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ISOLATION AND PURIFICATION OF PSORALEN AND BERGAPTEN FROM *FICUS CARICA* L LEAVES BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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Abstract

High-speed countercurrent chromatography was successfully applied for the first time for the separation of psoralen and bergapten from *Ficus carica* L leaves. The crude extract obtained by light petroleum (b.p.: 60°C–90°C) from the dried leaves of *Ficus carica* L. was separated with a two-phase solvent system of *n*-Hexane-ethyl acetate-methanol-water (1:1:1:1, v/v). Each peak fraction was analyzed by high-performance liquid chromatography. The method yielded 4.4 mg of psoralen at 99.1% purity and 2.1 mg of bergapten at 98.2% purity from 400 mg of the crude extract in a single run. The two compounds were identified by ¹H-NMR, ¹³C-NMR and MS.

Keywords

High-speed countercurrent chromatography; *Ficus carica* L leaves; psoralen; bergapten

INTRODUCTION

Ficus carica L, one of the oldest plant species in China, is abundant in Shandong, Xinjiang, Jiangsu province and so on. Its fruits are delicious and can be eaten by human. Its leaves are commonly used to cure hemorrhoid and clear away heart ache (Chinese Medical Dictionary, 1986). Furthermore, it has also been shown to possess anti-hypertension and anti-cancer effects (Yin et al. 1997 and 1998). Psoralen and bergapten are two of major active components of *Ficus carica* L leaves. The chemical structures of these two compounds are shown in Fig. 1. Psoralen has anti-tumor, anti-bacterial and anti-viral activities while bergapten is used to cure vitiligo, psoriasis, and alopecia areata (Chinese Medical Dictionary, 1986).

The preparative separation of psoralen and bergapten from *Ficus carica* L leaves by classical methods is tedious and time-consuming, requiring multiple chromatographic steps on silica

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gel column chromatography (Yang et al. 2002). So, an efficient method for the preparative isolation and purification of psoralen and bergapten from *Ficus carica* L leaves is needed.

High-speed countercurrent chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support and has an excellent sample recovery (Ito, 2003). It has been successfully applied to isolate and purify a number of natural products.

The present paper describes an HSCCC method for separation of psoralen and bergapten from a light petroleum crude extract of *Ficus carica* L leaves. The optimum conditions were obtained, which led to the successful separation of psoralen and bergapten with the purity of each at over 98%. The identifications of psoralen and bergapten were performed with ^1H -NMR, ^{13}C -NMR and MS.

EXPERIMENTAL

Materials

The dried *Ficus carica* L leaves were provided by Henan Hengxiang Inc., Xiangcheng city, Henan province.

Preparation of Crude Extract Sample

The dried *Ficus carica* L leaves were ground to powder (about 30 mesh). The powder (400 g) was marinated with 2000 ml light petroleum (b.p.: 60°C-90°C) for 48 h at room temperature. After filtration, the extract was evaporated to paste by rotary vaporization at 45°C under reduced pressure. About 6 g of the crude extract was obtained and stored in a refrigerator at 4°C for the subsequent HSCCC separation.

Selection of the Two-Phase Solvent System

The solvent system was selected according to the partition coefficients (K) of the target compounds. The K values were determined by HPLC as follows: add a small amount of the sample (typically a few milligrams or less depending on its extinction coefficient or absorptivity) to the two mutually equilibrated solvent phases (2 ml each) in a test tube. Thoroughly mix with a vortex to equilibrate the contents. After settling, pipette and deliver an equal volume of each phase into a separate test tube, and dilute with an equal volume of methanol. Analyze each phase by HPLC to determine the peak area of the target compound. The partition coefficient (K) is expressed as the solute absorbance in the upper phase divided by that of the lower phase, or $K = A_{\text{upper}}/A_{\text{lower}}$.

