield compound **2** (200 mg). The *n*-BuOH extract (145 g) was fractioned in a glass column using macroporous resin (Tianjin Nankai University Chemical Factory, China; 80 cm × 10 cm i.d.) as adsorbent and eluted with water, 50% ethanol and 95% ethanol to afford a 50% ethanol fraction (70 g), which was chromatographed over a glass column using silica gel (70 cm × 10 cm i.d.) as adsorbent and eluted with a gradient solvent of $CHCl_3$–MeOH–H_2O (8:2:0.2 to 5:5:0.5, v/v/v) to give five fractions. Fraction 3 was purified on glass column using silica gel (40 cm × 3.5 cm i.d.) as adsorbent to afford compound **3** (200 mg).

2.4. Preparation of solutions

2.4.1. Preparation of standard solutions

The standard stock solutions of compounds **1** (550 μg/ml), **2** (575 μg/ml) and **3** (515 μg/ml) were prepared in MeOH and stored in the refrigerator. The working solutions were prepared by appropriate dilution of the stock solutions with MeOH, and the resulting concentrations of compounds **1**, **2** and **3** were 22, 115 and 128.8 μg/ml, respectively.

2.4.2. Preparation of sample solutions

The powder of *S. involucrata* (0.5 g) was refluxed with MeOH (70 ml) for 4 h. The extracted solutions were prepared by the method of weight relief, by which the weight lost in the extraction procedure was compensated. Collected 50 ml filtrate and evaporated it to dryness, and the residue was dissolved in MeOH and transferred to 5 ml volumetric flask.

All solutions were filtered through a 0.45 μm nylon filters (Whatman, UK) before inject into the valve of HPLC. The injection volume was 20 μl.

3. Results and discussion

3.1. Structural elucidation

11β,13-Dihydrodehydrocostuslactone-8-*O*-[6′-*O*-acetyl-β-D-glucoside] (**1**): $C_{23}H_{32}O_9$; colourless needles; mp 169–171 °C; hydroxamic acid test and Molish reaction

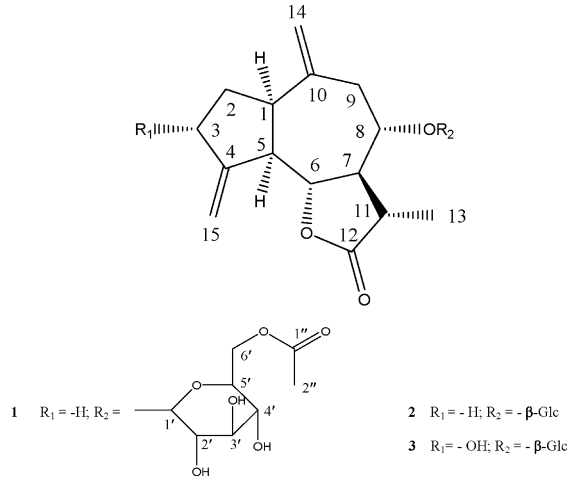

Fig. 1. Structures of compounds **1**, **2** and **3**.

positive; $[\alpha]_D^{20}$ −15.3° (c 0.058; MeOH); UV λ_{max}^{MeOH}: 203.8 nm; IR ν_{max}^{KBr}: 3434, 3010, 2800, 1750, 1660, 1460, 1373, 1080, 1037 cm^{-1}; HR-FABMS, *m/z*: 453.2109$[M+H]^+$ (calculated for $C_{23}H_{33}O_9$ 453.2124); ^{1}H NMR (500 MHz, DMSO-d_6): 5.03 (s, 1H, 15-H_a), 4.96 (s, 1H, 15-H_b), 4.90 (s, 1H, 14-H_a), 4.81 (s, 1H, 14-H_b), 4.36 (d, *J* = 7.5 Hz, 1H, 1′-H), 4.31 (dd, *J* = 12.0, 2.0 Hz, 1H, 6′-H_a), 4.04 (dd, J = 12.0, 7.0 Hz, 1H, 6′-H_b), 3.90 (t, *J* = 10.0 Hz, 1H, 6-H), 3.64 (ddd, *J* = 10.0, 8.0, 5.0 Hz, 1H, 8-H), 3.38 (m, 1H, 5′-H), 3.16 (t, *J* = 7.0 Hz, 1H, 3′-H), 3.06 (m, 1H, 4′-H), 2.99 (m, 1H, 2′-H), 2.89 (m, 1H, 1-H), 2.76 (br. t, *J* = 9.5 Hz, 1H, 5-H), 2.75 (dd, *J* = 13.0, 5.0 Hz, 1H, 9-H_a), 2.67 (dd, *J* = 10.5, 7.5 Hz, 1H, 11-H), 2.44 (m, 1H, 3-H_a), 2.39 (m, 1H, 3-H_b), 2.21 (dd, *J* = 13.0, 8.0 Hz, 1H, 9-H_b), 2.18 (q, *J* = 10.0 Hz, 1H, 7-H), 1.99 (s, 3H, 2″-H), 1.82 (m, 1H, 2-H_a), 1.74 (m, 1H, 2-H_b), 1.29 (d, *J* = 7.0 Hz, 3H, 13-H); ^{13}C NMR (125 MHz, DMSO-d_6): 46.5 (C-1), 29.3 (C-2), 31.8 (C-3), 152.0 (C-4), 52.1 (C-5), 79.2 (C-6), 53.1 (C-7), 83.0 (C-8), 43.9 (C-9), 145.4 (C-10), 40.1 (C-11), 178.7 (C-12), 15.9 (C-13), 113.6 (C-14), 108.6 (C-15), 103.6 (C-1′), 73.6 (C-2′), 76.8 (C-3′), 70.1 (C-4′), 73.5 (C-5′), 63.6 (C-6′), 170.1 (C-1″), 20.6 (C-2″); the structure of **1** was shown in Fig. 1; key HMBC correlations were shown in Fig. 2.

