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High-speed countercurrent chromatography (HSCCC) and silica gel column chromatography were used to separate and purify alkaloids from Chinese herbal medicine *Euchresta tubulosa* Dunn. The purpose of this study is to provide a system mode for rapid separation of alkaloids from natural products. In the experiment, the eluent of silica gel column chromatography was screened by thin layer chromatography (TLC) to obtain four components with different polarity. Then, the two-phase solvent systems of different components were selected and purified by HSCCC. Four alkaloids with relatively high content were obtained by this mode successfully, including matrine (28 mg), oxymatrine (32 mg), N-formyl cytosine (24 mg), and cytosine (58 mg). The purity was higher than 91% by high performance liquid chromatography-ultraviolet (HPLC-UV) and their chemical structures were identified by nuclear magnetic resonance (NMR) and electron ionization mass spectrometry (EI-MS). The results showed that the combination of HSCCC and silica gel column chromatography could make alkaloids from natural products separate systematically.

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Article

Systematic Separation and Purification of Alkaloids from *Euchresta tubulosa* Dunn. by Various Chromatographic Methods

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Abstract: High-speed countercurrent chromatography (HSCCC) and silica gel column chromatography were used to separate and purify alkaloids from Chinese herbal medicine *Euchresta tubulosa* Dunn. The purpose of this study is to provide a system mode for rapid separation of alkaloids from natural products. In the experiment, the eluent of silica gel column chromatography was screened by thin layer chromatography (TLC) to obtain four components with different polarity. Then, the two-phase solvent systems of different components were selected and purified by HSCCC. Four alkaloids with relatively high content were obtained by this mode successfully, including matrine (28 mg), oxymatrine (32 mg), *N*-formyl cytisine (24 mg), and cytisine (58 mg). The purity was higher than 91% by high performance liquid chromatography–ultraviolet (HPLC-UV) and their chemical structures were identified by nuclear magnetic resonance (NMR) and electron ionization mass spectrometry (EI-MS). The results showed that the combination of HSCCC and silica gel column chromatography could make alkaloids from natural products separate systematically.

Keywords: chromatographic methods; *Euchresta tubulosa* Dunn.; alkaloids; systematic separation; chemical structures

1. Introduction

Euchresta tubulosa Dunn. (Leguminosae, Euchresta) is the evergreen shrub mainly distributed in Sichuan, Chongqing, Hunan and Hubei [1]. It is also known as Odougen, Hudoulian, Dougan, Opium Seven in china. In Japan, it was used to treat ulcers and arrhythmias [2]. In Wu-ling Mountains, it was often used to treat sore throat and abdominal pain by Tujia minority. Alkaloids the main active ingredients of *E. tubulosa* Dunn. are increasingly used in clinical treatment [3] with anti-tumor [4], antiviral and antibacterial [5], anti-inflammatory and analgesic [6], cardiovascular effect [7], insecticidal [8]. Matrine and oxymatrine are recently proved to have anti-cancer potentials, such as inhibiting cancer cell proliferation, inducing cell differentiation [9]. Cytisine is a low-cost medication found to increase the likelihood of smoking cessation. [10]. *N*-formyl cytisine has also been studied for medicinal purposes [11]. At present, alkaloids in congeneric plants (*Euchresta*) were separated only by a single method such as silica gel column chromatography, thin-layer chromatography and sephadex LH-20 [12,13], which have obvious defects, and it is difficult to achieve the purpose of systematic separation and purification.

The silica gel column can be selected according to the quality of the crude extract, so a large amount of crude extract can be preliminarily separated. However, the sample size of HSCCC is relatively small. Alkaloids are generally alkaline, and it is easy to cause deathly adsorption on silica gel [14], so HSCCC was considered to avoid the loss of alkaloids, a form of liquid–liquid distribution technology invented

by ITO [15]. Finally, the four alkaloids with higher relative content including matrine, oxymatrine, *N*-formyl cytosine and cytosine were obtained by combining the two chromatographic methods, as shown in Figure 1. *N*-formyl cytosine was obtained for the first time from *E. tubulosa* Dunn. HSCCC is used in this plant for the first time, too.

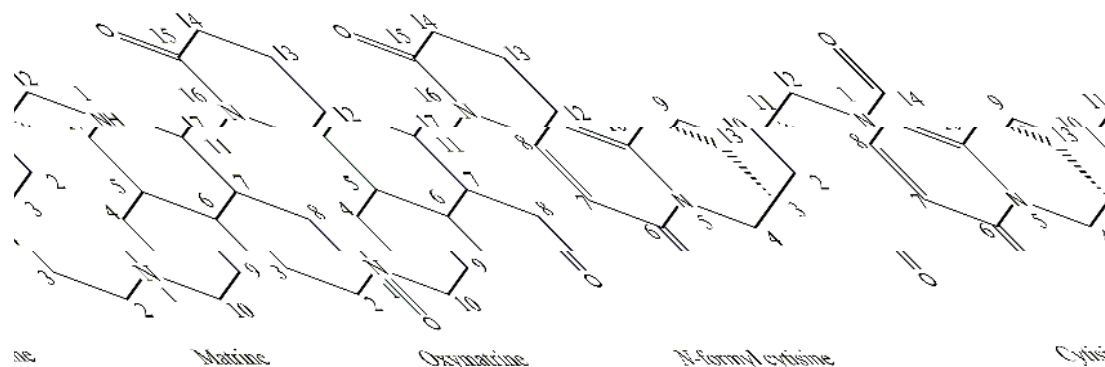


Figure 1. Chemical structures of alkaloids from *Euchresta tubulosa* Dunn.

