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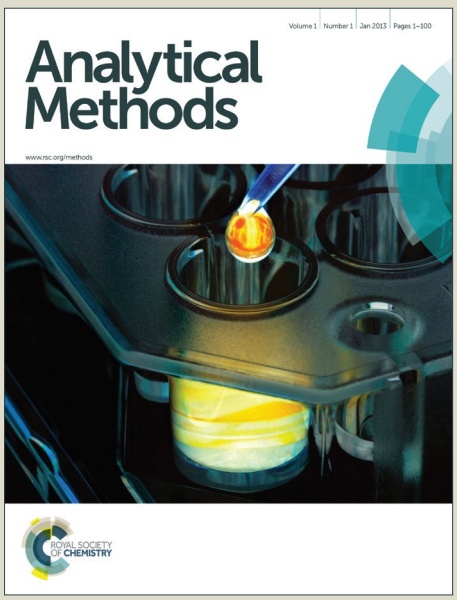

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ARTICLE

Determination of Phenanthrenes and Stilbenoid in the Ethyl Acetate Extract of *Thunia alba* (Lindl) by HPLC-DAD

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The target of this work was to determine the amount phenenthrenes and stilbenoid in the ethyl acetate extract of *Thunia alba* (Lindl). Compounds lusianthridin (**1**), coelonin (**2**) (two phenenthrenes) and thunalbene (**3**) (a stilbenoid) were isolated from *Thunia alba* (Lindl) by column chromatography and SephadexLH-20. Their structure were elucidated on the basis of spectral data (UV, MS, ^{1}HNMR, ^{13}C NMR) with refs. Then, three compounds were used as standards for HPLC determination. Method validation was performed with linearity, limit of detection (LOD), limit of quantification (LOQ), precision, stability, repeatability, recovery, selectivity and robustness. The experimental results showed the linearity of the method was in the range from 0.06 to 0.80 μg/ml with regression coefficients ranging from 0.9987 to 0.9996. The limits of detection were 0.007, 0.009 and 0.013 μg/ml and the limits of quantification were 0.027, 0.022 and 0.033μg/ml for compounds **1**, **2** and **3**, respectively. The relative standard deviation(RSD) of precision, stability and repeatability were in the range from 0.67% to 2.82%. The average recoveries (n=6) ranged from 96.81% to 102.69% and the RSD values varied from 1.59% to 1.81%. The method validation showed that the established method is accurate, simple and repeatable and can be used to provide a reference for the quality control of *Thunia alba* (Lindl).

1. Introduction

Chinese herbal medicine has played an indispensable role in preventing and treating human diseases for a long time, and thus has already attracted global attention[1-2]. The Orchidaceae with over 700 genera and 25, 000 species is one of the largest plant families on earth, which has important ecological, economic, cultural and medicinal values. The genus *Thunia* comprises of approximately 7 species, all of which are distributed in Sichuan, Xizang, Yunnan, Bhutan, India, Sikkim, Indonesia, Malaysia, Myanmar, Nepal, Thailand and Vietnam[3]. *T. alba*, *T. bensoniae* and *T. marshalliana* are the representative species of the genus *Thunia*. All of these species, only *Thunia alba* (Lindl) distributed in China and was used as medical plant. *Thunia alba* (Lindl) was locally known as the "Rock Angle" or "Jie-gu-dan" in Dali of Yunnan Province and was used for the treatment of cough, pneumonia, bronchitis and duodenal ulcer[4- 5]. The isolation of compounds of diverse structural types from *Thunia alba*(Lindl) has been reported , including stilbenoids, bibenzyls, phenanthrenes, 9, 10-dihydrophenanthrenes, phenanthropyrans and simple aromatic compounds[6-12]. Our chemical research on *Thunia alba* (Lindl) has resulted in the isolation of three known compounds (two phenanthrenes and a stilbenoid), lusianthridin (4, 7-dihydrox-2-methoxy-9, 10-dihydrophenanthrene)[13, 14](**1**), coelonin (2, 7-dihydrox-4-methoxy-9, 10-dihydrophenanthrene)[13, 15](**2**), and thunalbene (3, 3' dihydrox-5-methoxy-stilbene)[16](**3**). Their structures are presented in **Figure 1**.

