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Short communication

Isolation of high purity 1-[2′,4′-dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone from *Acronychia pedunculata* (L.) Miq. by high-speed counter-current chromatography

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Abstract

Following an initial clean-up step on silica, high-speed counter-current chromatography (HSCCC) was used to purify an aryl ketone, 1-[2′,4′-dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone from an extract of the stem bark of the shrub *Acronychia pedunculata*. The two-phase solvent system used was composed of *n*-heptane–ethyl acetate–methanol–water at an optimized volume ratio of 4:1:4:1 (v/v/v/v). Target compound (58.1 mg) with a purity of 98.9% was obtained after HSCCC of 183.5 mg sample with a purity of 35.7% recovered after the silica clean-up step. Identification of the target compound was performed by ^{1}H NMR, ^{13}C NMR, two-dimensional NMR and LC–electrospray ionization MS.

Keywords: *Acronychia pedunculata*; Counter-current chromatography; Pharmaceutical analysis; Dihydroxydimethylbutenylmethoxyl phenylethanone

1. Introduction

Acronychia pedunculata (L.) Miq. is a small shrub widely distributed in Indo-Malayan and Southern China. The roots, stems, leaves, and fruits of this plant have been used in folk medicine for the treatment of diarrhoea, tussis, asthma, ulcers, itchy skin, scales, pain, and rheumatism, and as an antipyretic and antihemorrhagic agent as well as an aphrodisiac [1]. 1-[2′,4′-Dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone (Fig. 1) is one of the major ingredients of *Acronychia pedunculata* solvent extracts. This compound was recently shown to have significant cycloxygenase-2 (COX-2) inhibitory activity [2].

The separation and purification of the target compound using conventional methods such as column liquid chromatography requires several steps resulting in lower recovery [3,4].

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of sample components that is often the case with solid supports [5]. The high recovery and the high efficiency, up to 50,000 theoretical plates has been achieved, is why in recent years this method has been met with increasing popularity for the preparative separation of natural products.

This paper describes for the first time the purification to 98.9% purity of a COX-2 inhibitor from an *Acronychia pedunculata* extract by HSCCC with a recovery of more than 98%.

2. Experimental

2.1. Apparatus

ÄKTAbasic system (Amersham Biosciences, Uppsala, Sweden) composed of a P-900 pump, a UV-900 detector,

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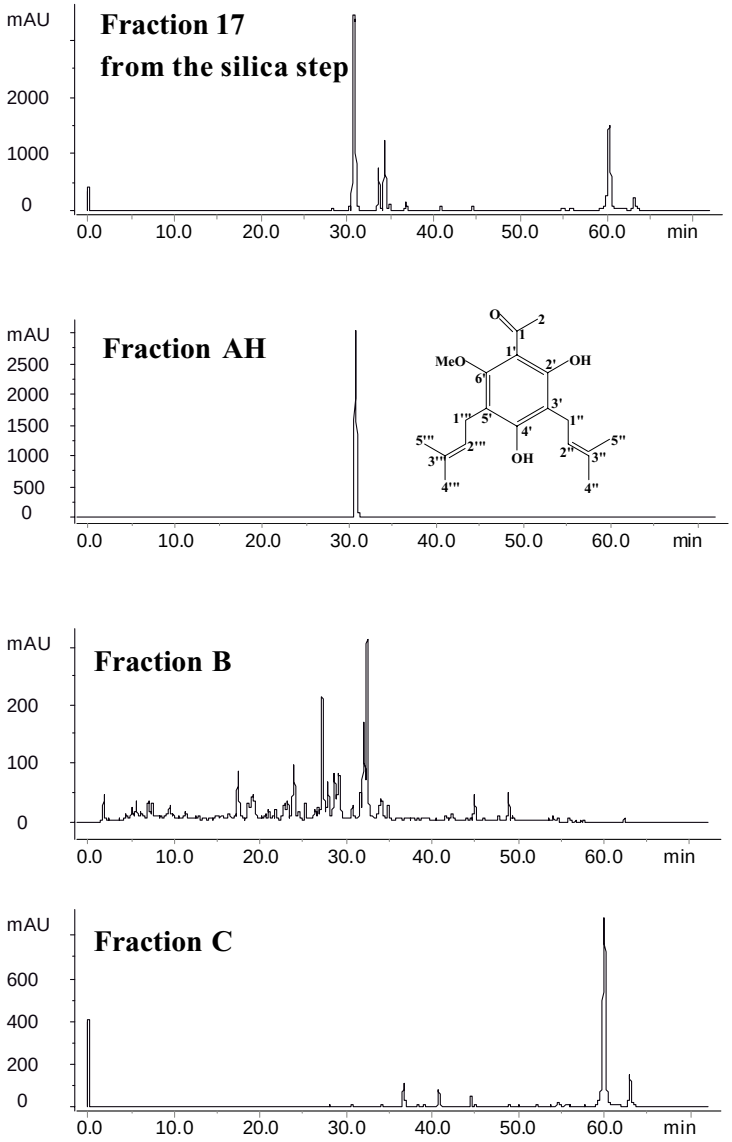

