Separation of Three Major Active Components from *Eleutherine americana* Merr. et K. Heyne Using High-speed Counter-current Chromatography Combined with Silica Gel Column Chromatography

Wanyu ZUO[1], Yalan LUO[1], Bingyu CHENG[1], Luyang LU[1], Jizhong ZHANG[1,2]*

1. College of Pharmacy and Food, Southwest Minzu University, Chengdu 610041, China; 2. Xizang University of Tibetan Medicine, Lhasa 850000, China

Abstract [**Objectives**] To establish an efficient and environmentally friendly separation and purification method for the large-scale preparation of the major active components—eleutherol, eleutherine, and isoeleutherine—from the ethnomedicinal plant *Eleutherine americana* Merr. et K. Heyne. [**Methods**] The sample of *E. americana* bulbs was initially extracted with ethanol, followed by three successive extractions with ethyl acetate-water (2∶1, *V/V*) to obtain the target component-enriched fraction. Eight solvent systems were systematically optimized, and a mixture of petroleum ether-ethyl acetate-ethanol-water (5∶5∶6∶4, *V/V/V/V*) was identified as the optimal solvent system for high-speed counter-current chromatography (HSCCC) separation under conditions of 900 rpm, 2 mL/min, and 35 ℃. The crude HSCCC product was further purified by silica gel column chromatography (200 - 300 mesh) using gradient elution with a solvent system of n-hexane-dichloromethane-ethyl acetate (varying from 10∶5∶1 to 4∶5∶1, *V/V/V*). UPLC-PDA (Agilent SB-C_{18} column) and nuclear magnetic resonance spectroscopy (600 MHz) were comprehensively employed to assess compound purity and confirm molecular structures. [**Results**] An optimized technique integrating HSCCC and silica gel column chromatography was established, successfully enabling the large-scale preparation of three bioactive components: eleutherol (purity 99%), eleutherine (purity 98%), and isoeleutherine (purity 98%). Structural identification results were consistent with those reported in the literature. Compared to traditional methods, the new approach demonstrated improved separation efficiency and reduced solvent consumption. [**Conclusions**] The combined separation method utilizing HSCCC and silica gel column chromatography established in this study demonstrates notable advantages, including high efficiency, environmental friendliness, and cost-effectiveness, enabling the large-scale preparation of the three major active components from *E. americana*. This approach outperforms conventional methods by offering higher separation efficiency, reduced solvent consumption, and superior product purity, providing a robust technical solution for the development and utilization of bioactive compounds from *E. americana*. Moreover, it offers a novel methodological reference for the isolation and purification of other natural products.

Key words *Eleutherine americana*, High-speed counter-current chromatography(HSCCC), Silica gel column chromatography, Separation and purification

