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SEPARATION OF AVERMECTIN COMPONENTS FROM *Streptomyces avemitilis* EXTRACTION USING HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

Three compounds of antibiotics - avermectins from fertilizing product of Streptomyces avemitilis are achieved by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of n-hexane--ethyl acetate-methanol-water (6:4:5:5, v/v) on a preparative scale. The separation conditions were: 1.5 mL/min (0 to 200 min) and 2.0 mL/min (200 to the end), 900 rpm and 20 °C based on the peak resolution. About 11.9 mg of avermectin B_{1a}, 1.0 mg of avermectin B_{1b} and 9.6 mg of avermectin B_{2a} from 50 mg of crude extract were obtained by one-step separation. The purities of the three compounds determined by HPLC were 99.7, 96.2 and 97.6%, respectively. Their chemical structures were identified by electron spray ionization mass spectroscopy (ESI-MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR).

Keywords: Streptomyces avemitilis; high-speed counter-current chromatography; avermectin B_{1a}; avermectin B_{1b}; avermectin B_{2a}.

Avermectins (AV) are a kind of macrolide antibiotics containing eight compounds with similar structure (Table 1), and they were first discovered by Merck laboratories from *Streptomyces avemitilis*, a new actinomyces species isolated from soil at the Kitasato Institute [1]. Avermectins were widely used in livestock and plant according to their efficient broad-spectrum anti-parasitic activity by affecting on glutamate-gated chloride channel of worms [2]. Among the eight components, B_1 (abamectin, mixture of avermectin B_{1a} and B_{1b}) was the most active constituent and B_2 (mixture of avermectin B_{2a} and B_{2b}) as a potential human drug was the most safe insecticide for mammals [1-4]. With the increasing application of avermectin in agriculture, the single component with high purity was necessary for residues determination and the potential toxicity test [5-7]. However, there were great difficulties in separating the “a” series and “b” series because of the structure similarity and low containing of “b” series. Some sophisticated methods have been reported for the separation of avermectin B_1. Using traditional multiple-crystallization technology [8-9], B_1 products were obtained with 70-80% avermectin B_{1a} and 5-10% avermectin B_{1b} and 10--25% other unclear compounds. Nevertheless, there has been no mention of results of crystallization of avermectin B_2.

High-speed counter-current chromatography (HSCCC), a unique liquid-liquid distribution chromatography technique, was an effective separation and purification technology for similar structure compounds including antibiotics [10-16]. In 1996, Ito *et al.* [17] applied HSCCC to separate ivermectin injection. In 2011, Koichi *et al.* [18] applied HSCCC/MS to separate and analyze abamectin and obtained high purity avermectin B_{1a} and B_{1b}. Nevertheless, isolation of avermectin B_2 by HSCCC has not been reported. In the present work an efficient method was established to separate high purity of avermectin B_{1a}, B_{1b} and B_{2a} from the fermentation broth of actinomycete *Streptomyces avemitili* with one step, and their chemical structures were identified further by electron spray ionization mass spectroscopy (ESI-MS), ^{1}H, ^{13}C nuclear magnetic resonance (NMR).

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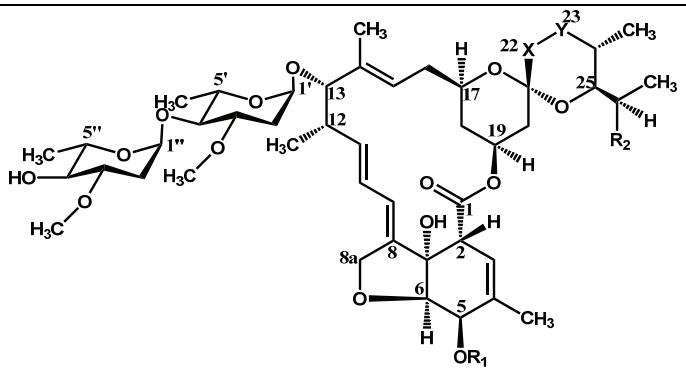
Table 1. Chemical structure of avermectins



Avermectin	Formula	R_1	R_2	X-Y
A_{1a}	$C_{49}H_{74}O_{14}$	CH_3	C_2H_5	CH=CH
A_{1b}	$C_{48}H_{72}O_{14}$	CH_3	CH_3	CH=CH
A_{2a}	$C_{49}H_{76}O_{15}$	CH_3	C_2H_5	CH_2-CH(OH)
A_{2b}	$C_{48}H_{74}O_{15}$	CH_3	CH_3	CH_2-CH(OH)
B_{1a}	$C_{48}H_{72}O_{14}$	H	C_2H_5	CH=CH
B_{1b}	$C_{47}H_{70}O_{14}$	H	CH_3	CH=CH
B_{2a}	$C_{48}H_{74}O_{15}$	H	C_2H_5	CH_2-CH(OH)
B_{2b}	$C_{47}H_{72}O_{15}$	H	CH_3	CH_2-CH(OH)