In this study, the elution system of total alkaloids was screened by TLC, then the crude extract was divided into different parts by silica gel column chromatography, finally the components with different polarity were separated by HSCCC. The purity of the compound was analyzed by HPLC-UV and the chemical structures were identified by NMR and EI-MS, respectively. The aim of this study is to establish a mode for the systematic separation and purification of alkaloids in natural products.

2. Materials and Methods

2.1. Materials

The root and stem of *E. tubulosa* Dunn., identified by Ai-Wen Dong, research fellow, Hunan Key Laboratory of Forest Chemical Engineering, Jishou University, were collected from Tianping Mountain Reserve of Badagongshan National Forest Park in Hunan Province. Analytical carbon tetrachloride ($\geq 99.5\%$), dichloromethane ($\geq 99.5\%$), *n*-butanol ($\geq 99\%$), methanol ($\geq 99.5\%$), chloroform ($\geq 99\%$), ammonia water (25% to 28%), hydrochloric acid (36% to 38%), triethylamine ($\geq 99\%$), ethanol (95%), petroleum ether (60–90 °C) and chromatographic methanol (99.9%) were purchased in Shanghai TAITAN Technology Co., Ltd., Shanghai, China. Water was distilled water made in laboratory. Silica gel and GF254 plates were purchased in Qingdao Marine Chemical, Inc., China.

2.2. Preparation of Crude Alkaloid Extract

The root and stem of *Euchresta tubulosa* Dunn. were ground into dry powder by high speed multifunctional grinder (YB-1250A, Zhejiang Yongkang Sufeng industry and Trade Co., Ltd., Zhejiang, China) and screened through a 60-mesh sieve. The dry powder (3 kg) was extracted by 95% ethanol hot reflux for three times with the ratio of material to liquid of 1: 6 (g: mL), each time for three hours, and the fixed temperature of water bath was 80 °C. After filtration, extract (826 g) was obtained by 55 °C rotary evaporation. Dilute hydrochloric acid and distilled water were used to prepare acid solution with pH 2.0, and the extract was dissolved with 1.0 L acid solution, filtered and degreased with petroleum ether. Then the pH of the solution was adjusted to 10 with ammonia water. Finally, the solution was extracted with chloroform at the ratio of 1:1 (*v/v*) until chloroform layer and bismuth potassium iodide had almost no precipitation reaction. The chloroform extract was concentrated and dried at 40 °C to obtain total alkaloid (15.2 g), which were preserved at low temperature for later separation and purification.

2.3. Preliminary Separation of Crude Alkaloid Extract

Total alkaloid (1 mg) was dissolved in methanol solution (2 mL). TLC was used to select the eluent consisting of petroleum ether, dichloromethane, ethyl acetate and methanol. The methods of coloring were the combination of iodine vapor coloring and ultraviolet lamp coloring. It was found that the eluent composed of CH₂Cl₂ and MeOH could effectively separate the total alkaloids. 60–80 mesh silica gel (16.0 g) was added to total alkaloid extract and mixed with petroleum ether as the sample. 100–200 mesh silica gel with a volume of 3/4 silica gel column (7.5 cm × 68 cm) was dissolved by dichloromethane to wet mount the column (exhaust bubble), then silica gel column was balanced by dichloromethane (triethylamine was added to adjust pH to 8) and stood overnight. The sample was eluted by gradient mode with CH₂Cl₂/MeOH (1: 0, 50: 1, 25: 1, 10: 1, 0: 1, 0: 1, *v/v*) to achieve the purpose of preliminary separation. TLC was used to monitor and merge the components with similar R_f value. Each component was evaporated and concentrated at 50 °C and stored at –20 °C for HSCCC separation.

2.4. Partition Coefficient Measurement

Selection of two-phase solvent system was based on the partition coefficient (*K*) of target compounds in each solvent system. *K* was calculated by HPLC-UV method. The specific method is as follows. The selected two-phase solvent system was configured with 3 mL of each phase, and then the sample (1 mg) was dissolved in the solvent system. After the distribution was completed, each phase (1 mL) was taken out and concentrated by 50 °C rotary evaporation separately. Then the residue was dissolved in methanol (1 mL), analyzed by HPLC-UV. The *K* of the target compound was calculated according to formula (1).

$$K = C_{\text{upper}}/C_{\text{lower}} \quad (1)$$

*C*_{upper}: peak area of target compound in the upper phase;

*C*_{lower}: peak area of target compound in the lower phase.