Isolation and Purification of Psoralen and Bergapten with HSCCC

A preparative HSCCC instrument (Model CCC-1000, Pharma-Tech Research Corp., Baltimore, Maryland, USA) was used for the separation of *Ficus carica* L leaves extract. It holds three multiplayer coil separation columns connected in series (diameter of tube: 2.6 mm and total volume: 325 ml) and a 10 ml sample loop. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (R) was 5 cm, and the β -value from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1600 rpm. The system was also equipped with a constant flow pump (Pharma-Tech Research Corp.), a model SPD-10A UV monitor (SHIMADZU, Kyoto, Japan). The data were collected with a model EC-2000 chromatography workstation (Dalian Yilite Apparatus CO. Ltd., Dalian, China). A two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:1:1:1 v/v) was applied for the HSCCC separation with the upper phase as the stationary phase and the lower phase as the mobile phase.

In each separation, the separation coil tube was first filled with the stationary phase at the rate of 6 ml/min, then the mobile phase was pumped into the head end of the column at the rate of 2 ml/min, while the HSCCC apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet. 400 mg of the *Ficus carica* L leaves crude extract in 5ml of upper phase and 5 ml of lower phase, was injected into the separation column through the sample loop. Afterward the mobile phase was delivered into the column to elute the component. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm, and collected by an ADVANTEC SF-3120 model fraction collector, collecting 4 mL of each fraction. Each peak fraction was collected according to the chromatogram and evaporated with N₂ gas. The residue was sealed and stored in a refrigerator at 4°C for the subsequent HPLC analysis and identification by MS and NMR.

HPLC Analysis

The HPLC equipment used in the study was Waters Alliance series HPLC system including a Waters 2695 Separations Module with an automated injector, a Waters 2996 photodiode array detector, a Waters HPLC workstation and an Agilent reverse C₁₈ column (150 mm×4.6 mm id, 10 μm). The crude extract of *Ficus carica* L leaves and each HSCCC peak fraction were performed at 30°C. eluting with acetonitrile- water (30:70 v/v) and the flow rate was 1.0ml/min. The effluent was monitored at 254 nm.

EIMS and NMR

EIMS experiments were performed on a ZAB-HS mass spectrometer (Micromass Inc., UK) analyzing ions up to m/z 220. ¹H- and ¹³C-NMR spectra were recorded in CDCl₃ on a Bruker ARX-400 (Bruker Inc., Switzerland) with 400MHz for ¹H- and 100.5MHz for ¹³C- measurements, respectively.

RESULTS AND DISCUSSION

Optimization of HSCCC Conditions

As shown in Fig. 2, HPLC analysis of the crude extract from *Ficus carica* L leaves contained two major compounds where the purity of psoralen (peak A) and bergapten (peak B) in crude extract is 69.3% and 30.7%, respectively, based on HPLC peak area percentage. In order to achieve excellent separation of these two compounds by HSCCC, two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water was tested at various volume ratios to optimize K values: A volume ratio at 1:1:1.2:0.8, the K_{psoralen} was 0.56 and the K_{bergapten} was 0.67 which gave the separation time too short to produce the good resolution between the two peaks. The stationary phase retention at flow rate of 2 mL/min was 74% (Fig. 3a). When the volume ratio of 1:1:1:1 was used, the K_{psoralen} was 1.33 and the K_{bergapten} was 1.67 which gave a longer separation time, but yielded much better peak resolution. The stationary phase retention at flow rate of 2 mL/min was 70% (Fig. 3b). The crude extracts from *Ficus carica* L leaves were separated and purified under the above optimum HSCCC conditions. Psoralen was obtained at 180 min to 230 min, while bergapten was obtained at 240 min to 270 min (Fig. 3b). The purity of psoralen and bergapten was determined by HPLC.

HPLC Analysis and Identification of HSCCC Peak Fractions

The purity of each peak fraction of HSCCC separation was determined by HPLC. The HPLC chromatogram of each fraction was shown in Fig. 4 (a and b). The purity of psoralen (peak A in Fig. 4a) and bergapten (peak B in Fig. 4b) was 99.1% and 98.2%, respectively, based on HPLC peak area percentage.