1 Introduction

The term "Hongcong" is a common reference to the dried bulbs of *Eleutherine americana* Merr. et K. Heyne, which are also referred to as "Wanniang" or "Baibuhuanyang". This plant belongs to the Iridaceae family and is distributed in Yunnan, Guangxi, and other regions of China. As a medicinal and edible plant widely used by ethnic minorities such as the Dai and Yi, it is employed in the treatment of palpitations, dizziness, dysentery, hemoptysis, and hematemesis. Its pharmacological effects include clearing heat and detoxifying, promoting diuresis and dampness removal, activating blood circulation and resolving stasis, and hemostasis. This herb is officially documented in the *Yunnan Provincial Standards for Traditional Chinese Medicinal Materials* (Volume III: Dai Ethnic Medicine, 2005 edition). Modern research indicates that the primary active components of *E. americana* are naphthols, naphthoquinones, and anthraquinones, with the naphthol compound eleutherol, as well as the naphthoquinones eleutherin and isoeleutherin, being the most extensively studied compounds[1]. These components exhibit a broad range of biological activities and pharmacological effects, with their primary therapeutic benefits reflected in the following aspects. Firstly, they demonstrate significant antibacterial effects[2-4]. They can effectively inhibit the growth and reproduction of various pathogenic bacteria by disrupting bacterial cell membranes, among other mechanisms. In particular, they exhibit strong inhibitory effects against common pathogenic microorganisms such as *Staphylococcus aureus*, *Aspergillus niger*, and *Pyricularia oryzae*. Secondly, they can effectively inhibit melanin production, reduce skin pigmentation, and exhibit certain antifungal activity against dermatophytes. Thirdly, in terms of cardiovascular protection[4-5], they can dilate coronary arteries, improve blood flow, and exert antihypertensive effects by inhibiting angiotensin-converting enzyme (ACE). Additionally, regarding their anti-inflammatory effects, these components can suppress inflammatory cytokines, alleviate inflammatory responses, and reduce symptoms such as redness, swelling, heat, and pain[6-7]. Moreover, their antioxidant mechanism primarily involves direct free radical scavenging through electron or proton transfer, as well as the regulation of signaling pathways by inhibiting NF-κB/iNOS and activating Nrf2. These effects are achieved through the synergistic interactions of multiple components and multi-target mechanisms[7-9]. Furthermore, studies have shown that the active components of *E. americana* can inhibit topoisomerase II-mediated

Received: August 16, 2025 Accepted: November 12, 2025
Supported by Natural Science Foundation of Tibet Autonomous Region, Science and Technology Department of Tibet (XZ202501ZR0118).
Wanyu ZUO, master's degree candidate. *Corresponding author. Jizhong ZHANG, doctoral degree, professor.

transient double-strand cleavage of DNA and suppress TCF/β-catenin transcription, thereby exerting an anti-tumor effect. These mechanisms contribute to inhibiting the proliferative activity of human leukemia cell lines[10-11]. However, due to the similarity of polarity exhibited by these three components (exhibiting adjacent retention times in UPLC), conventional separation methods (*e.g.* silica gel column chromatography) require repeated gradient elution coupled with recrystallization, resulting in cumbersome procedures, prolonged cycles[12], and low yields. These limitations severely hinder pharmacological research and restrict the development of bioactive compounds from *E. americana* for practical applications.

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatography technique that operates without a solid stationary phase. This technique achieves continuous separation by maintaining a dynamic equilibrium between two-phase solvents. By eliminating sample loss or deactivation caused by solid-phase adsorption, HSCCC is particularly suitable for isolating compounds with similar polarities[13]. In recent years, HSCCC has been successfully applied to the separation of natural products such as flavonoids and alkaloids[14-15]. However, the application of HSCCC technology to isolate the three major active components from *E. americana* has not been reported yet. This study marks the first application of HSCCC for this purpose. Through the systematic optimization of extraction conditions and solvent systems, combined with silica gel column chromatography, a rapid, efficient, and environmentally friendly separation method was established. This approach provides critical technical support for advancing research on the pharmacodynamic material basis of *E. americana*, enhancing its quality standards, and facilitating industrial-scale development.

2 Materials and methods

2.1 Instruments HSCCC TBE-300B, Tauto Biotech Co., Ltd.; Waters Acquity UPLC H-Class, Waters, USA; Agilent SB-C_{18} Column (3.0 mm × 100 mm, 1.8 μm); Nuclear Magnetic Resonance Spectrometer AVII-600 MHz, Bruker; Digital Thermostatic Water Bath HH-2, Guohua Electric Appliance Co., Ltd.; IKA RV10 Rotary Evaporator, Gongyi Yinyu Instrument Factory; Circulating Vacuum Pump, Gongyi Yinyu Instrument Factory; Vortex Mixer XW-80A, Shanghai Jingke Industrial Co., Ltd.; KQ5200E Ultrasonic Cleaner (40 kHz, 250 W), Kunshan Ultrasonic Instrument Co., Ltd.