2.5. Selection of Two-Phase Solvent System

A key factor for the success of HSCCC is the selection of two-phase solvent system, which enables the target compound to have an appropriate *K* range of 0.5–2.0. Because too small *K* value will lead to HSCCC peak faster, poor separation and too large *K* value will lead to a wider range of peak, poor purity. According to the research and development of literature [16,17], it was found that dichloromethane system was mostly used in the separation of alkaloids by HSCCC. However, considering the difficulty of sample recovery and the convenience of experiment, it was decided not to use acidic and ionic solutions to change the solubility of water to target alkaloids, and only to add other organic reagents to change the solubility of organic to target alkaloids. The organic solvents including n-hexane, cyclohexane, petroleum ether, carbon tetrachloride, ethyl acetate and n-butanol which can change the solubility of alkaloids were investigated. The suitable organic solvents were selected according to the system stability and stratification time. It was found that carbon tetrachloride and n-butanol could significantly change the solubility of organic phase. The system was stable, and the stratification time was less than 20 s. HSCCC also has other different elution modes to solve the problem of inappropriate *K*, such as dual mode, reversed mode elution or expelling solution from column [18]. However, this paper only discusses the feasibility of systematic separating alkaloids from natural products by combining various chromatographic methods, so only the forward elution mode from beginning to end was considered. The applicability of other modes will be analyzed by readers according to specific problems.

2.6. HSCCC Separation

Before each separation, the water bath temperature was set at 27 °C by low constant temp bath (DC-0506, Tauto Biotechnology, Shanghai, China), and the column of HSCCC (TBE-300B, i.d. of the tubing = 1.5 mm, total volume = 285 mL and a 20 mL sample loop, Tauto Biotechnology, Shanghai,

China) should be fully filled with the upper phase water (stationary phase) at 15 mL/min by the constant pump (TBP1002ST, Tauto Biotechnology, Shanghai, China) until the outlet effluent phase was 30 mL. Then the speed of the instrument was set to 900 rpm/min (0–1000 rpm/min) and the wavelength of the ultraviolet detector (TBD2000-UV, Tauto Biotechnology, Shanghai, China) was turned on to 254 nm at the same time. The lower phase (mobile phase) was pumped at 2.0 mL/min until the baseline of the ultraviolet detector was stable and there were no bubbles, and the outflow liquid appeared dual phase; then, the sample was ready to be injected through the injection valve. Finally, the samples were collected manually according to the frontal group of chromatograms in the chromatographic workstation (WH500-USB, Tauto Biotechnology, Shanghai, China). Each component was concentrated by 40 °C rotary evaporation and stored at −20 °C for purity analysis by HPLC-UV.

2.7. HPLC-UV Analysis and Structural Identification of HSCCC Peak Fractions

The sample was analyzed by HPLC-UV (Agilent 1260, Agilent, USA) with a YMC-Triart C₁₈ EXRS (250 mm × 4.6 mm, i.d., 1.5 mm). The mobile phase was methanol and water. The column temperature was 30 °C, the flow rate was 0.8 mL/min. Injection volume: 20 µL; wavelength: 254 nm. Elution conditions of component 1: 0 min (10%B)–35 min (95%B); elution conditions of component 2: 0 min (10%B)–35 min (95%B); elution conditions of component 3: 0 min (10%B)–5 min (10%B)–25 min (95%B); detector: 1260 Quat Pump VL (G1311C).

NMR experiments were carried out using a Bruker AVANCE III NMR spectrometer (600 Hz, Bruker Biospin, Germany) with CDCl₃ or MeOD as the solvent. The MS analyses were performed by EI mass spectrometer (DSQ, Thermo, Massachusetts, USA).

3. Results

In the experiment, the powder of *Euchresta tubulosa* Dunn. was extracted by hot reflux method. The total alkaloid was degreased by petroleum ether and enriched by chloroform extraction. The flow chart of alkaloid preparation is shown in Figure 2. The total alkaloid was preliminarily separated into four components by silica gel column chromatography using gradient elution. The alkaloid with high relative content in each component was separated by HSCCC. The flow chart of systematic separation is shown in Figure 3.



Figure 2. Preparation flow chart of total alkaloids.