EXPERIMENTAL

Apparatus

The semi-preparative HSCCC instrument applied in the present research is a Model TBE-300A high-speed counter-current chromatograph (Tauto Biotechnique Company, Shanghai, China), with a mutilayer coil planet centrifuge equipped with a polytetrafluoroethylene (PTFE) preparative coil (diameter of tube, 1.6 mm; total volume, 280 mL). A manual injection valve with a 20 mL sample loop was used to introduce the sample into the coil system. The two-phase solvent system was delivered by a Model TBP-50A pump (Tauto Biotechnique, Shanghai, China). The β value of the preparative column varied from 0.5 at the internal terminal to 0.8 at the external terminal (β = = r/R, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 to 1000 rpm. The continuous monitoring of the effluent was operated with a model UVD-200UV Monitor, a multi-wavelength UV-Vis monitor for simultaneous monitoring of up to four wavelengths at 254, 280, 340 and 365 nm (Shanghai Jinda Biochemy Apparatus Co. Ltd., Shanghai, China). A HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was employed to control the separation temperature. The data were collected with a model V4.0 Chromatogram Workstation (Shanghai Jinda Biochemy Apparatus Co. Ltd., Shanghai, China).

HPLC was applied with an Agilent 1200 series HPLC-VWD system consisting of a G1322A degasser, a G1311A QuanPump, a sample injector with a 20 μL injection loop, a thermostated column compartment and a G1314B UV-Vis detector (Agilent Technologies, CA, USA). The system was controlled by Agilent Chemstation software (Agilent Technologies, USA).

The structure was identified with an electrospray ionization mass spectrometer (ESI/MS) (Finnigan Advantage LCQ, Thermo, USA) and a Varian INOVA 400-MHz-FT-nuclear magnetic resonance (NMR) spectrometer (Varian, USA).

Reagents

The avermectin fertilizing product was provided from Shenghua BIOK Co. Ltd., (Zhejiang, China). Acetonitrile, methanol (TEDIA) used for HPLC analysis were of chromatographic grade. *n*-hexane, ethyl acetate (Gaojing Fine Chemical Industry Co. Ltd., Zhejiang, China), methanol (Damao Chemical Reagent Factory, Tianjin, China) used for HSCCC separation and the preparation of crude extracts were all of analytical grade. All the pure water used in the experiment was produced by Barnstead TII super Pure Water System (Thermo Fisher Scientific, MA, USA).

Preparation of crude extract of avermectin

Approximately 20 g of the avermectin fertilizing product was added to 20 mL methanol to mill for 10 min, and then settled for 10 h at 25 °C. The supernatant fluid was filtered and concentrated to dryness by rotary evaporator under reduced pressure and further dried under vacuum. The residue was stored in a freezer at 4-5 °C for further separation.

Determination of partition coefficients (*K*) and selection of two-phase solvent system

Several two-phase solvent systems were tested for HSCCC separation. The selection of two-phase solvent was depended on the partition coefficients (*K*) of avermectins. The *K*-values were determined as follows: approximately 1 mg of avermectin crude extract was added into a 10 mL test tube to which 2 mL of each phase of the pre-equilibrated two-phase solvent system was previously added. The tube was shaken to distribute the sample in the two phases thoroughly. Then an equal volume (1 mL) of upper and lower phase was transferred and evaporated, respectively. The residue was dissolved in 1 mL of HPLC mobile phase for HPLC analysis. The peak area of the upper phase was recorded as A_U and that of the lower phase, as A_L. The *K*-value was calculated according to the equation $K = A_U/A_L$.

HSCCC Separation

A mixture of *n*-hexane-ethyl acetate-methanol-water (6:4:5:5, v/v) was prepared in a separatory funnel and set at room temperature until there were two clearly separated phases. Both phases were degassed by using an ultrasonic bath for 45 min. The upper phase was chosen as the stationary phase and the lower phase as the mobile phase. The multilayer coiled column was first filled with the stationary phase. Then the mobile phase was pumped into the column at a suitable flow rate at first into the head of the column. In the meantime, the HSCCC apparatus was rotated at work revolution speed, and the system was kept at steady temperature. After the mobile phase emerged and the volume was unchanged, the liquid-liquid equilibrium was established in the column. The crude sample (50 mg) was dissolved in 20 mL of mixture of stationary and mobile phases (1:1, v/v), then injected into the separation column. The effluent was continuously monitored with UV detector at 254 nm and peak fractions were collected according to the chromatogram.