2.2 Reagents *E. americana* bulbs were purchased from Menglun Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, and authenticated by Associate Professor Lu Luyang at the College of Pharmacy and Food, Southwest Minzu University. The solvents employed in this study comprised cyclohexane, ethyl acetate, and ethanol of analytical grade, as well as chromatographic methanol of chromatographic grade.

2.3 Methods

2.3.1 Optimization of solvent extraction process. Two equal portions of ethanol extract derived from dried *E. americana* bulb powder were separately extracted with ethyl acetate and chloroform. The extracts were dissolved in methanol and analyzed by UPLC to compare peak areas. Subsequently, four extraction cycles were performed to determine the optimal extraction frequency.

2.3.2 UPLC chromatographic conditions. Separation was performed on an Agilent SB-C_{18} column (3.0 mm × 100 mm, 1.8 μm) using a mobile phase of acetonitrile (A) and water (B) with a gradient elution: 40% – 50% A (0 – 3 min) and 50% – 80% A (3 – 7 min). The flow rate was maintained at 0.25 mL/min, with detection performed at a wavelength of 254 nm. The column temperature was controlled at 35 ℃, and the injection volume was set to 2 μL.

2.3.3 Optimization of the extraction solvent system. In the selection of solvent systems for HSCCC, the partition coefficient (K) serves as a critical parameter. It is defined as the ratio of the mass concentration of the target compound in the stationary phase to that in the mobile phase. The ideal K value ranges between 0.5 and 2, while the ideal resolution (R) falls within 1.2 – 1.5. It is evident that elevated R values are indicative of enhanced separation efficiency and increased purity of the compound.

Solvent systems were prepared according to the ratios listed in Table 1, thoroughly mixed, and allowed to equilibrate until phase separation occurred. Ethanol extract from *E. americana* was weighed and transferred into screw-cap test tubes, followed by the addition of equal volumes of upper and lower phases. The tubes were vigorously vortexed to ensure the complete dissolution of the extract in both phases. After equilibration, precise volumes of the upper and lower phases were separately collected, evaporated to dryness, and subsequently redissolved in chromatographic-grade methanol. The solutions were filtered through a 0.22 μm membrane and analyzed by UPLC. The partition coefficient (K) of the target compounds was calculated for each solvent system.

The calculation formula is: $K = \dfrac{\text{Upper phase concentration}}{\text{Lower phase concentration}}$

Table 1 Solvent systems with varying ratios

No.	Solvent system	Solvent ratio
1	Petroleum ether-Ethyl acetate-Ethanol-Water	5∶5∶6∶4
2	Petroleum ether-Ethyl acetate-Ethanol-Water	8∶3∶5∶5
3	n-Hexane-Ethyl acetate-Ethanol-Water	6∶4∶5∶5
4	n-Hexane-Ethyl acetate-Ethanol-Water	8∶3∶5∶5
5	Cyclohexane-Ethyl acetate-Ethanol-Water	8∶3∶5∶5
6	Cyclohexane-Ethyl acetate-Ethanol-Water	11∶3∶5∶5
7	Cyclohexane-Ethyl acetate-Ethanol-Water	10∶2∶5∶5
8	Cyclohexane-Ethyl acetate-Ethanol-Water	12∶2∶5∶5

2.3.4 Preparation of extraction samples. A measured quantity of dried *E. americana* bulbs was crushed and passed through a No. 4 sieve. The resulting powder was then extracted by hot reflux extraction with 95% ethanol three times (1 h each time). The filtrates were combined and concentrated under reduced pressure to obtain the ethanol extract concentrate. A precise amount of the ethanol extract was subjected to three successive extractions with

ethyl acetate-water (1 ∶ 2, *V/V*). The ethyl acetate extracts were combined and concentrated under reduced pressure to yield the ethyl acetate extract concentrate.