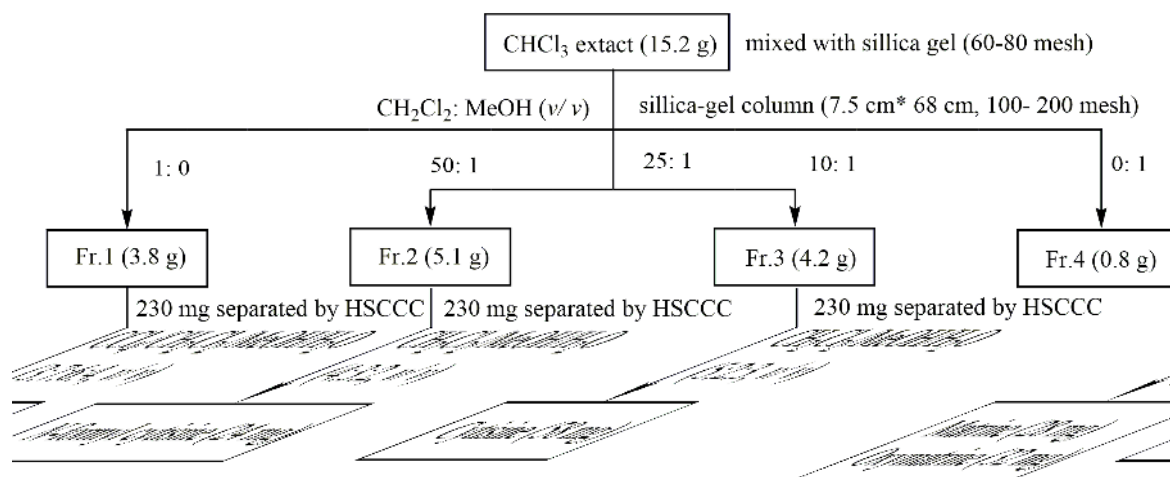


Figure 3. Flow chart of separation and purification.

3.1. Selection of Two-Phase Solvent System

The K values of a series of two-phase solvent systems composed of five solvents with different volume ratios (carbon tetrachloride/dichloromethane/*n*-butanol/ methanol/water) were measured. Finally, carbon tetrachloride/dichloromethane/methanol/water (3:7:6:4, v/v), dichloromethane/methanol/water (4:3:2, v/v) and dichloromethane/methanol/water (5:2:3, v/v) were selected as the two-phase separation systems of HSCCC. Table 1 shows the K value (0.5–2.0) of the target compound in the selected solvent system. The results showed that the dichloromethane system was suitable for the separation of alkaloids from *Euchresta tubulosa* Dunn.

Table 1. The K values of target components measured in different solvent systems.

Solvent System	K Values		
	Carbon Tetrachloride/Dichloromethane/Methanol/Water (3:7:6:4, v/v)	Dichloromethane/Methanol/Water (4:3:2, v/v)	Dichloromethane/Methanol/Water (5:2:3, v/v)
Matrine	0.52	–	–
Oxymatrine	0.88	–	–
<i>N</i> -formyl cytosine	–	0.92	–
Cytosine	–	–	1.11

3.2. Systematic Separation

According to gradient elution of silica gel column chromatography, total alkaloid was divided into four components with different polarity. Four components were preserved at low temperature for subsequent HSCCC separation.

For the separation of component 1, carbon tetrachloride/dichloromethane/methanol/water (3:7:6:4, v/v) was used as the two-phase solvent system for HSCCC. According to HPLC-UV analysis, two compounds with high content were identified as target compounds. According to the relevant HSCCC separation conditions, the instrument was debugged. The retention rate of stationary phase was 73% and two purified peaks were obtained, marked as peaks 1 and 2 in Figure 4A. They were identified as matrine and oxymatrine in turn. The residual liquid in the column was blown out by nitrogen gas and concentrated for subsequent separation and purification.