HPLC Analysis, mass spectrometry, ^{1}H NMR and ^{13}C-NMR identification of the fractions

The crude avermectin extract and each purified fraction separated by HSCCC were analyzed by HPLC. The HPLC analysis was performed with an Extend C_{18} column (5 μm, 250 mm×4.6 mm i.d., Agilent, USA).

The purified fraction of avermectins obtained from the semi-preparative HSCCC separation was analyzed by ESI-MS, ^{1}H-NMR and ^{13}C-NMR, respectively. ^{1}H-NMR and ^{13}C-NMR spectra were measured in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. All the experiments were run at room temperature.

RESULTS AND DISCUSSION

Optimization of HPLC method

Different ratios of mobile phase (methanol-acetonitrile-water) were tested. The results indicated that the best separation of peak 1 was obtained at the lower proportion of organic reagent. The mobile phase consisted of methanol, acetonitrile and water in the initial ratio of 30:45:25 (v/v) with the flow rate of 1.0 mL/min. The gradient elution mode was as follows: 0-18 min, methanol/acetonitrile/water (30:45:25, v/v); 18-19 min, methanol/acetonitrile/water (30:45:25, v/v) to (50:35:15, v/v); 19-40 min, methanol/acetonitrile/water (50:35:15, v/v). The detection wavelength was set at 245 nm. The HPLC chromatogram of the crude extract is shown in Figure 1a. Peaks 1-3 correspond to B_{2a} (retention time: 12.3 min), B_{1b} (13.0 min) and B_{1a} (33.2 min), and they were 32.4, 5.9 and 45.2% of the total peak area, respectively.

Optimization of suitable two-phase solvent system

An ideal two-phase solvent system was critical for HSCCC separation which mainly involved four aspects listed below [19]. Firstly, sufficient stationary phase should be retained while the mobile phase passed through the system. Secondly, for satisfactory retention of the stationary phase, the settling time of the solvent systems should be considerably less than 30 s. Thirdly, *K* of the target compound should be close to 1 for ensuring the retention time of target component and an acceptable *K*-value is in the range of 0.5-2.0. Finally, the separation factor between two components ($\alpha = K_2/K_1$, $K_2 > K_1$) should be higher than 1.5 in semi-preparative HSCCC equipment.

In our experiments, several groups of solvent systems were evaluated and the partition coefficients were measured as listed in Table 2. According to the *K*-values, the results indicated that the *K*-values of the solvent systems 1 to 6 were too low (<0.5) and that of solvent system 7 was too high (>2). Comparing the *K* and α values of solvent system 8 to 11, system 10 was selected as the ideal system. The short settling

