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Full Length Article

Isolation of Herbicidal Substance, α-terthienyl, from the Root of *Flaveria bidentis* using HSCCC and HPLC

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Abstract

Flaveria bidentis (L.) Kuntze is a serious invasive plant with allelopathic properties in Hebei, China. To obtain the herbicidal substance from the root extracts of *F. bidentis* effectively and rapidly, the isolation and purification method was optimized by using high-speed counter-current chromatography (HSCCC). The results showed that the two-phase solvent system of n-hexane-ethyl acetate-methanol-water (6:4:5:5, v/v/v/v) could separate the root extracts and get three components. The results of herbicidal activity screening for the three components indicated that component 3 had the strongest herbicidal activity against *Digitaria sanguinalis* and *Arabidopsis thaliana*. Component 3 was further separated and purified using high-performance liquid chromatography (HPLC) for structural determination, nuclear magnetic resonance (NMR) and mass spectrometry (MS) information validated the herbicidal componient 3 as α-terthienyl. © 2016 Friends Science Publishers

Keywords: HSCCC; HPLC; *Flaveria bidentis* (L.) Kuntze; α-terthienyl; Herbicidal activity

Introduction

Flaveria bidentis (L.) Kuntze, belonging to the *Flaveria Juss*. (Asteraceae), is an important invasive plant in China (Wei *et al.*, 2011) and caused a serious impact on the native ecosystem and the safety of agricultural production (Cabrera and Juliani, 1976; Pereyra de Santiago and Juliani, 1972; Xie *et al.*, 2014). Previous studies have shown that flavonoids (Guglielmone *et al.*, 2002; 2005), patuletin-3-O-glucoside (Wei *et al.*, 2011), astragalin (Wei *et al.*, 2011), hyperoside (Xie *et al.*, 2014), and 6-methoxykaempferol-3-O-galactoside (Gidda and Varin, 2006) with pharmaceutical potency were the important secondary metabolites of *F. bidentis* (L.) Kuntze. (Furumoto *et al.*, 2007; Xie *et al.*, 2010). The extract of *F. bidentis* (L.) Kuntze has been reported to have an insecticidal, fungicidal, and antiviral activity too (Guglielmone *et al.*, 2005). The purity of the active ingredient in plants is the basis for further study on the activity and development of its products. Therefore, it is significant to develop an economic method for separating and purifying ingredients.

High-speed counter-current chromatography (HSCCC) (Chen *et al.*, 2014; Shi *et al.*, 2009) is a valuable chromatographic technique by eliminating the irreversible adsorption of the sample without the need of solid support. This method has been widely applied in the separation and preparation of natural products (Assimopoulou *et al.*, 2009; Zhou *et al.*, 2005; Liang *et al.*, 2015). HSCCC by time-controlled collection method has been successfully used in isolation and purification of the α-terthienyl from *F. bidentis* (L.) Kuntze. The two-phase solvent system in the HSCCC chose n–hexane and acetonitrile (1:1,v/v) as solvent (Wei *et al.*, 2012). Previously, we have not obtained the effective components using the aforementioned method to isolate the root extracts of *F. bidentis* (L.) Kuntze because the different organs of *F. bidentis* (L.) Kuntze have different types of secondary metabolites.

Our previous studies have reported that the extraction of several organic solvents from *F. bidentis* (L.) Kuntze has strong herbicidal activity (Xu *et al.*, 2008). In order to obtain the herbicidal substance from the root of *F. bidentis* effectively and rapidly, the root extracts of *F. bidentis* were first isolated using HSCCC. The main active fraction was then further separated and purified by HPLC. The herbicidal compound achieved a rapid and effective separation and purification. A high purity of α-terthienyl was obtained by combining the advantages of the two kinds of chromatography methods. Simultaneously, this method will provide a reference in separation and purification of the active ingredient in other plants.

Materials and Methods

Reagents

All organic solvents utilized for HSCCC were analytically pure and supplied by Tianjin Fuchen Chemical Factory

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(Tianjin, China). The methanol used for the HPLC analysis was chromatographically pure and supplied by Beijing Dikma Technology Co., Ltd. (Beijing, China).

Plant Material

Flaveria bidentis (L.) Kuntze plants were harvested from the suburbs of Baoding in September, 2013. The weeds, *Digitaria sanguinalis* and *Arabidopsis thaliana*, were germinated and grown in pot with vermiculite under the controlled conditions at 25°C with 3800 lx of light and 70% of RH. *D. sanguinalis* was treated with extracts at the 2- to 3-leaf stage, while *A. thaliana* at the 4- to 6-leaf stage.

