Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1075 (2005) 127–131
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Isolation and purification of four flavonoid constituents from the flowers of *Paeonia suffruticosa* by high-speed counter-current chromatography

Xiao Wang [a,b], Chuange Cheng [a], Qinglei Sun [a], Fuwei Li [a], Jianhua Liu [a], Chengchao Zheng [b,*]

[a] *Test Center, Shandong Academy of Sciences, 19 Keyuan Street, Jinan, Shandong 250014, China*
[b] *College of Life Sciences, Shandong Agricultural University, 18 Daizong Street, Taian, Shandong 271018, China*

Received 16 January 2005; received in revised form 29 March 2005; accepted 1 April 2005
Available online 27 April 2005

Abstract

Four flavonoids, apigenin-7-*O*-neohesperidoside, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside and kaempferol-7-*O*-glucoside have been isolated and purified for the first time from the flowers of *Paeonia suffruticosa* by high-speed counter-current chromatography with a two-phase solvent system composed of ethyl acetate–ethanol–acetic acid–water (4:1:0.25:5, v/v). Then, 5 mg apigenin-7-*O*-neohesperidoside, 4 mg luteolin-7-*O*-glucoside, 9 mg apigenin-7-*O*-glucoside and 2.5 mg kaempferol-7-*O*-glucoside could be obtained after injecting 40 mg sample and their purities were 94, 97, 97 and 96%, respectively. All these constituents were identified by mass spectrometry and nuclear magnetic resonance.

Keywords: Plant materials; *Paeonia suffruticosa*; Counter-current chromatography; Flavonoids

1. Introduction

The flowers of *Paeonia suffruticosa Andr*. (*P. suffruticosa Andr*.) are used in Chinese folk medicines for the treatment of diseases related mainly to irregular menstruation and dysmenorrhea [1,2]. Our preliminary investigation by TLC using authentic standards led to the detection of flavonoids with antioxidant activity in the polar extracts of the flowers of *P. suffruticosa Andr*. [3]. In order to provide a better understanding of the pharmacological function and further exploit the important plant resource, isolation and structure elucidation of the lead structures is essential. We herein report an efficient method for the preparative isolation and purification of four flavonoids from *P. suffruticosa Andr*. by HSCCC followed by the identification of these components using high-performance liquid chromatography (HPLC)–electrospray ionization mass spectrometry (ESI-MS), electron ionization mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR).

2. Experimental

2.1. Reagents

Organic solvents including ethanol, hexane, *n*-butanol, acetone, ethyl acetate, and methanol were all of analytical grade and were purchased from Guangcheng Chemical Factory, Tianjin, China. Methanol used for HPLC analysis was of chromatographic grade and purchased from Siyou Tianjin Chemical Factory, Tianjin, China.

2.2. Preparation of crude sample

Flowers of *P. suffruticosa Andr*. were collected in Heze (China) during the late spring of 2003. The fresh flowers (5 kg) of *P. suffruticosa Andr*. were extracted three times with 95% aqueous ethanol (25 l). Then, the extract was combined and evaporated to dryness under reduced pressure,

* Corresponding author. Present address: College of Life Sciences, Shandong Agricultural University, 18 Diazong Street, Taian, Shandong 271018, China. Tel.: +86 538 824 2894; fax: +86 538 822 6399.
E-mail address: cczheng@sdau.edu.cn (C. Zheng).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.04.017

which yielded 125 g of residue. Then, 110 g of the residue was dissolved with 2 l water. After filtration, the aqueous solution was extracted three times with 5 l of water-saturated light petroleum (b.p. 60–90 °C) and ethyl acetate successively which yielded 15 g of light petroleum extract and 26 g of ethyl acetate extract after being combined and evaporated to dryness under reduced pressure. Twenty grams of ethyl acetate extract was further subjected to polyamide chromatography (400 g of polyamide, Taizhou Chemical Factory, Zhejiang, China) by eluting stepwise with aqueous ethanol (0, 30, 60 and 90%, v/v) to obtain four fractions. Fraction two (30% aqueous ethanol) was evaporated to dryness under reduced pressure and yielded 850 mg of yellow powder. Portions of this partially purified sample of *P. suffruticosa Andr*. flowers was subjected to HSCCC.