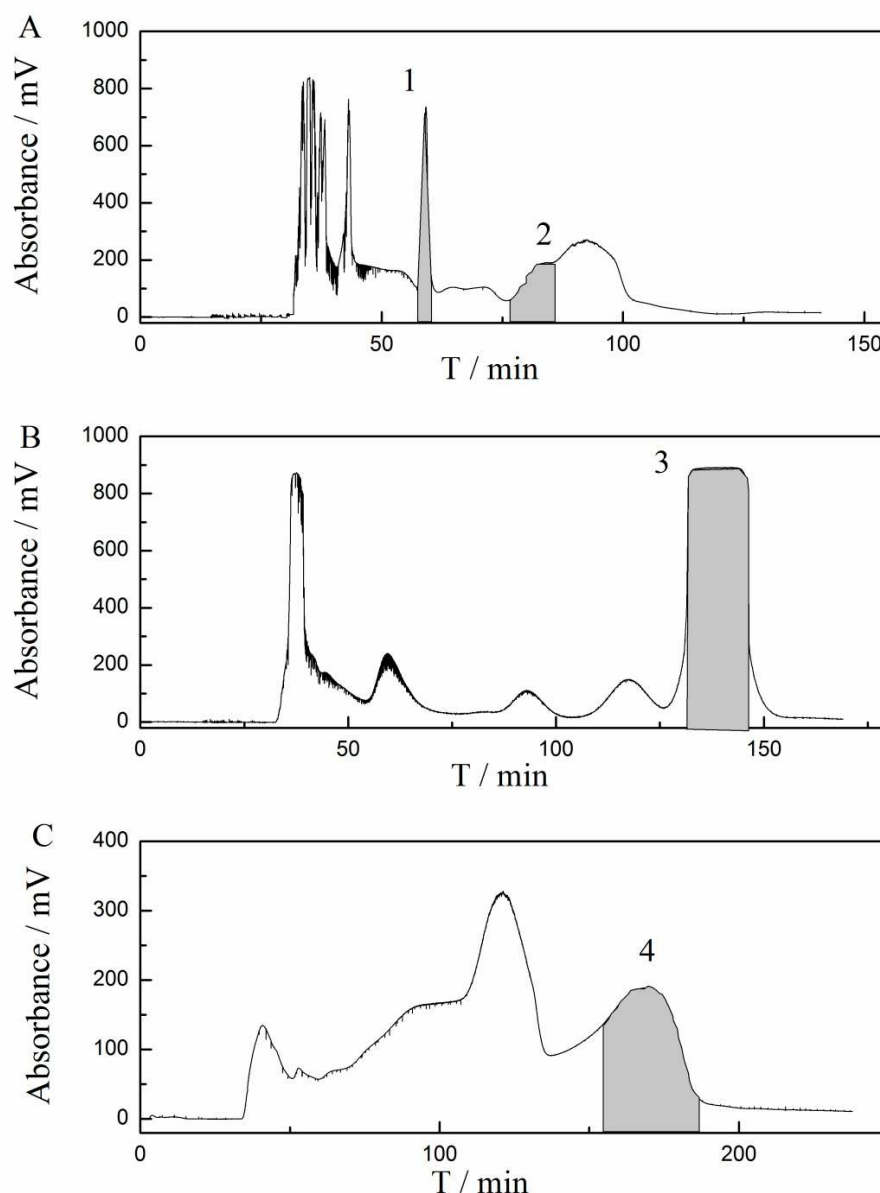


Figure 4. HSCCC chromatograms of three fractions of Fr.1 (A), Fr.2 (B) and Fr.3 (C); solvent systems: carbon tetrachloride/dichloromethane/methanol/water (3:7:6:4, *v/v*, A), dichloromethane/methanol/water (4:3:2, *v/v*, B) and dichloromethane/ methanol/ water (5:2:3, *v/v*, C); flow rate: 1.8 mL/min; revolution speed: 900 rpm; temperature: 25 °C; sample size: 230 mg.

For the separation of component 2, dichloromethane/methanol/water (4:3:2, *v/v*) was used as the two-phase solvent system for HSCCC. According to HPLC-UV analysis, three compounds with high content were identified as target compounds. According to the relevant HSCCC separation conditions, the instrument was debugged. The retention rate of stationary phase was 70% and one purified peak was obtained, marked as peaks 3 in Figure 4B. It was identified as *N*-formyl cytosine. The purity of other compounds for further purification was less than 80%. The residual liquid in the column was blown out by nitrogen gas and concentrated for subsequent separation and purification.

For the separation of component 3, dichloromethane/methanol/water was used as the two-phase solvent system for HSCCC. According to HPLC-UV analysis, two compounds with high content were identified as target compounds. According to the relevant HSCCC separation conditions, the instrument was debugged. The retention rate of stationary phase was 70% and one purified peak was obtained, marked as peak 4 in Figure 4C. It was identified as cytosine. The purity of other compounds

for further purification was less than 80%. The residual liquid in the column was blown out by nitrogen gas and concentrated for subsequent separation and purification.

Component 4 was analyzed by TLC and HPLC-UV. It was found that the types of compounds were more complex and the quality was less. In the future, the quality of component 4 will be enriched and separated by chromatography.

3.3. HPLC-UV Analysis

The purity of components (1–3) and compounds separated by HSCCC was analyzed by HPLC-UV under suitable conditions. According to the peak area normalization method, the purity of each compound was higher than 91%. The HPLC-UV chromatograms are shown in Figure 5.