The ^{1}H and ^{13}C NMR spectra of compound **1** were similar to those of **2**. By comparing the spectra of compound **1** with

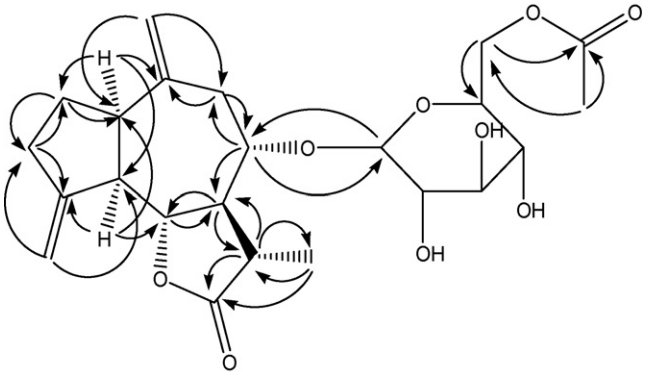

Fig. 2. Key HMBC correlations of compound **1** (H to C).

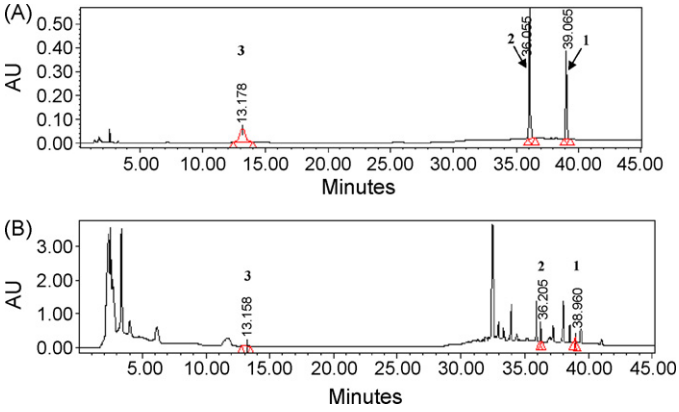

Fig. 3. Chromatograms for analysis of compounds **1**, **2** and **3**. (A) Standard solution and (B) sample solution of *S. involucrata*. HPLC conditions: Agilent Zorbax SB-C_{18} (250 mm × 4.6 mm i.d., 5 μm) column controlled at 25 °C. The mobile phase was a mixture of acetonitrile and water in the mode of gradient elution and the detected wavelength was 209.8 nm. (**1**) Compound **1**, (**2**) compound **2** and (**3**) compound **3**.

that of **2**, the ^{1}H NMR spectrum of **1** exhibited a singlet methyl signal at *δ* 1.99 which did not exist in that of **2**, and lacked an H signal belonging to hydroxyl; the ^{13}C NMR spectrum of **1** exhibited two C signals (*δ* 170.1, 20.6) which did not exist in that of **2**, and the C-6′ of **1** shifted downfield from *δ* 61.2 to *δ* 63.6, meanwhile, the C-5′ of **1** shifted upfield from *δ* 77.1 to *δ* 73.5. Combined with the MS spectrum, the formula of **2** ($C_{21}H_{30}O_8$) was subtracted from that of **1** ($C_{23}H_{32}O_9$), then the residue of formula was C_2H_2O. So the two carbon signals (*δ* 170.1, 20.6) of **1** belonged to acetyl. Therefore, the structure of **1** was deduced as Fig. 1. And it was verified by HMBC correlations (see Fig. 2).

11β,13-Dihydrodehydrocostuslactone-8-*O*-β-D-glucoside (**2**, $C_{21}H_{30}O_8$) and 3α-hydroxyl-11β,13-dihydrodehydrocostuslactone-8-*O*-β-D-glucoside (**3**, $C_{21}H_{30}O_9$) were identified by direct comparison the spectrum data with the literature [8].