The structural identification of psoralen and bergapten was carried out by MS, ^1H -NMR and ^{13}C -NMR Spectra as follows: EIMS m/z 186,158,130, 102,87,75,51,32. It showed the molecular ion at m/z 186, which is in agreement with the molecular formula $\text{C}_{11}\text{H}_6\text{O}_3$ of psoralen (Kim, 2006). And the EIMS: m/z 216,201,188,173,145,108,89,74. It showed the molecular ion at m/z 216, which is in agreement with the molecular formula $\text{C}_{12}\text{H}_8\text{O}_4$ of bergapten (Liu et al., 2004).

Psoralen: ^1H -NMR (400MHz, CDCl_3) δ (ppm): 6.38(3C-H), 7.81 (4C-H), 7.67 (5C-H), 7.47 (8C-H), 7.70 (2'C-H), 6.84 (3'C-H); Psoralen: ^{13}C -NMR, (100.5MHz, CDCl_3) δ (ppm): 161.0 (2-C), 114.6 (3-C), 144.0 (4-C), 119.8 (5-C), 124.8 (6-C), 156.4 (7-C), 99.8 (8-C), 152.0 (9-C), 115.4 (10-C), 146.9 (2'-C), 106.3 (3'-C), the results were similar to those in reference [6].

Bergapten: ^1H -NMR (400MHz, CDCl_3) δ (ppm): 6.27 (3C-H), 8.15 (4C-H), 7.13 (8C-H), 7.59 (2'C-H), 7.02 (3'C-H), 4.27 (OCH_3 -H); Bergapten: ^{13}C -NMR, (100.5MHz, CDCl_3) δ (ppm): 161.2 (2-C), 112.5 (3-C), 139.2 (4-C), 149.5 (5-C), 112.6 (6-C), 158.3 (7-C), 93.8 (8-C), 152.7 (9-C), 106.4 (10-C), 144.7 (2'-C), 105.0 (3'-C), 60.0 (5 OCH_3 -C), the results were similar to those in reference [7].

In conclusion, HSCCC was successfully used for the isolation and purification of psoralen and bergapten from *Ficus carica* L leaves. 4.4 mg of psoralen at 99.1% purity and 2.1 mg of bergapten at 98.2% purity can be obtained from 400 mg of the crude extract in one step separation.

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(1) Psoralen

Fig.1.
Structures of psoralen and bergapten

Fig.2. HPLC analysis chromatogram of crude extract with light petroleum from *Ficus Carica* L leaves

HPLC Conditions: column: Agilent reverse C₁₈ column (150 × 4.6 mm id, 10 μm); column temperature: 30°C; mobile phase: acetonitrile-water (30:70 v/v); flow rate: 1.0 ml/min; detection wavelength: 254 nm.

Fig.3. Chromatogram of the crude extract from *Ficus Carica L* leaves by HSCCC

Two-phase solvent system: *n*-hexane-ethyl acetate-methanol-water at 1:1:1.2:0.8 (Fig.3 a) and 1:1:1:1 (Fig.3 b); stationary phase: upper phase; mobile phase: lower phase; elution mode: head to tail; the flow rate of mobile phase: 2 ml/min; revolution speed: 800 rpm; retention of the stationary phase: about 74% (*n*-hexane-ethyl acetate-methanol-water at 1:1:1.2:0.8) and 70% (*n*-hexane-ethyl acetate-methanol-water at 1:1:1:1); detection wavelength: 254 nm; sample size: 400 mg crude extract sample dissolved in 5 ml of upper phase and 5 ml of lower phase; separation temperature: room temperature.

Fig.4. HPLC analysis of the psoralen and bergapten separated and purified by HSCCC
HPLC Conditions: column: Agilent reverse C₁₈ column (150 × 4.6 mm id, 10 μm); column temperature: 30°C; mobile phase: acetonitrile-water (30:70 v/v); flow rate: 1.0 ml/min; detection wavelength: 254 nm.