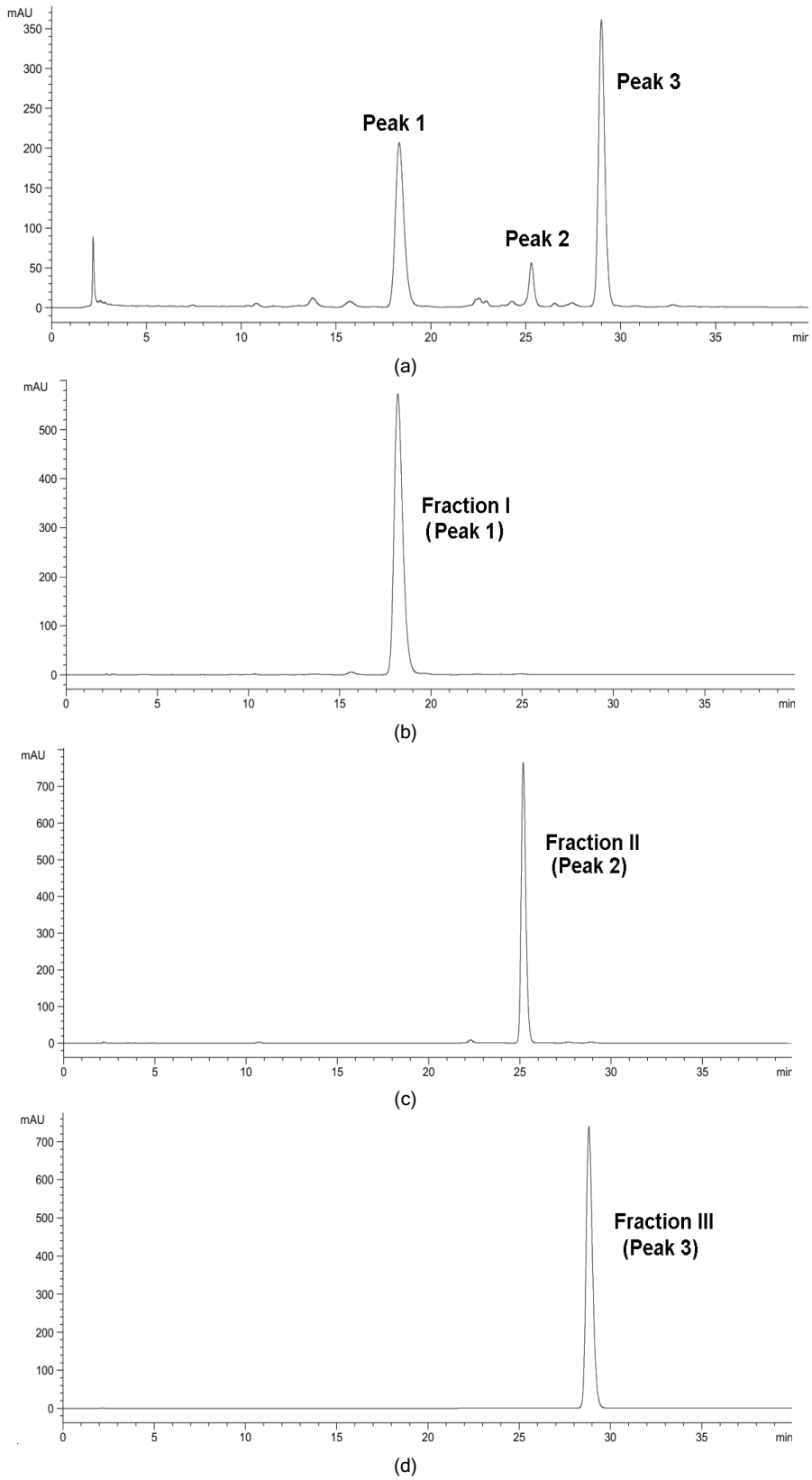


Figure 1. HPLC Chromatograms of the crude sample and HSCCC fractions from Streptomyces avemitilis. Column: Extend C_{18} column (5 μm, 250 mm×4.6 mm i.d., Agilent, USA); column temperature: 25 °C; the mobile phase: water-methanol-acetonitrile (25:30:45, v/v) at 0 to 18 min, water/methanol/acetonitrile (15:50:35, v/v) at 19 to 40 min; flow rate: 1.0 mL/min; detection: 245 nm. a) Methanol extraction from S. avemitilis, b) avermectin B_{2a} (peak 1), c) avermectin B_{1b} (peak 2) and d) avermectin B_{1a} (peak 3).

Table 2. Partition coefficients (K) and separation factors (α) of avermectin components

No.	Solvent system (v/v)	Setting time, s	Peak No. 1		2		3
			K^a	α^b	K	α	K
		Chloroform-*n*-butanol-methanol-water					
1	4:0.2:3:2	13.4	0	-	0	-	0
2	4:1:3:2	30.7	0	-	0	-	0
3	4:2:1:2	25.2	0.004	0.75	0.003	1.33	0.004
		n-Hexane-ethyl acetate- acetonitrile					
4	5:1:5	7.8	0	-	0	-	0
5	5:2:5	8.6	0	-	0	-	0
6	4:2:5	8.9	0.029	1.28	0.037	1.35	0.05
		n-Hexane-ethyl acetate-methanol-water					
7	1:5:1:5	20.5	∞	-	∞	-	∞
8	4:6:5:5	17.2	1.92	1.92	3.69	1.37	5.06
9	5:5:5:5	13.9	1.04	2.00	2.08	1.47	3.05
10	6:4:5:5	12.8	0.78	2.27	1.77	1.63	2.89
11	7:3:4:5	9.3	0.39	2.51	0.98	1.64	1.61

$^a K = A_U/A_L$ (A_U: peak area of upper phase; A_L: peak area of lower phase); $^b \alpha = K_2/K_1$, $K_2 > K_1$

time (12.8 s), the suitable *K*-values (0.78, 1.77 and 2.89) and α values (2.27 and 1.63) indicated high retention of stationary phase, suitable retention time and good resolution of separated compounds. Moreover, more time was required to elute target compounds in chloroform-*n*-butanol-methanol-water system due to the high solubility of avermectin in chloroform. On the other hand, although *n*-hexane-ethyl acetate-acetonitrile system had good solubility of target compounds, *K*-value was unsatisfied for HSCCC separation because of unequal solubility of target compounds in upper and lower phase. The best separation and sample solubility was obtained with the solvent system of *n*-hexane-ethyl acetate-methanol-water. In this case, the HPLC method was used to test the *K*-value as the peak area of target compound in the upper phase versus the lower phase.