Preparation of the Root Extracts from *F. bidentis*

100 g of the powdered fibrous root was extracted with 500 mL of methanol for 24 h at room temperature, followed by the extraction of the fibrous root using a microwave extractor for 30 min, and then filtered. The filtrate was dehydrated with anhydrous sodium sulfate, and then evaporated under reduced pressure at 50°C to remove the solvent. The final crude sample of 9.80 g was obtained for further isolated by HSCCC.

Preparation of the Two-phase Solvent System

Based on the chromatographic theory, the necessary condition for HSCCC separation is that the sample should have an appropriate partition coefficient ($0.5 < K < 2.0$) between two phases. Partition coefficient can be measured by HPLC, electrochemistry (EC), and thin layer chromatography (TLC) methods and so on (Ito, 2005). Here we chose the HPLC method. According to the screening method of the solvent system created by Ito, the samples were mainly distributed in the non-aqueous phase in the chloroform system. In this study, five two-phase solvent systems and they composed of four same solvents with different ratio were chosen. The four solvents were n-hexane–ethyl acetate–methanol–water with mixture ratios of 5:5:5:5, 6:4:5:5, 7:3:5:5, 8:2:5:5 and 9:1:5:5 by volume, respectively. The solvents were mixed and equilibrated in a separating funnel. When the mixture was thorough equilibrated, the two phases were separated and degassed for further application.

Preparation of the Sample Solutions

The root extracts (50 mg) were dissolved in the mixture of two phases (5 mL of each phase) and the sample solution was ready for further isolation and purification.

Measurement of Partition Coefficients

The root extracts (5 mg) were dissolved in pre-equilibrated two-phase solvent system (3 mL of each phase) in a beaker (10 mL). After the sample solution thorough equilibrated, 2 mL of each phase was separated and removed the solvents. Dissolving the residue in methanol (1 mL) and analyzing it with HPLC. The peak area of each component in the upper phase divided by that in the lower phase could defined as the partition coefficient (K) value of the component.

Separation Procedure of HSCCC

HSCCC (Shanghai Tauto Biotechnology Co., Ltd., Shanghai, China) was equipped with a TBE-300A centrifuge, which consisted of a polytetrafluoroethylene multilayer coil (total volume, 300 mL), a TBP-5002 pump was used to pump the solvent into the column, and a TBD-2000 UV detector was used for monitoring the effluent. The sample was introduced into the column by an injection valve with a loop of 20 mL.

Initially, the multilayer coiled column was complete full of the upper phase as stationary phase, then the apparatus began to rotate. Pumping the lower phase into the column at a flow rate of 2 mL/min when the rate of apparatus reached to 900 r/min. After the system reaching to the hydrodynamic equilibrium, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a TBD-2000 UV detector at 214 nm. According to the chromatogram, each peak fraction was collected and removed the solvents under reduced pressure. The concentrate was stored at 0°C for the herbicidal activity determination.

Herbicidal Bioassay

The effect of herbicidal active substances on inhibiting the growth of *D. sanguinalis* and *A. thaliana* was determined based on the foliar treatment method. Herbicidal active substances were sprayed on the leaves of *D. sanguinalis* and *A. thaliana*, and each treatment was repeated by three times. The symptoms were then monitored at 24 h post-treatment and compared with those of the untreated control. The grading standard about the inhibitional effect was shown in Table 1.

HPLC Analyses and Structural Identification of the Compounds

The system of HPLC was Agilent 1200 (PaloAlto, CA, USA), which consisted of an autosampler, degasser, binary pump, thermostated column compartment and ultraviolet detector.

HPLC analyses were carried out with Zorbax Eclipse XDB-C18 column (155 × 4.0 mm, 5-μm inner diameter) with the flow rate of 1 mL/min, the detection wavelength of 340 nm and methanol–water (90:10, v/v) as the mobile phase, the injection volume was 20 μL.

MS, ^{1}H–nuclear magnetic resonance spectra, and CHNSO elemental analyzer (EA) (Vario EL cube) were used to identify the structure of this herbicidal substance. The Bruker AV500 NMR was used for measuring the NMR spectra, and the internal standard was tetramethylsilane (TMS).

Results

HPLC Analyses of the Root Extracts

The root extracts were dissolved in methanol and detected by HPLC. The results showed that they mainly included four peaks, which were numbered from I to IV. The retention times were 1.534, 3.148, 4.509 and 5.559 min, respectively (Fig. 1).

Determination of the Optimal Solvent System for HSCCC

The proper retention which is required to be greater than 40% and the suitable partition coefficient, which should be between 0.5 and 2 are of great importance for the successful separation process in HSCCC. The K value of the four substances in the root extracts was measured. By comparison, the K value of the four substances was closer to the suitable K value range (Table 2) in the solvent system of n-hexane–ethyl acetate–methanol–water (6:4:5:5, v/v/v/v). Thus, it was the optimal solvent system and could be used for the following separation.