Fig. 1. HPLC analysis of fraction 17 from the silica clean-up step and of HSCCC fractions AH, B, and C (the column residue). Column: 250 mm × 4.6 mm i.d. Sephasil C_{18} 5 μm ST; column temperature: room temperature; mobile phase: ACN gradient 50–100% in distilled water was applied for 52 min followed by another 20 min elution with 100% ACN; flow-rate: 1.0 ml/min; UV wavelength: 217 nm.

a Frac-900 fraction collector, an INV-907 injection valve, two PV-908 valves, a M-925 mixer, a 50 ml Superloop, and a Unicorn 4.10 work station, was connected to a HSCCC apparatus and to an analytical HPLC column in parallel.

A HSCCC-TBE300A apparatus from Tauto Biotech., Shenzhen, China, was connected to the ÄKTAbasic system via an EIC-900 box (Amersham Biosciences). The HSCCC apparatus is based on the coil planet centrifuge principle and is equipped with three PTFE multiple layer coils, each 50 m in length and 1.6 mm in i.d. with a total capacity of 320 ml. Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 1000 rpm, the optimum speed of 800 rpm was used invariably in the present study.

LC–electrospray ionization (ESI) MS analysis was done by coupling an ÄKTAbasic system to a Finnigan LCQ Ion-trap MS (La Jolla, USA) in negative ionization mode.

^{1}H, ^{13}C, DQF-COSY and HETRES spectra were recorded using a JNM-EX400 NMR spectrometer (JEOL, Japan) using $C^{2}HCl_3$ as the solvent.

2.2. Reagents

Acronychia pedunculata (L.) Miq., stem bark was collected in March 2000 at Gampaha, Sri Lanka and was identified by the botanist, Professor B.A. Abeywickrama, of the Department of Botany, University of Colombo. Voucher specimen is kept in the Department of Chemistry, University of Colombo, Sri Lanka.

All organic solvents used for HSCCC were analytical grade and acetonitrile (ACN) used for HPLC, LC–MS was gradient grade.

2.3. Preparation of sample solution

Acronychia pedunculata stem bark powder (400 g) was extracted for 24 h in a Soxhlet apparatus using dichloromethane. Rotatory evaporation concentration of the extract gave 34.6 g dry powder, 8.5 g of which was chromatographed on a 500 mm × 40 mm (total bed volume 500 ml) silica column (230–400 mesh, Merck, Darmstadt, Germany). Before sample application the powder was dissolved in 32 ml dichloromethane, and mixed with 16 g dry silica. The mixture was evaporated to dryness in a fume cupboard at room temperature, suspended in 40 ml *n*-hexane–ethyl acetate (9:1) and applied on the top of the silica column equilibrated in the same solvent mixture. The sample was eluted successively with 500 ml of each of *n*-hexane–ethyl acetate mixtures (9:1), (8:2) and (7:3). Thirty fractions of 50 ml were collected during the entire chromatography. All fractions were analyzed by HPLC. Fractions 17–19 contained the target compound and were evaporated separately to dryness under reduced pressure. Sample solutions for HSCCC were prepared by dissolving the dry powders in the lower phase solvent to a concentration of 10 mg/ml.