2.3.5 Preparation of two-phase solvent system and sample solution. Based on the optimization results of the HSCCC solvent system, a solvent system consisting of petroleum ether-ethyl acetate-ethanol-water (5 ∶ 5 ∶ 6 ∶ 4, *V/V/V/V*) was selected. Approximately 2 000 mL of the solution was prepared in a separatory funnel, thoroughly mixed, and then allowed to stand overnight. The separated upper and lower phases were ultrasonically degassed for 15 – 20 min, with the upper phase serving as the stationary phase and the lower phase as the mobile phase. An appropriate amount of the ethyl acetate extract of *E. americana* bulbs was dissolved in the lower phase using ultrasonication, after which the mixture was filtered for subsequent use.

2.3.6 Preliminary HSCCC separation. The low-temperature thermostat circulation device was activated and set to 35 ℃. Once the temperature stabilized, the upper phase was pumped into the helical tube of the HSCCC column to serve as the stationary phase at a flow rate of 20 mL/min. Once the upper phase had flowed out uniformly (20 – 50 mL), the rotation speed was adjusted to 900 rpm, and the flow rate was reduced to 2 mL/min. Subsequently, the lower phase (mobile phase) was introduced.

After equilibrium was established between the upper and lower phases (indicated by stable outflow), the prepared sample solution was injected into the HSCCC sample valve. The eluent was monitored by a detector (wavelength 254 nm), and fractions were collected accordingly. These fractions were dried under reduced pressure to obtain dried crystalline powder samples.

2.3.7 Silica gel column chromatography separation of HSCCC crude extract. The sample was dissolved in a small amount of dichloromethane and mixed with 20 g of silica gel (200 – 300 mesh) for dry loading column packing. The column was then compacted by suction. Gradient elution was performed sequentially with n-hexane-dichloromethane-ethyl acetate (10 ∶ 5 ∶ 1; 8 ∶ 5 ∶ 1; 6 ∶ 5 ∶ 1; 4 ∶ 5 ∶ 1; 2 ∶ 5 ∶ 1), with TLC employed for monitoring. Fractions exhibiting identical compositions were combined and concentrated under reduced pressure to obtain Fractions 1, 2, and 3.

Fraction 1 was repeatedly subjected to silica gel column chromatography (n-hexane-dichloromethane-ethyl acetate, ranging from 10 ∶ 5 ∶ 1 to 4 ∶ 5 ∶ 1), followed by recrystallization to yield Compound A. Fraction 2 was dissolved in a small amount of dichloromethane, mixed with an appropriate amount of silica gel, and subsequently loaded onto a silica gel column. Gradient elution with n-hexane-dichloromethane-ethyl acetate (ranging from 10 ∶ 5 ∶ 1 to 4 ∶ 5 ∶ 1) was performed, and identical fractions were combined and concentrated under reduced pressure to obtain Compound B. Fraction 3 was dry-loaded onto a silica gel column and subjected to gradient elution with n-hexane-dichloromethane-ethyl acetate (ranging from 10 ∶ 5 ∶ 1 to 4 ∶ 5 ∶ 1). Identical fractions were combined and concentrated under reduced pressure to obtain Compound C.

2.3.8 Purity analysis and structural analysis. An appropriate amount of the sample was dissolved in chromatographic-grade methanol and filtered through a 0.22 μm microporous membrane. The purity of the collected fractions was analyzed by UPLC under the chromatographic conditions described in Section **2.3.1**. The chemical structures of the target compounds were determined based on ^{1}H-NMR, ^{13}C-NMR data, and comparison with literature references.

3 Results and analysis

3.1 HSCCC

3.1.1 Extraction process. The peak area analysis indicated that the chloroform extract exhibited a larger peak area compared to the ethyl acetate extract. However, due to the higher toxicity of chloroform, ethyl acetate was ultimately selected as the extraction solvent because of its superior safety profile. By observing the color changes in the organic phase and accurately weighing the dried product, it was found that the organic phase became significantly lighter after the third extraction, accompanied by a marked decrease in yield. Therefore, the optimal number of extraction cycles was determined to be three.

