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Short communication

Application of high-speed counter-current chromatography for preparative separation of cyclic peptides from *Vaccaria segetalis*

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ABSTRACT

Following an initial clean-up step on silica gel, high-speed counter-current chromatography (HSCCC) was used to separate cyclic peptides from an extract of the seeds of *Vaccaria segetalis*. The two-phase solvent system used for HSCCC separation was composed of petroleum ether–ethyl acetate–methanol–water at an optimized volume ratio of 0.5:3.5:1:5. From 190 mg of crude extract, 38.0 mg of segetalin B and 28.5 mg of segetalin A were obtained with purities of 98.1% and 95.6% as determined by HPLC, respectively. The chemical structures of the target compounds were confirmed by high resolution electrospray ionization time of flight MS (HRESI-TOF-MS) and ^{1}H NMR analyses.

1. Introduction

Vaccaria segetalis (Neck.) Garcke (Caryophyllaceae) is an annual herb widely distributed in Asia, Europe and North America [1]. Wang-Bu-Liu-Xing, the dried seeds of *V. segetalis*, is a well-known traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia, which has been widely used to promote diuresis, facilitate milk secretion, activate blood circulation and relieve carbuncles in Chinese medical practice [2,3]. In phytochemical studies, varieties of compounds were isolated including cyclic peptides, triterpene saponins, alkaloids, phenolic acid, flavonoids and steroids from the seeds of this herb [1,4]. Cyclic peptides comprise a class of naturally occurring molecules, which exhibit a range of biological activities [5]. Cyclic peptides obtained from the seeds of *V. segetalis* have become a subject of interest because of bioactivities including oestrogen-like activity [6,7], vasorelaxant activity [8]. Although these cyclic peptides can be chemically synthesized [9,10], a large amount of organic solvents were used and also toxic waste in organic synthesis was produced. Therefore, development of an efficient method for the preparation of these compounds from the natural sources is warranted.

High-speed counter-current chromatography (HSCCC), as a support-free liquid–liquid partition chromatography, eliminates the irreversible adsorptive loss of the samples onto solid support and has an excellent sample recovery [11]. Compared to other liquid–liquid techniques, HSCCC has its own advantages such as shorter separation time, wider range of selection of solvent systems and quantitative material recovery. This method has been successfully applied to separate and purify various natural products [12–14]. In this paper, an efficient new method for separation and purification of cyclic peptides segetalin B and segetalin A (Fig. 1) from the Chinese medicinal plant *V. segetalis* is reported.

2. Experimental

2.1. Reagents and materials

Acetonitrile used for HPLC analyses was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China). The water used in mobile phase mixtures was treated with a Milli-Q water purification system (Millipore, USA). Petroleum ether (60–90 °C), ethyl acetate, $CHCl_3$ and methanol for preparation of crude samples and HSCCC separation were all of analytical grades (Juye Chemical Factory, Jinan, China).

The dried seeds of *V. segetalis* were purchased from a local drug store. The botanical identification was made by Dr. Li Jia, Shandong University of Traditional Chinese Medicine, China.

2.2. Apparatus

Preparative CCC was carried out using a Model GS10A-2 high-speed counter-current chromatograph (Beijing Emilion Science & Technology Co., Beijing, China). The multi-layer coil planet centrifuge was prepared by winding 1.6 mm i.d. PTFE tubing coaxially