Phenanthrenes and bibenzyls are most characteristic as chemical marker for *Thunia alba* (Lindl), which have been shown to have various biological activities such as anti-inflammatory[17], anti-oxidation[18], anti-bacterial agent[19], anti-allergy[20], antialgal and antitrypanosomal activities[21-22], vasorelaxant effects[23] and anticancer[24-26]. Phenanthrenes, monbarbatain A, B, C and D had exhibited free radical scavengining of the 2,2'-diphenylpicrylhydrazyl (DPPH) by Ming-hui Yang reported[13]. Some refs indicated that the compounds moscatilin, loddigesiinol D and pholidotol A, B can intensively inhibit NO activity[27]. Lusianthridin was found to exert cytotoxic effects both in vitro and in vivo. The significant activities on A549 human lung carcinoma (ED_{50}: 7.7μg/ml); SK-OV-3 human ovary adenocarcinoma (ED_{50}: 9.4μg/ml) and HL-60 human promyelocytic leukaemia (ED_{50}: 9.8μ/ml) cell lines were demonstrated[28].

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High-performance liquid chromatographic (HPLC) was widely used for the analysis of chemical constitutes. HPLC-DAD chromatographic method is a suitable tool for the separation and quantification of phenolic compounds in plant extracts because of its versatility, precision and relatively low cost. For medicinal plant analysis, using HPLC-DAD method, the identification and quantification of bioactive and marker compounds in complex matrices can be realized even structurally similar natural products based on their chromatographic retention data, spectral characteristics and quantitative information[29-31]. In 2006, Li Yang reported the method on simultaneous determination of phenols (bibenzyl, phenanthrene, and fluorenone) in Dendrobium species by high-performance liquid chromatography[32]. Subsequently, analytical methods of 9, 10-dihydrophenanthrenes in *Bletilla striata (Thunb.) Reichb.f.* and *Pholidota chinensis* were reported[33-34]. However, it was rarely reported the determination of phenanthrenes in *Thunia alba*(Lindl). Especially, the less work on analysis of compound **3** by HPLC has been previously reported. In addition, the aim of our study was to develop a time-saving and simple HPLC method with a DAD detector for the determination of the three compounds, which was used for analysis of different genus *Thunia* plant and different substitutes or false to ensure the quality and the clinical efficacy.

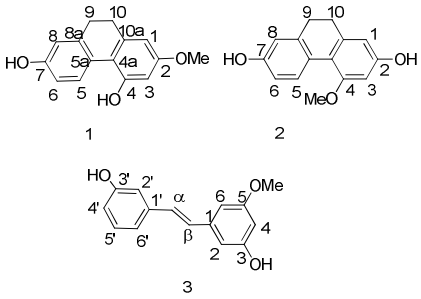

Figure 1. Structures of compounds **1**, **2** and **3**

2. Experimental

2.1 Reagents and materials

Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Acetonitrile was purchased from Tedia (Ohio, USA). Phosphoric acid was purchased from Nanjing Chemical Factory (Nanjing, China). Sephadex LH-20 (GE Healthcare, Sweden), silica gel (200-300 mesh) and silica gel GF_{254} sheets (020-0.25 mm) (from Qingdao Marine Chemical Factory) were used for column chromatography and TLC, respectively. Deionized water was prepared by passing distilled water through a Mili-Q system (Milipore, Milford, MA, USA). All other reagents were of analytical grade and were bought from commercial sources. The rhizomes of *Thunia alba* (Lindl) were collected in Wenshan and Lincang of Yunnan Province and were deposited in School of Pharmaceutical Sciences and Chemistry of Dali University. The identification was performed by Prof. Jiang-Miao Hu, Kunming Institute of Botany, Chinese Academy of Sciences.

2.2 Extraction and isolation

The crushed rhizomes of *Thunia alba* (Lindl)(10.0 Kg) were extracted in the cold-soaked way with 90% ethanol for 48 h each time, and it was repeated for 5 times. Then the combined ethanol extract was concentrated under reduced pressure at 45 ℃ to afford a brown residue (252 g). The residue was diluted with H_2O and partitioned with petroleum ether, EtOAc and *n*-BuOH, successively. The EtOAc fraction (60.8 g) was subjected to silica gel column chromatography and eluted with a $CHCl_3$-CH_3OH (10:0→0:10, v/v) gradient to afford 8 fractions (A-H). Fractions B and C were futher purified by column chromatography, eluted with chloroform-acetone (60:1→0:1) and were subsequently subjected to Sephadex LH-20 to give compounds **1** (10.7 mg), **2** (9.6 mg) and **3** (9.5 mg). The NMR data of compounds **1**, **2** and **3** were given in **Table 1**. Three compounds were purified by semi-preparative HPLC and the purity was determined to be more than 98% by normalization of the peak areas detected by HPLC analysis.