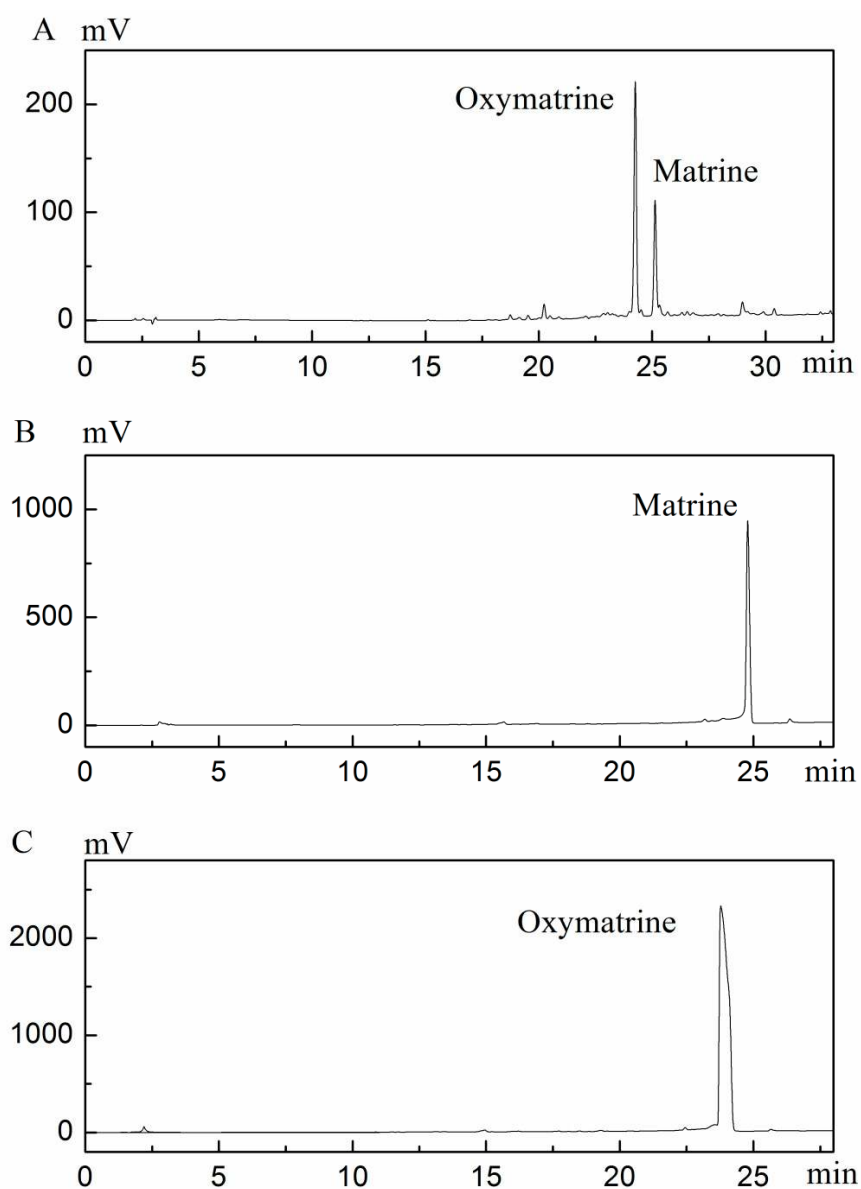


Figure 5. Cont.