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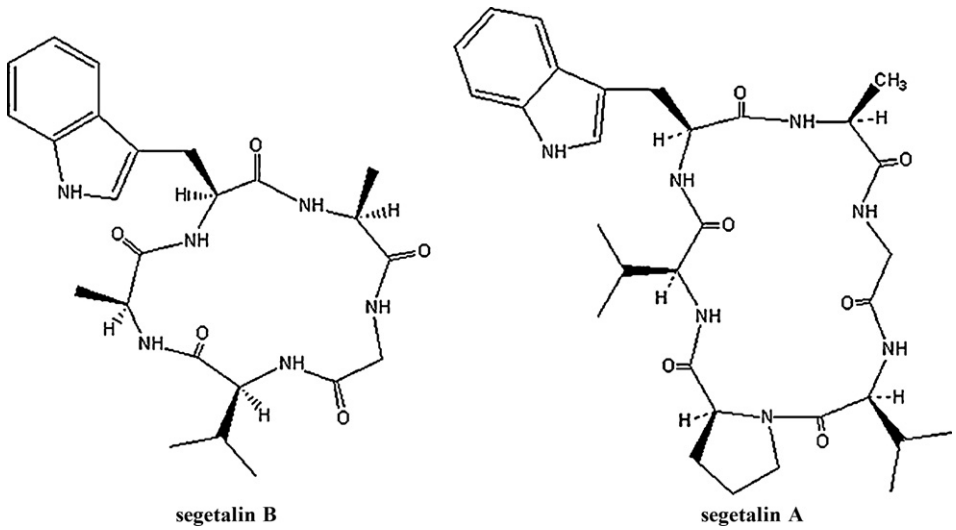

Fig. 1. Chemical structures of cyclic peptides from *Vaccaria segetalis*.

onto the column holder with a total capacity of 230 mL. The β value of this preparative column ranged from 0.5 at the internal to 0.8 at the external part ($\beta = r/R$, r is the distance from the coil to the holder shaft and R is the distance between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 to 1000 rpm, and 800 rpm was used in the present study. The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Emilion Science & Technology Co., Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Emilion Science & Technology Co., Beijing, China) at 254 nm. A manual sample injection valve with a 20-mL loop was used to introduce the sample into the column. A Model 3057-11 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The HPLC system used throughout this study consisted of a Waters 600 pump, a Waters 600 controller (Waters, USA), a sample injector (Rheodyne, USA) with a 20-μL loop, and a Waters 996 photodiode array detector (Waters, USA). Evaluation and quantification were made on an Empower pro data handling system (Waters, USA).

2.3. Preparation of the crude extract

The powdered seeds of *V. segetalis* (10.0 kg) were first extracted by reflux in 60 L of petroleum ether for three times. Then the power was extracted by reflux in 60 L ethyl acetate three times to give an ethyl acetate extract, and this was concentrated at 60 °C under reduced pressure. The ethyl acetate soluble fraction (79.0 g) was subjected to silica gel column chromatography using a gradient system of $CHC1_3$–MeOH (1:0–0:1, v/v). TLC protosite reaction with ninhydrin reagent was used to detect cyclic peptide compounds as Ref. [15] described, the fractions eluted by $CHC1_3$–MeOH (9:1, v/v) presented purplish red spots, which indicated that these fractions contain cyclic peptide compounds. Then the fractions were combined and concentrated under reduced pressure. 2.3 g of crude extract was obtained, a portion of which was used for further HSCCC separation.

2.4. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (K) of the target components. The K values were determined by HPLC analyses as following [16,17]: 1 mg of crude sample was weighted in a 10 mL test tube to which 3 mL of each phase of the equilibrated two-phase solvent system was added. The test tube was caped and shaken vigorously for several minutes to thoroughly equilibrate the sample between two phases. Then, equal volume (100 μL) of the upper and lower phase was separately evaporated to dryness under a gentle stream of nitrogen. The residues were dissolved with methanol to 1 mL and analyzed by HPLC to determine K value of each component. The peak area of the upper phase was recorded as A_U (area of upper phase) and that of the lower phase was recorded as A_L (area of lower phase). The K value was calculated according to the following equation: $K = A_U/A_L$.

2.5. Preparation of two-phase solvent system and sample solution

The selected two-phase solvent system of petroleum ether–ethyl acetate–methanol–water was prepared by adding all the solvents to a separation funnel according to the volume ratios of 0.5:3.5:1:5 and thoroughly equilibrated by shaking repeatedly. Then, the two phases of the selected solvent system were separated shortly and degassed by sonication prior to use.

The sample solution for HSCCC separation was prepared by dissolving 190 mg of crude extract in the mixture of 3 mL of the upper phase and 3 mL of the lower phase of the solvent system used.