2.3 Wavelength selection

Compounds **1**, **2** and **3** were detected by a spectral scan between 200 and 400nm to obtain the optimal absorption wavelength. The results of the UV spetra indicated that the maximum absorbance peaks of the compounds **1**, **2** and **3** were 212 and 279nm, 208 and 279nm, and 230 and 299nm, respectively. Thus, the 279nm peak was selected as the wavelength for compounds **1** and **2,** and 299nm was used as the wavelength for compound **3,** because its absorption was much greater at higher wavelength.

2.4 Chromatographic conditions

The HPLC analysis of the three compounds was conducted on an Agilent LC-1200 apparatus (Palo Alto, USA) equipped with a quaternary pump, a vacuum degasser, an autosampler, a DAD detector and chromatographic data were processed by Agilent Chem Station Software. The column used for the analysis was a ZORBAX SB-Aq, with a 4.6 mm internal diameter, 250 mm length and 5 μm particle size. The mobile phase consisted of acetonitrile (A) and 0.05% phosphoric acid aqueous solution (B) was carried with a linear elution gradient as follows: 0 min, 20%A; 5 min, 20%A; 30 min, 28%A; 30-40 min, 28%A; 50 min, 30%A; 50-65 min, 30%A; 75 min, 20%A. The flow rate was 1 ml/min. The column temperature was 25 ℃. An injection volume of 15ul was used for all standards and samples.

2.5 Sample preparation

The dried rhizomes of *Thunia alba* (Lindl) were powdered to a homogeneous size and passed through a no. 60 mesh size. A 100.0g sample of powder was added to a 3000 ml round-bottom flask and 2000 ml 95% ethanol (1/20, v/v) was added. The mixture was refluxed for 3.0 h, 2.0 h and 1.0 h at 60 ℃. The extract solution was

combined and filtered by a Buchner funnel, followed by concentrating at 45 °C by a rotary evaporator to afford a residue. The residue was subsequently extracted with petroleum ether, EtOAc and *n*-BuOH. The EtOAc extract was evaporated by a vacuum freeze drier to obtain the powder. The sample solution was prepared by dissolving the EtOAc extract in methanol.

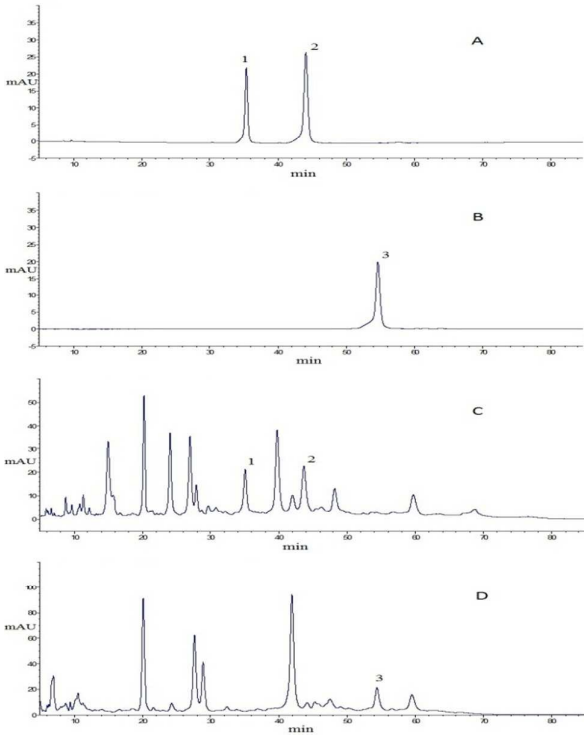

Figure 2 HPLC chromatograms at 279 nm and 299 nm for the standard solutions (A, B) and sample solutions (C, D)

The methanol standard solution containing compounds **1**, **2** and **3** was prepared by dissolving in methanol to final concentrations of 40 μg.ml^{-1}, 30 μg.ml^{-1}, and 30 μg.ml^{-1} for compounds **1**, **2** and **3**, successively. All solutions were filtered through a 0.22 μm membrane filters and stored at 4 °C before the HPLC analysis. The HPLC chromatograms are shown in **Figure 2**.