Separation Results of HSCCC

Using the optimal solvent system, the sample solutions were separated into three peaks in the chromatogram. The three peaks were numbered sequentially as 1, 2 and 3 (Fig. 2). The effluents of the three peaks were concentrated, and the herbicidal activity of the concentrates was determined. The results showed that peak 3 had a strong herbicidal activity, and the inhibition rates on the growth of crabgrass and Arabidopsis were more than 95% and 90%, respectively. Therefore, the purity of peak 3 was detected by HPLC.

HPLC Analyses of the Purity of Peak 3

The purity of peak 3 was analyzed using HPLC and the results showed that it mainly included two components: components i and ii, with retention times of 4.069 and 4.968 min, respectively (Fig. 3).

A partition coefficient varying from 0.5 to 2.0 is necessary in using the HSCCC. The resolution pertaining to the ratio of the partition coefficient is extremely important because it determines whether the two substances could be baseline separated. Often, when the resolution was 1.5 or greater, the baseline separation of the two substances could be achieved. When the resolution was less than 1.5, a certain degree of overlap occurring between the peaks of the two substances, which could not be baseline separated. Peak 3 is the mixture of component I and component II, because the resolution was less than 1.5.

The Results of Herbicidal Bioassay

The herbicidal activity of component I and component II was determined. The results showed that component II had a higher herbicidal activity (Fig. 4). The growth of control weeds treated with methanol was not suppressed, but that treated with compound ii was significantly inhibited. The levels of the inhibitional effect on *D. sanguinalis* and *A. thaliana* were 5 degree and 4 degree, respectively. Component II was prepared using HPLC and detected at different chromatographic conditions (methanol: water (70:30, v/v) was the mobile phase, detection wavelength was 365 nm, and flow rate was 0.5 mL/min). The results showed that there was only a single peak with the retention time of 6.121 min, which indicating that compound ii was a single compound.

Table 1: Grading standard about the inhibitional effect through foliar treatment

Degree	Inhibition rate of growth (%)
0	The same as control
1	＜25
2	25～50
3	50～75
4	75～95
5	＞95

Table 2: Partition coefficient (K) of the compounds in different solvent systems

Solvent systems	Partition coefficient (K)			
n–hexane–ethyl acetate–methanol–water (v/v/v/v)	I	II	III	IV
5:5:5:5	0	2.02	17.12	13.82
6:4:5:5	0.01	1.04	2.32	1.84
7:3:5:5	0.02	0.91	7.99	3.20
8:2:5:5	0.01	1.16	17.65	5.42
9:1:5:5	0.01	0.66	3.18	1.17

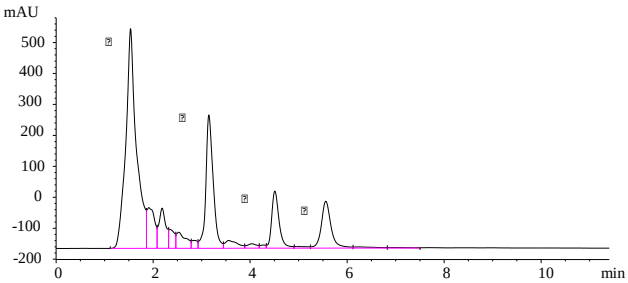

Fig. 1: High-performance liquid chromatogram of the root extracts. The mobile phase was methanol–water (90:10), and the detection wavelength was 214 nm

Structural Identification of Compound II

MS, ^{1}H-NMR, and EA were used to identify compound II. MS m/z: 246.949, ^{1}H-NMR ($CDCl_3$): δ 7.22 (dd, J = 5.1, 1.1 Hz, ^{1}H), 7.18 (dd, J = 3.6, 1.2 Hz, ^{1}H), 7.08 (s, ^{1}H), 7.04–7.00 (m, ^{1}H). The elemental analysis indicated that the content of C, H, N, and S in compound ii was 58.165%, 3.355%, 0.145% and 37.280%, respectively. The atomic number of C, H, N and S in compound ii was 11.97, 8.29, 0.026 and 2.88, respectively. The element N could be