Optimization HSCCC flow-rate, revolution speed and temperature

The chromatographic conditions including the mobile phase flow rate, column revolution speed and temperature were screened according to resolution of peak 1 and the stationary phase retention. The flow rate range of 1.5-2 mL/min, revolution speed range of 800-900 rpm and temperature range of 15-25 °C were tested, respectively. The results showed that the best separation and an ideal retention of the stationary phase (75.9%) could be obtained at flow-rate of 1.5 mL/min from 0 to 200 min and of 2.0 mL/min from 200 min to the end, revolution speed of 900 rpm and temperature of 20 °C. Under the optimal HSCCC conditions, three avermectin compounds were obtained with one-step elution for less than 7 h (Figure 2).

The sample injection size of 50-100 mg was also investigated under the optimum HSCCC conditions. With the sample amount increasing, the stationary phase retention was stable while the resolution of peak 1 decreased and 50 mg was thought to be suitable (Figure 3).

Separation of HSCCC

Under the optimal conditions, fraction I (150-180 min), fraction II (265-289 min), and fraction III (321-386 min) were obtained respectively as shown in Figure 2. From 50 mg of the crude sample, 9.6 mg of compound 1 with 99.7% HPLC purity (fraction I), 1.0 mg of compound 2 with 96.2% HPLC purity (fraction II) and 11.9 mg of compound 3 with 97.6% HPLC purity (fraction III) were obtained (Figure 1b-d) were further confirmed to be avermectin B_{2a}, avermectin B_{1b} and avermectin B_{1a}, respectively, by ESI-MS and NMR analysis.

Confirmation of chemical structures

Although Merck identified the structures of avermectin compounds in 1981, there is no integrated ^{1}H--NMR data of avermectin B_{1a}, B_{1b} and B_{2a} published.

In this work, the structure identification of peak fractions was performed by ESI-MS, ^{1}H- and ^{13}C-NMR.

Compound 1. The mass of the compound was observed at *m/z* 913.5 ($[M + Na]^+$), ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR ($CDCl_3$, 100 MHz) chemical data displayed in Tables 3 and 4 were consistent

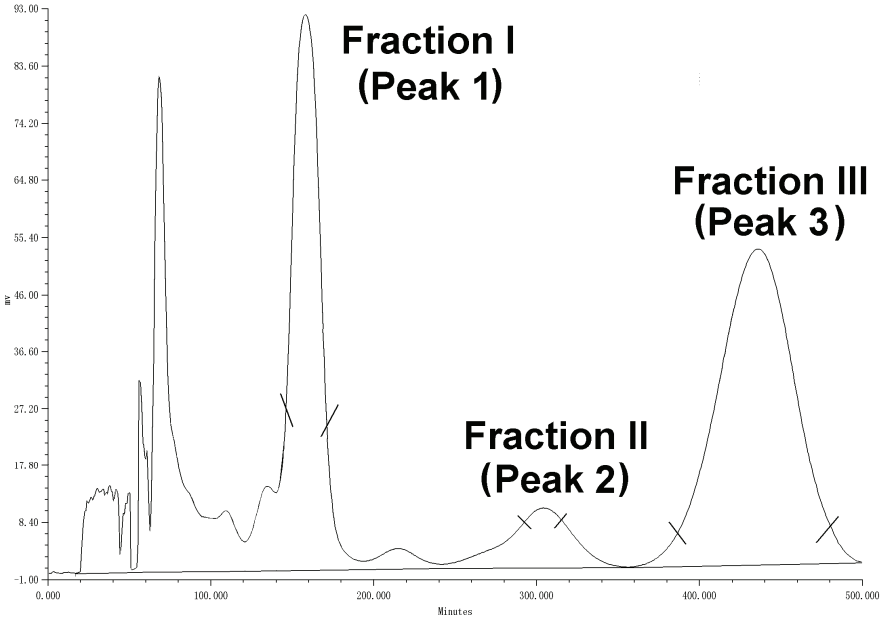


Figure 2. HSCCC Chromatograms of the crude sample. Flow rate: 1.5 mL/min (0 to 200 min) and 2.0 mL/min (200 min to the end), revolution speed: 900 rpm, temperature: 20 °C; stationary phase: upper organic phase; detection wavelength: 254 nm; sample size: 50 mg.