3.1.2 Solvent systems. The partition coefficients (K) and resolution values (R) of eleutherinoside A, eleutherinoside B, and isoeleutherinoside B in red shallot across different solvent systems were calculated, with the results presented in Table 2.

As shown in Table 2, the solvent system consisting of petroleum ether-ethyl acetate-ethanol-water (5 ∶ 5 ∶ 6 ∶ 4, *V/V/V/V*) exhibited moderate K values and optimal peak elution times. The ratio of partition coefficients (R) varied from 1.2 to 1.5, indicating good separation between the compounds. Additionally, this system required a smaller solvent volume, resulting in lower costs. Therefore, the petroleum ether-ethyl acetate-ethanol-water (5 ∶ 5 ∶ 6 ∶ 4, *V/V/V/V*) system was selected as the optimal solvent system for the isolation of the target compounds.

Table 2 Partition coefficients (K) and resolution (R) of eleutherol, eleutherin, and isoeleutherin from *Eleutherine americana*

Solvent system	K			$R1$	$R2$
	Eleutherol	Eleutherin	Isoeleutherin		
Petroleum ether-Ethyl acetate-Ethanol-Water (5 ∶ 5 ∶ 6 ∶ 4)	1.38	1.82	1.20	1.15	1.32
Petroleum ether-Ethyl acetate-Ethanol-Water (8 ∶ 3 ∶ 5 ∶ 5)	1.06	1.26	0.91	1.16	1.19
n-Hexane-Ethyl acetate-Ethanol-Water (6 ∶ 4 ∶ 5 ∶ 5)	1.60	1.30	1.50	1.15	1.07
n-Hexane-Ethyl acetate-Ethanol-Water (8 ∶ 3 ∶ 5 ∶ 5)	0.97	1.36	0.85	1.14	1.40
Cyclohexane-Ethyl acetate-Ethanol-Water (8 ∶ 3 ∶ 5 ∶ 5)	1.71	2.13	1.27	1.25	1.34
Cyclohexane-Ethyl acetate-Ethanol-Water (11 ∶ 3 ∶ 5 ∶ 5)	1.49	1.99	1.20	1.24	1.34
Cyclohexane-Ethyl acetate-Ethanol-Water (10 ∶ 2 ∶ 5 ∶ 5)	1.24	1.63	0.96	1.29	1.31
Cyclohexane-Ethyl acetate-Ethanol-Water (12 ∶ 2 ∶ 5 ∶ 5)	0.66	0.86	1.14	1.31	1.33

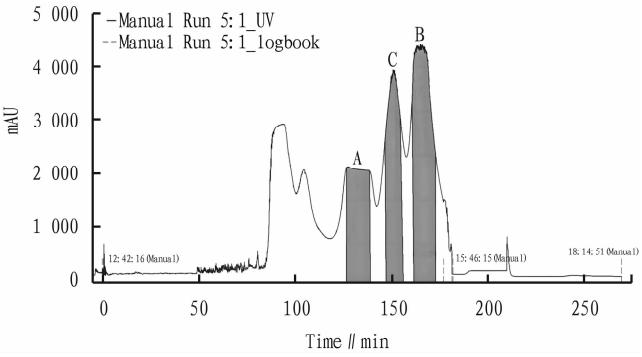
3.2 HSCCC and silica gel column chromatography separation results After drying under reduced pressure, Compound A (20 mg), Compound B (310 mg), and Compound C (38 mg) were obtained. Compound A appeared as pale yellow needle-shaped crystals and showed co-elution with the eleutherol reference standard in HPLC analysis, thereby confirming its identity as eleutherol. Compound B appeared as yellow needle-shaped crystals and co-eluted with the eleutherin reference standard in HPLC, identifying it as eleutherin. Similarly, Compound C appeared as yellow needle-shaped crystals and co-eluted with the isoeleutherin reference standard in HPLC, confirming its identity as isoeleutherin.