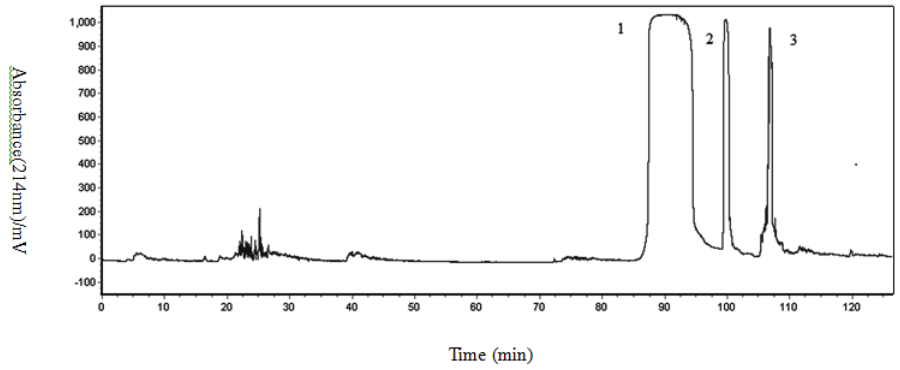

Fig. 2: Separation result of HSCCC

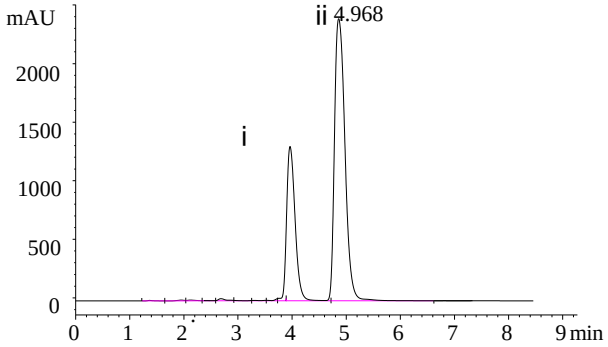

Fig. 3: High-performance liquid chromatogram of peak 3. The mobile phase was methanol–water (90: 10), and the detection wavelength was 340 nm

Fig. 4: Herbicidal activity of compound II. Control, weeds treated with methanol. Treatment, weeds treated with 100 mg/L of compound II

ignored. Therefore, the molecular formula of compound ii was $C_{12}H_8S_3$. Compound ii was determined as α-terthienyl in accordance with the above date.

Discussion

It requires a complex series of separation methods to obtain the high purity active compounds from natural products. Especially the products generally present a narrow range of polarity and physicochemical properties, thereby the difficulty of separating is increasing (Do *et al.*, 2014). Now, the methods for isolation of natural product include HPLC, HSCCC, planar chromatography, supercritical fluid extraction, pressurized liquid extraction and crystallization (Cannell, 1998), and HPLC (preparative and semipreparative) is the most widely used. The combination of the above separation methods is currently the most commonly used method for separating natural products. The highly polar antioxidants were firstly isolated and purified from Chirita longgangensis by combination of macroporous resin and HSCCC (Duan *et al.*, 2014). Phosphodiesterase inhibitors were separated from Eucommia ulmoides bark by combination of preparative HPLC and HSCCC (Shi *et al.*, 2013). In this study, we obtained the high-purity α-terthienyl by combining the HPLC and HSCCC.

The insecticidal and antibacterial activity of α-terthienyl had been reported (Guillet *et al.*, 2000) and the photoactivated toxicity of α-terthienyl to insects, nematodes, microorganisms, human blood cells, plants and other living organisms and organizations posed a great concern to the people (Dosdall *et al.*, 1992; Diamond *et al.*, 2006). A report had indicated that α-terthienyl had the allelopathic properties with potency as a natural weed-control agent (Glen *et al.*, 1982). Our research reported that the α-terthienyl from the root of *F. bidentis* (L.) Kuntze has a strong herbicidal activity against *D. sanguinalis* and *A. thaliana*. This study revealed that the inhibitory effect of α-terthienyl on the growth of weeds was stronger whereas that in the high temperature and strong light. The above phenomenon may be due to the photoactivated toxicity of α-terthienyl. Moreover, the herbicidal mechanism requires further study. The discovery of α-terthienyl with herbicidal activity will lead to the development of a new plant herbicide.

Conclusion

This study is the first to report on the isolation of an herbicidal substance in the *F. bidentis* (L.) Kuntze by combination of HSCCC and HPLC. The root extracts were separated into three components by HSCCC, and the two-phase solvent system was n-hexane–ethyl acetate–methanol–water (6:4:5:5, v/v/v/v). The component with herbicidal activity was then further separated and purified by using HLPC. Finally, the strongest herbicidal substance was obtained and identified as α-terthienyl. The α-terthienyl had a strong inhibition on the growth of *D. sanguinalis* and *A. thaliana*. Simple, rapid and solvent saving and with a good practical value, this isolation method will provide reference for the separation and purification of the active ingredient in other plants, and the herbicidal active substance α-terthienyl will lay a foundation for the development of new botanical herbicides.

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