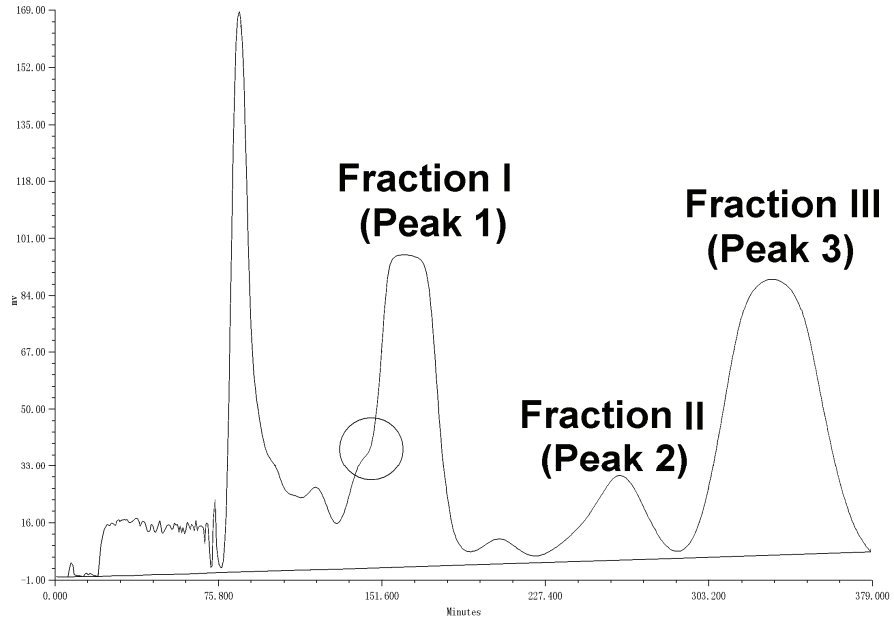


Figure 3. HSCCC Chromatograms of the crude sample. Flow rate: 1.5 mL/min (0 to 200 min) and 2.0 mL/min (200 min to the end), revolution speed: 900 rpm, temperature: 20 °C; stationary phase: upper organic phase; detection wavelength: 254 nm; sample size: 100 mg.

with B_{2a}. These findings confirmed that the compound is avermectin B_{2a} [20].

Compound ***2***. The mass of the compound was observed at *m*/*z* 881.6 ($[M + Na]^+$), ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR ($CDCl_3$, 100 MHz) chemical data displayed in Tables 3 and 4 were consistent with B_{1b}. These findings confirmed that the compound is avermectin B_{1b} [20].

Compound ***3***. The mass of the compound was observed at *m*/*z* 895.5 ($[M+Na]^+$), ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR ($CDCl_3$, 100 MHz) chemical data displayed in Tables 3 and 4 were consistent with B_{1a}. These findings confirmed that the compound is avermectin B_{1a} [20].

Table 3. ^{1}H-NMR data of avermectin B_{2a}, B_{1b} and B_{1a} in deuterated chloroform