2.6. HSCCC separation

The multilayer-coiled column was first entirely filled with the upper phase as stationary phase. The lower aqueous phase was then pumped into the head end of the column at a suitable flow-rate of 1.5 mL/min while the apparatus was rotated at 800 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (190 mg of the crude extract in 6 mL of both phases) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and analyzed by HPLC. After the separation was completed, the stationary phase was pumped out of the column with pressurized nitrogen gas and collected into a graduated cylinder to measure the retention volume. The retention of stationary phase (S_f) was defined as $S_f = V_S/(V_M + V_S)$ (V_M and V_S are separately the phase volume of mobile and stationary phase).

2.7. HPLC analyses

The crude sample which was pre-purified by silica column and each purified fraction from the HSCCC separation were analyzed by HPLC on a Welch Materials C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) with the column temperature at 25 °C. The mobile phase, a solution of acetonitrile–0.1% HCOOH (30:70, v/v), was set at a flow-rate of 1.0 mL/min. All solvents were filtered through a 0.45 μm filter prior to use. The effluent was monitored at 280 nm by a DAD detector. The purity of the isolated constituent was determined based on the peak area normalized to all observed HPLC peak area.

2.8. HRESI-TOF-MS for identification

The MS instrument used to perform the study was an electrospray ionization quadrupole-time of flight tandem mass spectrometry (Agilent Technologies). The data recorded was processed with the accurate Masshunter Qualitative Analysis Software B02.00 ChemStation (Aglient, USA). Spectra were acquired over the *m*/*z* 100–2000 range at a scan rate of 1 s per spectrum.

2.9. NMR for identification

The ^{1}H NMR spectra of segetalin A and segetalin B in DMSO-d_6 were recorded on a Varian-600 NMR spectrometer at a frequency of 599.7. TMS was an internal standard. Chemical shifts are reported in ppm and coupling constants in Hz.

3. Results and discussion

3.1. Optimization of HPLC conditions

The optimum HPLC condition aimed at chromatogram with good resolution of adjacent peaks within a short analysis time, especially when numerous samples were to be analyzed. Several elution systems were tested in HPLC separation of crude sample, such as isocratic elution of methanol–0.1%HCOOH, acetonitrile–0.1%HCOOH, methanol–acetonitrile–0.1%HCOOH, etc. The results indicated that when acetonitrile–0.1%HCOOH (30:70, v/v) was used as mobile phase in isocratic elution mode, the major peaks were obtained and each peak achieved baseline separation. The HPLC chromatogram of pre-purified crude extract by silica column was shown in Fig. 2A, which contained several peaks, and peak I and II corresponded to segetalin B and segetalin A, respectively.

3.2. HSCCC separation of the crude extract from the seeds of V. segetalis

In a HSCCC separation, the selection of two-phase solvent system is the critical step, and a good solvent system can provide an ideal partition coefficient (*K*) for the target compounds. The key of solvent optimization is first to find a solvent combination in which the samples are freely soluble, and then, to adjust this solvent combination to ensure that the *K* values of the target compounds is close to 1 [18,19]. If the *K* value is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in the loss of peak resolution; if it is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [20].

According to the physicochemical properties of segetalin B and segetalin A [21] and the rule of selecting a two-phase solvent system in Ref. [11] described, we designed a series of two-phase solvent systems composed of petroleum ether, ethyl acetate, methanol and water by changing the volume ratios to achieve an efficient resolution of the target compounds. Table 1 shows the *K* values of the different solvent systems. It can be seen that petroleum ether–ethyl acetate–methanol–water with volume ratios of 1:3:1:5 and 0.5:3.5:1:5 were suitable for separation of compounds I and II. So these solvent systems were tested for HSCCC separation.