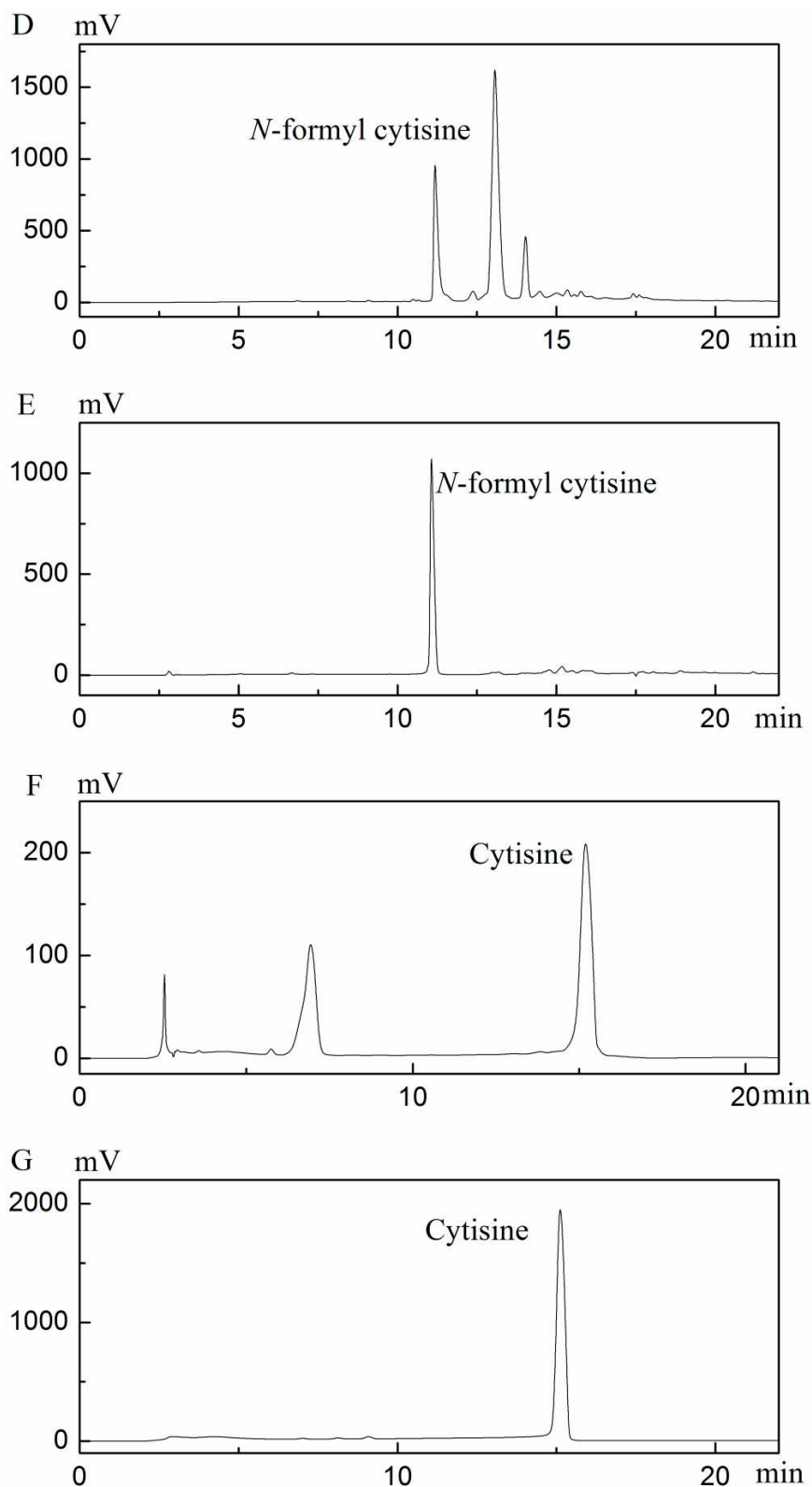


Figure 5. HPLC-UV chromatograms of component 1 (A), component 2 (D) and component 3 (F); HPLC-UV chromatograms of HSCCC peak fraction (B, C, E and G). Chromatographic column: YMC-Triart C18 EXRS (250 mm × 4.6 mm, i.d., 1.5 mm); mobile phase: B (methanol)-A (water). Temperature: 30 °C; flow rate: 0.8 mL/min; injection volume: 20 µL; wavelength: 254 nm. Elution conditions of component 1: 0 min (10%B)–35 min (95%B); elution conditions of component 2: 0 min (10%B)–35 min (95%B); elution conditions of component 3: 0 min (10%B)–5 min (10%B)–25 min (95%B); detector: 1260 Quat Pump VL (G1311C).

3.4. The Structural Identification

Matrine (1): $C_{15}H_{24}N_2O$; white crystal; EI-MS, m/z 248 [M]⁺; the data of 1H -NMR and ^{13}C -NMR are summarized in Table 2.

Table 2. 1H -NMR and ^{13}C -NMR data of matrine and oxymatrine (MeOD).

NO.	Matrine		Oxymatrine	
	^{13}C	1H	^{13}C	1H
1	/	/	/	/
2	53.17	2.81 (t, 1H, 13.1 Hz) 1.96 (s, 1H)	68.02	3.36–3.29 (m, 1H) 3.07 (ddd, 1H, 7.7, 3.8, 1.9 Hz)
3	20.73	1.83–1.54 (m, 1H) 1.52–1.37 (m, 1H)	16.85	2.64–2.46 (m, 2H)
4	27.12	1.83–1.54 (m, 1H) 1.52–1.37 (m, 1H)	25.37	1.91–1.80 (m, 1H) 1.79–1.55 (m, 1H)
5	36.39	1.83–1.54 (m, 1H)	34.33	1.91–1.80 (m, 1H)
6	63.76	2.29–2.18 (m, 1H)	68.50	3.36–3.29 (m, 1H)
7	41.45	1.52–1.37 (m, 1H)	41.84	1.79–1.55 (m, 1H)
8	26.42	1.83–1.54 (m, 1H) 1.52–1.37 (m, 1H)	23.75	1.91–1.80 (m, 1H) 1.79–1.55 (m, 1H)
9	21.14	2.10–2.02 (m, 1H) 1.52–1.37 (m, 1H)	18.21	2.08 (d, 1H, 12.8 Hz) 1.79–1.55 (m, 1H)
10	57.25	2.77 (d, 1H, 11.3 Hz) 2.10–2.02 (m, 1H)	66.42	3.36–3.29 (m, 1H) 3.07 (ddd, 1H, 7.7, 3.8, 1.9 Hz)
11	57.19	3.80 (td, 1H, 9.7, 6.0 Hz)	53.56	3.36–3.29 (m, 1H)
12	27.73	1.83–1.54 (m, 1H) 1.89–1.80 (m, 1H)	27.88	1.42–1.33 (m, 1H) 2.33–2.23 (m, 1H)
13	18.93	1.94–1.90 (m, 1H) 1.83–1.54 (m, 1H)	16.73	1.79–1.55 (m, 2H)
14	32.79	2.40 (dt, 1H, 17.2, 3.7 Hz) 2.29–2.18 (m, 1H)	32.18	2.40 (d, 1H, 17.4 Hz) 2.33–2.23 (m, 1H)
15	169.46	/	171.40	/
17	43.30	4.37 (dd, 1H, 12.9, 4.5 Hz) 3.03 (t, 1H, 12.7 Hz)	42.19	4.32 (d, 1H, 5.1 Hz) 4.09 (t, 1H, 12.4 Hz)

Oxymatrine (2): $C_{15}H_{24}N_2O_2$; white powder; EI-MS, m/z 264 [M]⁺; the data of 1H -NMR and ^{13}C -NMR are summarized in Table 2.