Chemical unit	B_{2a}(1)	B_{1b}(2)	B_{1a}(3)
C2-H	3.29, *q* (1H), *J* = 2.4 Hz	3.30, *q* (1H), *J* = 2.0 Hz	3.30, *q* (1H), *J* = 2.4 Hz
C3-H	5.43, *s* (1H)	5.00, *d* (1H), *J* = 9.2 Hz	4.99, *d* (1H), *J* = 9.2 Hz
C4a-H	1.88, *s* (3H)	1.88, *s* (3H)	1.88, *s* (3H)
C5-H	4.30, *t* (1H), *J* = 7.2 Hz	4.30, *t* (1H), *J* = 6.4 Hz	4.30, *t* (1H), *J* = 5.6 Hz
C6-H	3.58-3.65, *m* (1H)	3.59-3.65, *m* (1H)	3.60-3.66, *m* (1H)
C8a-H	4.64-4.73, *m* (2H)	4.65-4.73, *m* (2H)	4.65-4.73, *m* (2H)
C9-H	5.83-5.89, *m* (1H)	5.85-5.88, *m* (1H)	5.85-5.89, *m* (1H)
C10-H	5.74, *d* (1H), *J* = 3.6 Hz	5.73-5.76, *m* (1H)	5.73, *d* (1H), *J* = 3.2 Hz
C11-H	5.73, *d* (1H), *J* = 2.0 Hz	5.73-5.77, *m* (1H)	5.75, *d* (1H), *J* = 2.4 Hz
C12-H	2.51-2.54, *m* (1H)	2.50-2.53, *m* (1H)	2.53, *t* (1H), *J* = 6.4 Hz
C12a-H	1.17, *d* (3H), *J* = 6.8 Hz	1.17, *d* (3H), *J* = 6.8 Hz	1.17, *d* (3H), *J* = 6.8 Hz
C13-H	3.96, br*s* (1H)	3.94, *s* (1H)	3.94, *s* (1H)
C14a-H	1.50, *s* (3H)	1.50, *s* (3H)	1.50, *s* (3H)
C15-H	4.97, *t* (1H), *J* = 7.6 Hz	5.43-5.45, *m* (1H)	5.42-5.46, *m* (1H)
C16-H	2.33-2.37, *m* (1H) 2.22, *dd* (1H), *J* = 12.8, 4.0 Hz	2.23, *dd* (1H), *J* = 12.0, 5.2 Hz 1.56-1.61, *m* (1H)	2.21-2.29, *m* (2H)
C17-H	3.76-3.79, *m* (1H)	3.90-3.97, *m* (1H)	3.86-3.92, *m* (1H)
C18-H	0.87, *q* (1H), *J* = 11.2 Hz 1.78, *d* (1H), *J* = 10.8 Hz	0.88, *q* (1H), *J* = 12.0 Hz 1.81, *d* (1H), *J* = 11.2 Hz	1.75-1.81, *m* (2H)
C19-H	5.29-5.38, *m* (1H)	5.41-5.48, *m* (1H)	5.42-5.46, *m* (1H)
C20-H	1.42, *t* (1H), *J* = 8.0 Hz 1.98-2.02, *m* (1H)	1.50, *t* (1H), *J* = 12.0 Hz 2.02, *dddd* (1H), *J* = 12.0. 6.0, 4.4, 1.6 Hz	1.48-1.53, *m* (1H) 2.02, *dd* (1H), *J* = 11.6, 4.8 Hz
C22-H	2.53, *t* (1H), *J* = 6.4 Hz 1.67, *dd* (1H), *J* = 14.4, 3.2 Hz	5.74-5.76, *m* (1H)	5.78, *d* (1H), *J* = 1.8 Hz
C23-H	1.98-2.02, *m* (1H)	5.56, *dd* (1H), *J* = 10.6, 2.8 Hz	5.56, *dd* (1H), *J* = 10.0, 2.8 Hz
C24-H	1.57-1.64, *m* (1H)	2.24-2.25, *m* (1H)	2.26-2.29, *m* (1H)
C24a-H	0.91, *d* (3H), *J* = 6.4 Hz	1.10, *d* (3H), *J* = 6.8 Hz	0.91, *d* (3H), *J* = 4.0 Hz
C25-H	3.55, *d* (1H), *J* = 11.6 Hz	3.34, *dd* (1H), *J* = 10.0, 2.0 Hz	3.51, *d* (1H), *J* = 10.0 Hz
C26-H	1.50-1.60, *m* (1H)	1.95, *dt* (1H), *J* = 6.8, 2.4 Hz	1.57-1.64, *m* (1H)
C26a-H	0.88, *d* (3H), *J* = 6.4 Hz	0.93 or 0.94, *s* (3H)	0.93, *d* (3H), *J* = 8.0 Hz
C27-H	1.42-1.57, *m* (2H)	0.93 or 0.94, *s* (3H)	1.47-1.61, *m* (2H)
C28-H	0.97, *t* (3H), *J* = 7.2 Hz	-	0.94, *d* (3H), *J* = 4.0 Hz
C1′-H	4.77, *d* (1H), *J* = 3.6 Hz	4.78, *d* (1H), *J* = 3.2 Hz	4.77, *d* (1H), *J* = 2.8 Hz
C2′-H	2.31-2.37, *m* (2H)	1.51-1.55, *m* (1H) 2.29-2.36, *m* (1H)	1.55-1.57, *m* (1H) 2.23, *dd* (1H), *J* = 12.4, 4.4 Hz
C3′-H	3.95-3.97, *m* (1H)	3.97, *d* (1H), *J* = 6.4 Hz	3.98, *d* (1H), *J* = 6.4 Hz
C3′a-H	3.44, *s* (3H)	3.43, *s* (3H)	3.43 or 3.44, *s* (3H)
C4′-H	3.23, *t* (1H), *J* = 9.2 Hz	3.25, *t* (1H), *J* = 9.2 Hz	3.25, *t* (1H), *J* = 9.2 Hz
C5′-H	3.80-3.85, *m* (1H)	3.83, *dd* (1H), *J* = 9.6, 6.4 Hz	3.84, *dd* (1H), *J* = 9.2, 6.4 Hz
C6′-H	1.28, *d* (3H), *J* = 8.8 Hz	1.28, *d* (3H), *J* = 6.4 Hz	1.28, *d* (3H), *J* = 6.0 Hz
C1″-H	5.40, *d* (1H), *J* = 3.6 Hz	5.40, *d* (1H), *J* = 3.2 Hz	5.40, *d* (1H), *J* = 4.0 Hz
C2″-H	2.31-2.37, *m* (2H)	1.51-1.55, *m* (1H) 2.29-2.36, *m* (1H)	1.55-1.57, *m* (1H) 2.34, *dd* (1H), *J* = 13.6, 5.2 Hz
C3″-H	3.45-3.51, *m* (1H)	3.45-3.52, *m* (1H)	3.47-3.53, *m* (1H)
C3″a-H	3.44, *s* (3H)	3.43, *s* (3H)	3.43 or 3.44, *s* (3H)
C4″-H	3.17, *t* (1H), *J* = 9.2 Hz	3.17, *t* (1H), *J* = 9.2 Hz	3.17, *t* (1H), *J* = 9.2 Hz
C5″-H	3.80-3.85, *m* (1H)	3.77, *dd* (1H), *J* = 9.2, 6.4 Hz	3.78, *dd* (1H), *J* = 9.2, 6.4 Hz
C6″-H	1.26, *d* (3H), *J* = 8.8 Hz	1.26, *d* (3H), *J* = 6.0 Hz	1.26, *d* (3H), *J* = 6.4 Hz