3.3 Purity analysis and structural analysis UPLC analysis revealed that the purities of the three compounds were 99% for eleutherol, 98% for eleutherin, and 98% for isoeleutherin. The ^{1}H-NMR and ^{13}C-NMR spectral data are presented as follows. Eleutherol: ^{1}H-NMR (400 MHz, CDCl3), δ: 9.65 (1H, s, 1-OH), 7.90 (1H, s, H-4), 7.59 (1H, d, J=8.4 Hz, H-5), 7.41 (1H, t, J=8 Hz, H-6), 6.94 (1H, d, J=7.6 Hz, H-7), 5.73 (1H, q, J=6.5 Hz, H-9), 4.12 (3H, s, 8-OCH3), 1.75 (3H, d, J=6.4 Hz, 12-CH3), ^{13}C-NMR (600 MHz, CDCl3), δ: 170.5 (C-3), 156.5 (C-8), 149.1 (C-9), 137.2 (C-4a), 127.9 (C-9a), 126.6 (C-6), 125.9 (C-3a), 123.6 (C-5), 117.5 (C-8a), 116.5(C-4), 106.2 (C-7), 77.4 (C-1), 56.4 (8-OCH3), 19.1 (1-CH3).

Eleutherin: ^{1}H-NMR (400 MHz, CDCl3), δ:7.75 (1H, d, J=7.6 Hz, H-6), 7.66 (1H, t, J=8Hz, H-7), 7.28 (1H, d, J=8.8 Hz, H-8), 5.01 (1H, q, J=6.4 Hz, H-1), 4.0 (3H, s, 9-OCH3), 3.9-4.0 (1H, s, H-3), 2.70 (1H, dd, JAB=18 Hz, JAX=3.2 Hz), 2.24 (1H, dd, JAB 1=10 Hz, JAX=18.8 Hz, H-4), 1.53 (3H, d, J=6.8 Hz, 1-CH3), 1.34 (3H, d, J=6.0 Hz, 3-CH3); ^{13}C-NMR (600 MHz, CDCl3) δ: 184.2 (C-10), 182.7 (C-5), 159.7 (C-9), 148. (C-10a), 139.4 (C-4a), 134.7 (C-5a), 134. (C-7), 119.7 (C-9a), 119.1 (C-6), 117.8 (C-8), 67.4 (C-1), 62.4 (C-3), 56.4 (9-OCH3), 29.5 (C-4), 19.7 (1-CH3), 21.2 (3-CH3).

Isoeleutherin: ^{1}H-NMR (400 MHz, CDCl3), δ: 7.73 (1H, d, J=7.6 Hz, H-6), 7.64 (1H, t, J=8 Hz, H-7), 7.27 (1H, d, J=8.4 Hz, H-8), 4.85 (1H, m, H-1), 3.99 (3H, s, 9-OCH3), 3.58 (1H, m H-3), 2.74 (1H, d, J=18 Hz, H-4), 2.20 (1H, ddd, J1=4 Hz, J2 =1 2 Hz, J3=16.4 Hz, H-4), 1.53 (3H, d, J=6.4 Hz, 1-CH3), 1.36 (3H, d, J=6.4 Hz, 3-CH3); ^{13}C-NMR (600 MHz, CDCl3), δ: 184.1 (C-10), 183.7 (C-5), 159.4 (C-9), 148.7 (C-10a), 139.9 (C-4a), 134.5 (C-5a), 113.9 (C-7), 120.3 (C-9a), 119.0 (C-6), 117.7 (C-8), 70.3 (C-1), 68.7 (C-3), 56.5 (9-OCH3), 29.9 (C-4), 20.8 (1-CH3), 21.24 (3-CH3).

The data were consistent with those reported in the literature[11]. The HSCCC chromatograms and UPLC chromatograms of the three major components from *E. americana* are shown in Figs. 1-4.



NOTE A. Eleutherol; B. Eleutherin; C. Isoeleutherin.