N-formyl cytosine (3): $C_{12}H_{14}N_2O_2$; light yellow oily; EI-MS, m/z 218 [M]⁺; The data of 1H -NMR and ^{13}C -NMR are summarized in Table 3. In the NMR spectrum of the compound, each group has two approximately coincident signals. Because the C-N on N is linked by a single bond, it is easy to change the configuration, and the NMR spectrum is split. It is known from the literature [19] that the paired signals in 1H -NMR and ^{13}C -NMR are the characteristics of *N*-formyl cytosine. Due to the existence of some double bond properties of N-C=O bond, the rotation of these compounds is blocked, which leads to the widespread phenomenon of NMR splitting [20].

Table 3. 1H -NMR and ^{13}C -NMR data of *N*-formyl cytosine and cytosine (MeOD).

NO.	<i>N</i> -Formyl Cytosine		Cytosine	
	^{13}C	1H	^{13}C	1H
1	/	/	/	2.37 (s, 1H, N-H)
2	51.90/45.84	3.44/3.42 (dd, 1H, 12.8, 2.5 Hz) 3.56/3.53 (d, 1H, 1.8 Hz)	51.76	3.07 (d, 1H, 12.0 Hz) 2.99–2.97 (d, 1H, 13.2 Hz)
3	26.89/26.52	2.55 (s, 1H)	27.51	2.39–2.35 (m, 1H)
4	48.65/48.42	3.85/3.88 (dd, 1H, 15.7, 6.0 Hz) 4.10/4.08 (d, 1H, 15.6, 3.6 Hz)	52.80	4.08 (d, 1H, 15.5 Hz) 3.93 (dd, 1H, 15.5, 6.8 Hz)
5	/	/	/	/
6	163.14/163.0	/	164.4	/
7	117.56/117.26	6.45/6.44 (d, 1H, 9.0 Hz)	115.38	6.45 (d, 1H, 9.0 Hz)
8	138.87/138.50	7.27/7.19 (m, 1H)	139.9	7.49 (dd, 1H, 9.0, 7.0 Hz)
9	104.96/105.70	6.08/6.02 (d, 1H, 6.8 Hz)	106.7	6.30 (d, 1H, 7.0 Hz)
10	147.93/147.88	/	151.7	/

Table 3. Cont.

NO.	N-Formyl Cytisine		Cytisine	
	¹³ C	¹ H	¹³ C	¹ H
11	33.65/34.38	3.10 (m, 1H)	35.11	3.03 (m, 1H)
12	53.20/46.89	4.46/4.36 (d, 1H, 13.6 Hz) 2.88 (m, 1H)	49.75	3.46 (m, 1H, 11.4 Hz) 2.99–2.97 (d, 1H, 12.0 Hz)
13	26.13/26.07	2.08/2.09 (dd, 2H, 8.3, 4.8 Hz)	25.32	2.10–1.93 (m, 2H)
14	160.90/161.01	7.83/7.60 (s, 1H)		

Cytisine (4): C₁₁H₁₄N₂O; white crystal; EI-MS, *m/z* 190 [M]⁺; the data of ¹H-NMR and ¹³C-NMR are summarized in Table 3.

The data of ¹H-NMR and ¹³C-NMR of four alkaloids were consistent with those described in the literature previously [21–24].

4. Conclusions

Four alkaloids with relatively high content including matrine, oxymatrine, *N*-formyl cytosine and cytosine in *Euchresta tubulosa* Dunn., one of the most important chinese medicinal herbs, were separated by silica gel column chromatography combined with HSCCC. *N*-formyl cytosine was obtained for the first time from *Euchresta tubulosa* Dunn. HSCCC is used in this plant for the first time. At present, there are few studies on the active compounds in *E. tubulosa* Dunn. In this paper, the results provide a successful mode for the systematic separation of alkaloids, providing theoretical basis for the separation of new active compounds from *E. tubulosa* Dunn. in the future. Because there are many kinds of compounds in natural products, it is difficult to separate the alkaloids systematically by a single separation method. This mode combines different separation mechanisms to improve the success rate and the systematicity of separation. Dichloromethane/methanol system was used for preliminary separation of total alkaloid and the dichloromethane/water system used in HSCCC separation can obtain appropriate *K* value. In fact, the two-phase solvent system of HSCCC has high flexibility. As long as the two-phase system can be formed, it can be used in HSCCC. In this paper, only the alkaloids with relatively higher content in each component were studied, but the researchers can carry out similar operations for other alkaloids according to this mode.

Author Contributions: W.-X.L., H.W. and A.-W.D. conceived and designed the experiments; W.-X.L. and H.W. performed the experiments; W.-X.L. analyzed the data; A.-W.D. contributed reagents/materials/analysis tools; W.-X.L. wrote the paper.

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Conflicts of Interest: The authors declare no conflicts of interest.

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