Table 4. ^{13}C-NMR data of avermectin B_{2a}, B_{1b} and B_{1a} in deuterated chloroform

Chemical unit	B_{2a}(1)	B_{1b}(2)	B_{1a}(3)
C-1	173.6	173.4	173.8
C-2	45.7	45.8	45.7
C-3	117.9	118.4	118.4
C-4	138.0	138.0	138.0
C-4a	20.0	19.9	20.0
C-5	68.1	67.7	67.7
C-6	79.3	79.4	79.4
C-7	80.4	80.5	80.5
C-8	139.6	139.7	139.7
C-8a	68.4	68.5	68.5
C-9	120.4	120.4	120.4
C-10	124.7	124.7	124.7
C-11	138.0	138.1	138.1
C-12	39.7	39.8	39.8
C-12a	20.2	20.2	20.2
C-13	81.6	81.9	81.9
C-14	135.6	135.1	135.2
C-14a	15.2	15.1	15.1
C-15	117.5	118.1	118.0
C-16	34.5	34.6	34.5
C-17	68.3	68.3	68.4
C-18	36.4	36.7	36.7
C-19	67.7	68.1	68.1
C-20	40.7	40.5	40.5
C-21	99.6	95.7	95.8
C-22	41.1	136.1	136.3
C-23	69.9	127.8	127.8
C-24	35.7	30.9	30.6
C-24a	13.7	21.1	16.4
C-25	70.8	77.3	75.0
C-26	35.1	28.4	35.2
C-26a	11.8	14.9	13.0
C-27	27.3	16.6	27.5
C-28	12.5	-	12.0
C-1′	94.8	94.9	95.0
C-2′	34.1	34.3	34.3
C-3′	79.1	79.1	79.1
C-3′a	56.4	56.4	56.4
C-4′	80.3	80.4	80.4
C-5′	67.2	67.3	67.3
C-6′	17.6	17.7	17.7
C-1″	98.5	98.5	98.5
C-2″	34.1	34.2	34.2
C-3″	78.1	78.2	78.2
C-3″a	56.5	56.5	56.5
C-4″	76.0	76.2	76.1
C-5″	67.6	68.3	68.4
C-6″	18.4	18.4	18.4

CONCLUSIONS

HSCCC was successfully used for separation and purification of avermectin B_{1a}, B_{1b} and B_{2a} from *Streptomyces avemitilis* with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (6:4:5:5, v/v). It is the first report of isolating avermectin B_{2a} with HSCCC. About 11.9 mg of B_{1a}, 1.0 mg of B_{1b} and 9.6 mg of B_{2a} were obtained from the 50 mg crude extracts at purity of 99.7, 96.2 and 97.6%, respectively. All of these products would be evaluated for their potential toxicity and pharmacology activity in our further work.

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NAUČNI RAD

RAZDVAJANJE KOMPONENTI AVERMEKTINA IZ *Streptomyces avemitilis* HSCCC HROMATOGRAFIJOM

Iz proizvoda Streptomyces avemitilis izdvojena su preparativno tri antibiotika avermektina pomoću HSCCC hromatografije sa dvofaznim sistemom rastvarača sastava n-heksan-etil-acetat-metanol-voda (6:4:5:5, v/v). Uslovi razdvajanja su bili sledeći: 1,5 mL/min (0 do 200 min) i 2,0 mL/min (od 200 min do kraja), 900 rpm i 20 °C u zavisnosti od rezolucije pika. Oko 11,9 mg avermektina B_{1a}, 1,0 mg avermektina B_{1b} i 9,6 mg avermektina B_{2a} je dobijeno separacijom u jednoj fazi iz 50 mg sirovog ekstrakta. Čistoće ove tri komponente određena HPLC metodom su bile 99,7, 96,2 i 97,6%. Njihove hemijske strukture su određene elektrosprej jonizacionom masenom spektroskopijom (ESI-MS), ^{1}H-NMR i ^{13}C-NMR spektroskopijom.

Ključne reči: Streptomyces avemitilis; HSCCC hromatografija; avermektin B_{1a}; avermektin B_{1b}; avermektin B_{2a}.