Fig. 1 HSCCC chromatograms of the three major components from *Eleutherine americana*

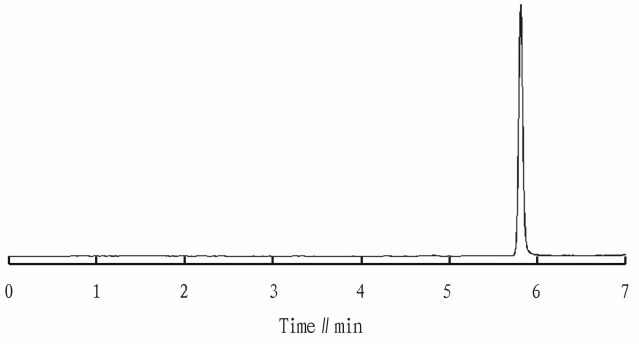


Fig. 2 UPLC chromatograms of eleutherol (A)

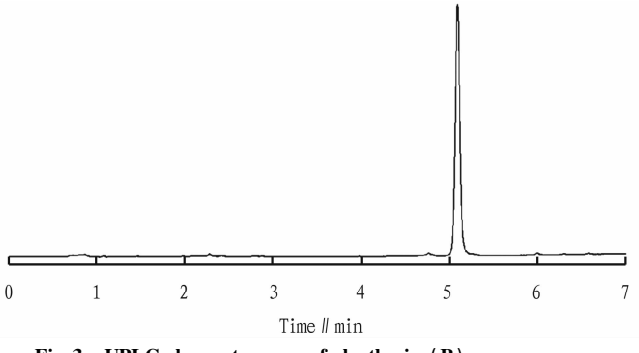


Fig. 3 UPLC chromatograms of eleutherin (B)

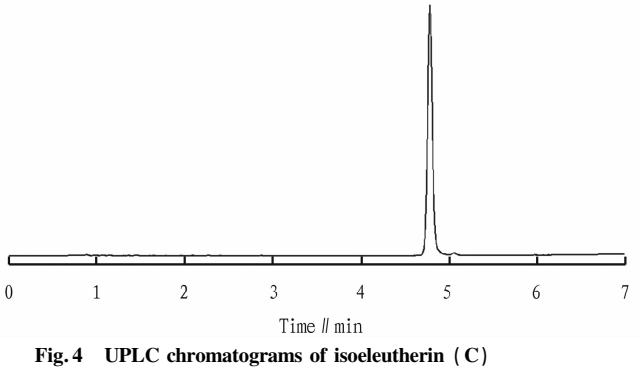


Fig. 4 UPLC chromatograms of isoeleutherin (C)

4 Conclusions and discussion

This study developed an efficient and environmentally friendly HSCCC separation method through systematic optimization, suc-

cessfully overcoming the technical bottleneck in isolating active components from *E. americana*. Utilizing an innovative solvent system composed of petroleum ether-ethyl acetate-ethanol-water (5∶5∶6∶4, *V/V/V/V*), this study successfully achieved high-efficiency separation of eleutherol, eleutherin, and isoeleutherin. Compared to traditional silica gel column chromatography, this method reduces the separation time to 3 h and simultaneously isolates three active components from the ethnomedicine *E. americana*, significantly improving efficiency. More importantly, by establishing a coupled technology integrating HSCCC and silica gel column chromatography, large-scale preparation from 2 kg of raw material yielded 20 mg of eleutherol (purity 99%), 310 mg of eleutherin (purity 98%), and 38 mg of isoeleutherin (purity 98%). This approach demonstrated enhanced production yields, reduced costs, and promising industrial application potential. This study not only provides a scientific basis for improving the quality standards of *E. americana*, but also establishes an eco-friendly separation strategy that offers new insights for the large-scale preparation of naphthoquinone natural products, which will significantly promote the modern development and utilization of ethnomedicine resources. Future research should further explore the combined application of HSCCC with other separation technologies, as well as investigate the *in vivo* metabolic mechanisms of active components from *E. americana* and develop novel drug delivery systems.

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