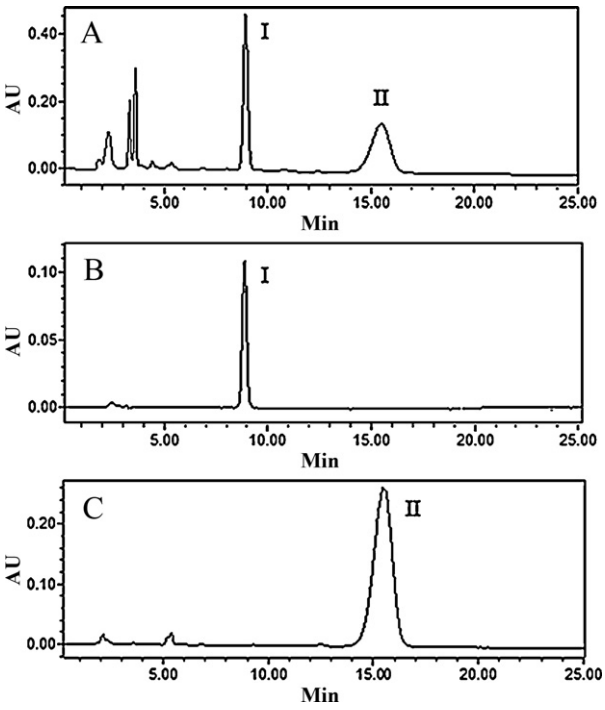

Fig. 2. HPLC chromatograms of the crude sample of cyclic peptides from *Vaccaria segetalis* and purified fractions from HSCCC separation. *Experimental conditions*: a Welch Materials C_{18} (250 mm × 4.6 mm, i.d., 5 μm); column temperature: 25 °C; mobile phase: acetonitrile–0.1% HCOOH (30:70, v/v); flow-rate: 1.0 mL/min; UV detection wavelength: 280 nm; injection volume: 10 μL. (A) Pre-purified crude sample; (B) peak I (segetalin B); (C) peak II (segetalin A).

Table 1
The *K* values of compound I (segetalin B) and compound II (segetalin A) in different two-phase solvent systems used in HSCCC.

Solvent system: petroleum ether–ethyl acetate–methanol–water	K_I	K_{II}
0:4:1:5	2.5	4.4
1:3:1:5	0.85	1.0
1:3:1.5:4.5	0.65	0.70
0.5:3.5:1:5	1.4	2.0

When petroleum ether–ethyl acetate–methanol–water (1:3.5:1:5, v/v) was used as two-phase solvent system, compound I and II were co-eluted and could not be separated well. When petroleum ether–ethyl acetate–methanol–water (0.5:3.5:1:5, v/v) was tested, good separation results could be obtained and the separation time was acceptable. As shown in Fig. 3, 190 mg of the crude sample was successfully separated by using the optimized solvent system of petroleum ether–ethyl acetate–methanol–water (0.5:3.5:1:5, v/v). The retention of the stationary phase was 65.0%, and the separation time was about 5 h in one separation run, and 38.0 mg of compound I and 28.5 mg of compound II were obtained from 190 mg of crude sample with the purity of 98.1% and 95.6%, respectively. The HPLC chromatograms of these pure compounds were also separately shown in Fig. 2B and C.

3.3. Identification of the isolated compounds

The structures of the HSCCC purified fractions were identified by HRESI-TOF-MS (operated in the positive ion mode in the range of *m*/*z* 100–2000) and ^{1}H NMR spectra with TMS as internal standard.

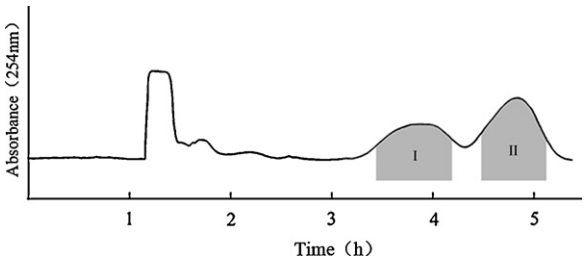

Fig. 3. HSCCC chromatogram of the crude sample of *Vaccaria segetalis*; solvent system: petroleum ether–ethyl acetate–methanol–water (0.5:3.5:1:5, v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 1.5 mL/min; revolution speed: 800 rpm; retention of stationary phase: 65.0%; sample size: 190 mg crude extract; detection at 280 nm; zone I: segetalin B, zone II: segetalin A.