3.2. Optimal chromatographic conditions

The acetonitrile–water system was selected as mobile phase after several trials with different mobile phases including acetonitrile–water, acetonitrile–water–acetic acid, methanol–water and methanol–water–acetic acid systems. The gradient elution mode was adopted because of their different lipophilic characteristics. Compared with the results of the ELSD, the DAD-UV detector was found more suitable because the sensitivity of ELSD was too low to detect the compound **1** in sample solutions. So the DAD-UV detector was selected and the detection wavelength was adjusted to 209.8 nm because of the UV characteristics of the investigated compounds. In order to identify the analytes in the samples, the retention time and UV spectra of the sample peaks were compared with those of the reference standards. The results showed excellent agreements between the standards and analytes.

Fig. 3 displayed the chromatograms of standard mixture solution and sample solution of *S. involucrata*, respectively. These three compounds were found to be well resolved for

quantification. The resolution was calculated as $Rs = 1.18 (t_2 - t_1)/(W_{0.5,1} + W_{0.5,2})$, where t_1 and t_2 were the retention times for two peaks, respectively. $W_{0.5,1}$ and $W_{0.5,2}$ were their half bandwidths of peaks. All of the values of Rs were over 2.5.

3.3. Optimal extraction time

According to the above sample preparation procedures, two repetitive samples from the same batch of the *S. involucrata* were extracted with MeOH for 2, 4 and 6 h, respectively, and analyzed with the established HPLC method. The contents of the investigated compounds were satisfactory when the samples were extracted for 4 h. Therefore, the optimal extraction time was selected to be 4 h.

3.4. Method validation

3.4.1. Linearity

Linearity of the assay for standards was determined with five data points over the range of compound **1** 0.066–0.44 μg (3, 5, 10, 15, 20 μl), compound **2** 0.23–2.30 μg (2, 6, 10, 15, 20 μl), and compound **3** 0.258–2.58 μg (2, 4, 8, 12, 20 μl). The calibrated curve was established by plotting the peak area against the injected quantity of the standards with linear regression analysis. Calibration curves showed that there was a linear correlation between peak areas (Y) and the quantity of the standards (X, μg). The linear calibration equations were $Y = 313{,}038X + 13{,}154$ ($r = 0.9999$) for **1**, $Y = 769{,}697X - 13{,}090$ ($r = 0.9999$) for **2** and $Y = 372{,}818X + 48{,}226$ ($r = 0.9998$) for **3**, respectively.

3.4.2. Precision

The system precision was determined by performing six times of replicated analysis of the same standard solution and evaluated by the R.S.D. (%) values of the peak area of the analytes. The R.S.D. (%) values were shown as follows: 0.74% (**1**), 0.57% (**2**) and 0.63% (**3**). Therefore, the system precision was considered to be satisfactory.

The method precision was determined by performing three concentration levels of marker compounds, and each concentration was analyzed in triplicates. The intra-day and inter-day precisions were determined by analyzing the samples within 1 day and 3 separate days, respectively. Table 1 showed the results of this investigation. The R.S.D. (%) values were all less than 1.5%, so the method precision was considered to be satisfactory.

3.4.3. Accuracy

The accuracy of the method was confirmed by the determination of recovery. Six repetitive samples from the same batch of the *S. involucrata* were spiked with the known amount of standards before extraction. The mixtures were extracted and analyzed under the above-mentioned conditions. The content of each guaianolide was determined by the corresponding calibration curve, and the content of each spiked standard was calculated by subtracting the detected amount of the corresponding guaianolide in the control from the total content. Recoveries were calculated by using the ratio of detected amount to that of marker added. The values of average recovery and R.S.D. (%) were listed in Table 2.

Table 1
Intra-day ($n = 3$) and inter-day ($n = 3$) precision of HPLC analysis of compounds **1**, **2** and **3**

Compounds	Concentration (μg/ml)	R.S.D (%)	
		Intra-day	Inter-day
1	22	0.65	1.14
	11	0.72	0.69
	5.5	0.79	1.42
2	115	0.93	0.94
	57.5	0.68	0.82
	28.8	0.61	0.96
3	128.8	0.78	1.01
	64.4	0.78	1.07
	32.2	1.14	1.29

Table 2
Values of average recovery and R.S.D. (%) of compounds **1**, **2** and **3** ($n = 6$)

Compounds	Recovery (%)	R.S.D. (%)
1	100.4	2.85
2	98.3	2.42
3	99.1	1.75

Table 3
Contents and R.S.D. (%) values of compounds **1**, **2** and **3** ($n = 6$)

Compounds	Content (mg/g)	R.S.D. (%)
1	0.08	0.92
2	0.79	0.65
3	0.49	1.08

3.5. Sample analysis

The newly established method was applied to determine the contents of the investigated compounds in *S. involucrata*. The results of chromatograms for content determination were shown in Fig. 3. The contents and R.S.D. (%) values were shown in Table 3.

4. Conclusion

This is the first report on the structural elucidation of a new guaianolide and the simultaneous determination of three guaianolides contained in *S. involucrata*. The established HPLC method was proved to be simple, precise and accurate. This proposed method is promising to improve the quality control of the related botanical drugs of *S. involucrata*.

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