2.6 Method validation

2.6.1 Calibration curve, limits of detection and limits of quantification

Different injection volumes (2 μl, 4 μl, 8 μl, 10 μl, 15 μl, and 20 μl) of the standards were loaded onto the HPLC instrument. Each calibration curve injection volume was performed in triplicate. The calibration curves of compounds **1**, **2** and **3** were constructed using the observed peak areas versus nominal concentrations of the analytes. The standard solution with the lowest concentration was further diluted to evaluate the LODs and LOQs. The LODs and LOQs were determined as signal to noise (S/N) ratios of 3 and 10, respectively. The results are shown in **Table 2**.

2.6.2 Precision, stability and repeatability

In order to ensure the precision of instrument, 15 ul of the sample solution was injected into the HPLC instrument. The relative standard deviation (RSD) of the peak areas for each compound was calculated. The RSD values were less than 1.92%. The intra-day and inter-day precision were obtained by analyzing the standards at three different concentrations of the compounds **1**, **2**, and **3** in six replicates in one day and on six consecutive days. The RSD values were less than 2.82%. The results are given in **Table 3**. The stability of the sample solutions were analyzed at time intervals of 0, 2, 4, 8, 12, 16, 20 and 24 h at room temperature. The RSD values were less than 2.26%. To test the repeatability of the assay, six independently prepared EtOAc extracts were analysed. The RSD values were less than 2.12%. The results are shown in **Table 4**.

2.6.3 Selectivity and robustness

The selectivity was determined by observing interferences from the sample. The chromatograms of the sample solutions indicated that the separation of three compounds was successfully achieved with good resolution and symmetric peak. Resolution of compounds **1**, **2** and **3** were 4.34, 1.51 and 2.71 by comparing the adjacent peaks in **Figure 2**. Symmetry factors of three compounds were 1.16, 0.95 and 1.22, respectively. The results indicated a good selectivity to determine of three compounds in EtOAc extract. To evaluate the robustness of the method, deliberate changes in chromatographic

Table 1 The NMR Data of compounds 1, 2 and 3 (in MeOD)

1			2			3		
Position	δ_H (ppm)	δ_C (ppm)	Position	δ_H (ppm)	δ_C (ppm)	Position	δ_H (ppm)	δ_C (ppm)
1	6.36(1H,d,*J*=2.6Hz)	105.2	1	6.31(1H,d,*J*=2.2Hz)	107.0	1		138.7
2		158.3	2		156.9	2	6.39(1H,s)	105.6
3	6.41(1H,d,*J*=2.4Hz)	100.5	3	6.36(1H,d,*J*=2.6Hz)	97.9	3		158.4
4		154.6	4		157.9	4	6.18(1H,s)	100.5
4a		114.9	4a		114.9	5		161.2
5		129.3	5	8.02(1H,d,*J*=9.3Hz)	128.5	6	6.48(1H,s)	103.3
5a		125.1	5a		125.1	1′		139.4
6	6.70(1H,dd,*J*=2.4,0.9 Hz)	112.5	6	6.56(1H,m)	112.2	2′	6.85(1H,s)	112.5
7		154.9	7		154.6	3′		157.3
8	6.72(1H,d,*J*=1.6Hz)	114.4	8	6.69(1H,m)	113.7	4′	6.58(1H,dd,*J*=8.2,1.8Hz)	114.4
8a		139.0	8a		139.4	5′	7.05(1H,t,*J*=7.8Hz)	129.2
9	2.73(2H,m)	29.8	9	2.71(2H,m)	29.8	6′	6.89(2H,d,*J*=3.7Hz)	117.9
10	2.93(2H,m)	30.5	10	2.84(2H,m)	30.5	OMe	4.75(3H,s)	54.3
10a		141.0	10a		140.4	OH-3,3′	3.68(2H,s)	
OMe	3.55(3H,s)	54.3	OMe	3.82(3H,s)	54.5	α	6.92(1H,m)	129.3
OH-4,7	4.85(2H,s)					β	6.90(1H,m)	129.4

[a]Overlapped signals

Table 2 Calibration equations, LOD (μg/ml) and LOQ (μg/ml) of the three compounds