2.4. Preparation of the two-phase solvent systems

Different proportions of *n*-heptane, ethyl acetate, methanol and water were mixed and thoroughly equilibrated in a separatory funnel at room temperature. The two phases were separated shortly before use.

2.5. Separation procedure

HSCCC was performed as follows. The multiple layer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the inlet column at a flow-rate of 2.0 ml/min, while the apparatus was run at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, 20 ml sample solution (10 mg/ml) was injected using a Superloop (Amersham Biosciences). The effluent from the tail end of the column was continuously monitored with UV detection at 217, 254 and 335 nm, respectively. Fractions of 5 ml were collected during the whole chromatography. After all desired peaks were eluted, the rotation and elution were stopped. The remaining column contents were emptied into a graduated cylinder using N_2 pressured at approximately 0.5 MPa. The retention of the stationary phase relative to the total column volume was calculated from the volume of the stationary phase collected from the column.

2.6. HPLC analysis and identification of 1-[2′,4′-dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone

The sample obtained after the silica clean-up step and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a 250 mm × 4.6 mm i.d. Sephasil C_{18} 5 μm ST column (Amersham Biosciences) at room temperature. An ACN gradient 50–100% in distilled water was applied for 52 min followed by another 20 min elution with 100% ACN. The flow-rate was 1.0 ml/min and the effluent was continuously monitored at 217, 254 and 335 nm, respectively. The concentration of the target compound was estimated by the peak area percent at 217 nm and was used as a reference in the crude sample analysis by the external standard curve method (ESCM).

For the LC–MS experiments, an Xterra MS C_{18} 100 mm × 2.1 mm, 3.5 μm column (Waters, USA) was used with the same gradient conditions as described above. Samples (10 μg) were injected and the flow rate was 0.2 ml/min.

The final identification of the target compound was carried out by LC–MS, ^{1}H NMR, ^{13}C NMR and two-dimensional (2D)-NMR (HETRES, DQF-COSY) spectra.

3. Results and discussion

A series of experiments were performed to determine the optimal solvent two-phase system for the HSCCC separation. The following systems were tested: *n*-heptane–dichloromethane–acetonitrile at volume ratios 10:3:7 and 10:1:9; *n*-heptane–ethyl acetate–methanol–water at volume ratios 5:4:5:4, 5:1:5:1, 10:1:10:1, 10:3:10:3 and 4:1:4:1. Among those the *n*-heptane–ethyl acetate–methanol–water system with the volume ratio 4:1:4:1 gave the best separation of the target compound. Three peak fractions denominated A, B, and C (the residue in the column) were recovered (Fig. 2).

The sample from the silica clean-up step and all three HSCCC fractions were analyzed by HPLC (Fig. 1). Fraction 17 from the silica clean-up step contained the target compound in a purity of 35.7% as estimated by ESCM. Peak fraction A was collected between 50–94 and included tube fractions 67–77 (AH) that were pooled separately. After evaporation to dryness fraction AH gave 58.1 mg target compound with 98.9% purity (as estimated by the peak area percentage). The combined side fractions 50–66 and 78–94 contained 9.2 mg target compound with a purity of 73.8% as estimated by ESCM.

The following ESI–MS, ^{1}H NMR, ^{13}C NMR and 2D-NMR data was used for the structural identification.