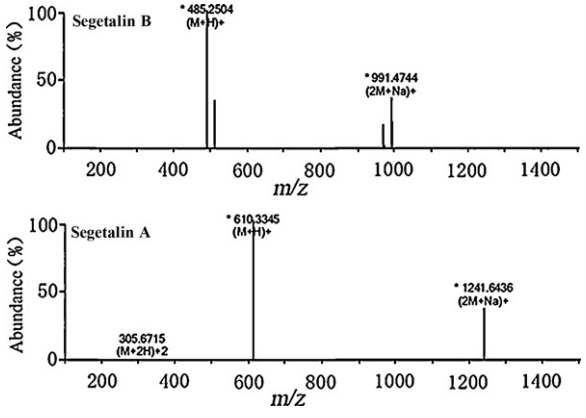

Fig. 4. HRESI-TOF-MS spectra of segetalin B and segetalin A $[M+H]^+$.

Segetalin B (peak I): Positive HRESI–MS, *m/z* 485.2504 $[M+H]^+$ (Fig. 4). ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 10.86 (1H, s), 8.44 (1H, brs),7.99 (3H, m), 7.75 (1H, d, J = 8.4 Hz), 7.54 (1H, d, J = 7.8 Hz), 7.33 (1H, d, J = 8.4 Hz), 7.12 (1H, d, J = 1.8 Hz), 7.06 (1H, t, J = 7.8 Hz), 6.97 (1H, t, J = 7.2 Hz), 4.22 (2H, m), 4.10 (2H, m), 3.92 (1H, dd, 6.9, 8.2), 3.30 (1H, m), 3.21 (2H, m), 1.99 (1H, m), 1.22 (3H, d, J = 8.4 Hz), 1.19 (3H, d, J = 7.2 Hz), 0.88 (3H, d, J = 7.2 Hz), 0.86 (3H, d, J = 6.0 Hz). After comparing the data with spectral information from literature [4,22], compound I was confirmed as segetalin B.

Segetalin A (peak II): Positive HRESI–MS, *m/z* 610.3348 $[M+H]^+$ (Fig. 4). ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 10.91 (1H, s), 8.97 (1H, d, J = 4.2 Hz), 8.52 (1H, d, J = 4.2 Hz), 7.55 (1H, d, J = 7.8 Hz), 7.43 (1H, t, J = 8.4 Hz), 7.32 (1H, d, J = 7.2 Hz), 7.32 (1H, d, J = 7.2 Hz), 7.14 (1H, s), 7.07 (1H, t, J = 7.8 Hz), 6.97 (1H, t, J = 7.2 Hz), 4.52 (1H, dd, J = 9.6, 4.8 Hz), 4.35(1H, d, J = 7.8 Hz), 4.26 (1H, t, J = 7.2 Hz), 4.13(1H, d, J = 9.6 Hz), 3.66–3.70 (2H, m), 3.58 (2H, m), 3.06–3.16 (3H, m), 2.11 (2H, m), 1.92 (2H, m), 1.67 (1H, m), 1.15 (3H, d, J = 6.0 Hz), 0.95 (3H, d, J = 5.4 Hz), 0.81 (3H, d, J = 7.2 Hz), 0.77 (3H, d, J = 6.0 Hz), 0.76 (3H, d, J = 6.0 Hz). After comparing the data with spectral information from literature [22,23], compound II was confirmed as segetalin A.

4. Conclusion

An efficient HSCCC method for separation and purification of two cyclic peptides, segetalin B and segetalin A, from the seeds of *V. segetalis* was developed by using petroleum ether–ethyl acetate–methanol–water (0.5:3.5:1:5, v/v) as the two-phase solvent system. Segetalin B (38.0 mg) at purity of 98.1% and segetalin A (28.5 mg) at purity of 95.6% could be obtained from 190 mg crude extract in a single run. The compounds obtained can be used as reference substances for chromatographic purposes as well as for further physiological studies.

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