Compounds	Calibration Equation y=ax+b	r^2	Linear range(μg/ml)	LODs	LOQs
1	y=2967.9x-6.7057	0.9995	0.08-0.80	0.007	0.027
2	y=5187.5x-74.287	0.9990	0.06-0.60	0.009	0.022
3	y=863.27x-38.409	0.9993	0.06-0.60	0.013	0.033

r^2 (correlation coefficient)

Table 3 Intra- and Inter-day precision for the assay of the three compounds

Compounds	Concentration (μg ml^{-1})	Intra-day RSD (n=6)%	Inter-day RSD (n=6)%
1	0.08	1.21	1.98
	0.40	1.50	1.72
	0.80	1.09	1.19
2	0.06	1.86	2.23
	0.30	2.00	2.82
	0.60	1.27	1.79
3	0.06	1.08	1.32
	0.30	0.94	1.14
	0.60	0.67	1.00

Table 4 Precision, stability and repeatability of the three compounds

Compounds	Precision (n=6) (RSD)(%)	Stability (n=6) (RSD)(%)	Repeatability (n=6) (RSD)(%)
1	1.78	1.25	1.25
2	1.33	1.93	2.12
3	1.92	2.26	1.62

Table 5 Recoveries of the 3 compounds (n=6)

Compounds	Original (μg)	Found [a] (μg)	Spiked (μg)	Recovery (%)	Mean (%)	RSD (%)
1	17.0200	137.3442	120	101.90	100.03	1.81
	17.3900	137.4885		100.57		
	17.3900	137.1451		98.59		
	17.4825	137.8767		102.25		
	17.2975	136.9378		97.92		
	16.8350	136.6532		98.92		
2	25.4288	114.9420	90	98.09	99.95	1.78
	25.9816	116.6806		102.69		
	25.9816	115.4427		97.93		
	26.1198	116.2645		100.55		
	25.8434	115.8038		99.85		
	25.1524	115.3033		100.60		
3	7.3784	97.1430	90	96.81	99.51	1.59
	7.5388	97.4380		98.66		
	7.5388	97.6085		100.92		
	7.5789	97.5792		100.00		
	7.4987	97.4742		99.67		
	7.2982	97.3730		101.02		

[a] Found is the sum of the original and spiked quantities

condition were carried out. The experimental results were found that the changes in flow rate (±4%), column temperature (±2°C), and mobile-phase composition (±2%) did not compromise the determination of the levels of three compounds.

2.6.4 Recovery

Recoveries were tested to investigate the accuracy of the method. Six independent EtOAc extract of 0.018 g were weighed accurately. Then, 3 ml of the mixed standard solutions was added, and the resultant samples were dissolved in ultrasonic water and diluted to 6 ml with the methanol. A volume of 15 μl was injected for analysis. The ratios of the determined and added amounts were used to calculate the recovery. The recovery rates were in the range from 96.81% to 102.69%, with RSD values less than 2.0%. The results are shown in **Table 5**.

2.7 Determination of three compounds in *Thunia alba* (Lindl)

Three compounds were determined to evaluate their contents in *Thunia alba* (Lindl) and were calculated using an external reference method according to the peak areas. The *Thunia alba* (Lindl) is mainly distributed in Wenshan and Lincang of Yunnan Province. In addition, the samples from both places were analysed and each sample was tested two times. The data are shown in **Table 6**.

Table 6 Contents of 3 compounds in *Thunia alba* (Lindl) from two locations(n=2)

Compounds	Contents in different locations(%)	
	Wenshan	Lincang
1	0.0925	0.0949
2	0.1382	0.1316
3	0.0401	0.0462

3. Conclusion

The phenanthrenes are a promising and expanding group of biologically active natural compounds. A fairly large number of phenanthrenes have been mainly reported in the Orchidaceae family. To our knowledge, it is the first report on determination of three compounds and this is the first report on validation of a method of analysis of thunalbene using HPLC-DAD. Our research found that the three compounds mainly exist in the EtOAc extract of *Thunia alba* (Lindl). Therefore, we chose the ethyl acetate extract of *Thunia alba* (Lindl) to determine the contents of the three compounds. This study has provided an efficient and accurate HPLC analytical method and also could be used for the fast determination of the phenanthrenes and stilbenoid in pharmaceutical products.

Acknowledgements

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