- LC–ESI-MS: *m*/*z* 317.16 $[M - H]^-$.
- ^{1}H NMR: (400 MHz, C^2HCl_3, ppm) δ 1.74 (3H, q, J = 1.2 Hz, CH_3-4″), 1.75, (3H, q, J = 1.2 Hz, CH_3-4‴), 1.81 (6H, q, J = 1.2 Hz, CH_3-5″, CH_3-5‴), 2.68 (3H, CH_3CO-2), 3.34 (2H, dq, J = 7.2, 1.2 Hz, CH_2-1″), 3.37

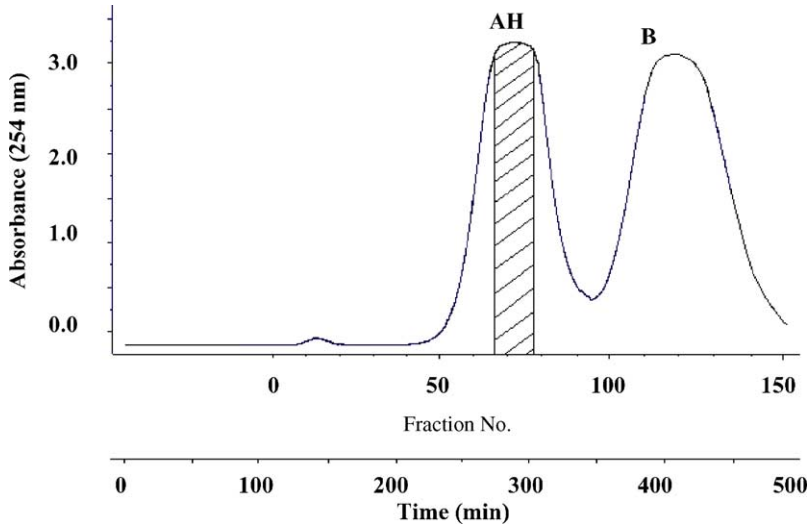

Fig. 2. HSCCC of fraction 17 from the silica clean-up step. Solvent system: *n*-heptane–ethyl acetate–methanol–water (4:1:4:1, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 183.5 mg dissolved in 20 ml of the lower phase; retention of the stationary phase: 79.4%. Pooled peak fractions including 67–77 (fraction AH) contains 58.1 mg 1-[2′,4′-dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone with a purity of 98.9%.

(2H, dq, $J = 7.2$, 1.2 Hz, CH_2-1‴) 3.70 (3H, s, 6′-OMe), 5.21 (2H, m, H-2″, H-2‴), 6.26 (1H, 4′-OH), 13.58 (1H, 2′-OH).

- ^{13}C NMR: (100 MHz, C^2HCl_3, ppm) δ 17.90 (C-5‴), 17.98 (C-5″), 21.82 (C-1‴), 22.77 (C-1″), 25.81 (C-4‴), 25.85 (C-4″), 31.1 (C-2), 62.81 (6-OMe), 108.98 (C-1′), 110.98 (C-3′), 112.65 (C-5′), 121.67 (C-2″), 122.26 (C-2‴), 134.51 (C-3″), 134.66 (C-3‴), 159.16 (C-6′), 160.67 (C-4′), 161.61 (C-2′), and 203.60 (C-1).
- 2D-NMR: supported the structural assignments.

After comparing the above data with spectral information from literature [4], the target compound was confirmed as being 1-[2′,4′-dihydroxy-3′,5′-di-(3″-methylbut-2″-enyl)-6′-methoxy] phenylethanone.

The result of our studies showed that HSCCC is a useful tool for the preparative separation of the target compound yielding high purity and high recovery.

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References

[1] T.-S. Wu, M.-L. Wang, T.-T. Jong, J. Nat. Prod. 52 (1989) 1284.
[2] W. Pathmasiri, H.E. El-Seedi, X. Han, J.-C. Janson, L. Bohlin, Phytochemistry, submitted for publication.
[3] V. Kumar, V. Karunaratne, M.-R. Sanath, K. Meegalle, Phytochemistry 28 (1989) 1278.
[4] L.-K. Sy, G.-D. Brown, Phytochemistry 52 (1999) 681.
[5] Y. Ito, CRC Crit. Rev. Anal. Chem. 17 (1986) 65.