2.3. HSCCC separation procedure

Preparative HSCCC was carried out using a Model GS10A-2, with a multilayer coil of 1.6 mm I.D. and 110 m in length with a total capacity of 230 ml. The β values of this preparative column range from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge) (Beijing Institute of New Technology Application, Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A manual sample injection valve with a 10 ml loop (for the preparative HSCCC) (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

In each separation, the multiplayer coiled column was first filled entirely with the upper organic phase as the stationary phase. Then, the lower aqueous phase was pumped into the head end of the column at a suitable flow-rate of 1.5 ml/min for Model GS10A-2, while the apparatus was rotated at a speed of 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting from the tail outlet, the sample solution was injected through the injection valve. The effluent from the tail end of the column was continuously monitored by UV detection at 254 nm, and the peak fractions were collected according to the chromatogram.

2.4. Preparation of two-phases solvent system and sample solution

The selected solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly prior to use. The aqueous phase was used as the mobile phase, while the organic phase was used as the stationary phase. The sample solution was prepared by dissolving the crude sample in the mixture solution of organic phase and aqueous phase (1:1, v/v) of the solvent system used for HSCCC separation.

2.5. HPLC analyses and identification of HSCCC fractions

The HPLC system used throughout this study consisted of a Waters 660 pump, a Waters 660 controller (Waters, USA), a sample injector (Rheodyne, USA) with a 10 μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium^{32} workstation (Waters).

The crude sample and each purified fraction from the preparative HSCCC separation were analyzed by HPLC with a Shim-pack VP-ODS column (250 mm × 4.6 mm, I.D.) at 280 nm and column temperature of 25 °C. The mobile phase, a solution of methanol and 0.05% formic acid (50:50, v/v), was set at a flow-rate of 1 ml/min. The effluent was monitored by a photodiode array detector.

The identification of HSCCC peak fractions was carried out, respectively, by EI-MS on an Agilent 5973N mass spectrograph, by LC–ESI-MS on an Agilent 1100/MSD and by ^{1}H and ^{13}C NMR spectra on a Varian NMR spectrometer.

3. Results and discussion

3.1. HSCCC separation

Successful separation by HSCCC largely depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K) for the targeted sample [4–6]. Small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening [7–9]. In this case, the K value was determined after partitioning the sample between the two solvent phases, and aliquots of the upper and lower layers were analyzed by HPLC. From these two chromatograms, the K value of each component was determined by computing the ratio of the peak areas between the corresponding peaks. Several two-phase solvent systems were tested and their K values were measured and summarized in Table 1.

First, the two-phase solvent systems of ethyl acetate–*n*-butanol–ethanol–water (3:0.6:1:5, v/v) and *n*-butanol–ethanol–water (4:1:5, v/v) were used. However, the resulted K values were too high and target compounds needed long time to elute and resulted in a poor resolution. Then the two-phase solvent system of ethyl acetate–methanol–water (5:1.5:5, v/v) and ethyl acetate–methanol–acetone–water (5:1:0.5:5, v/v) were employed, which resulted suitable K values, but the retention of the stationary phase were small (about 25 and 28%, respectively). When the two-phase solvent system was changed into ethyl acetate–ethanol–acetic

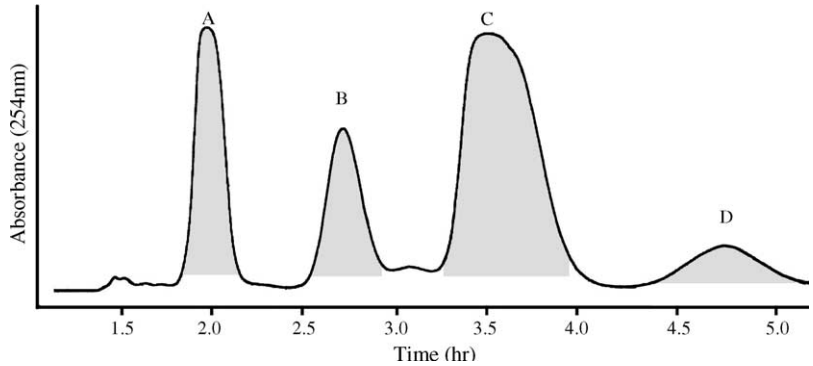

Fig. 1. HSCCC chromatogram of the crude extract. *Conditions*: revolution speed, 800 rpm; solvent system, ethyl acetate–ethanol–acetic acid–water (4:1:0.25:5, v/v); stationary phase, upper organic phase; mobile phase, lower aqueous phase; flow-rate, 1.5 ml/min; sample size, 40 mg; injection volume, 10 ml; retention of the stationary phase, 34%.

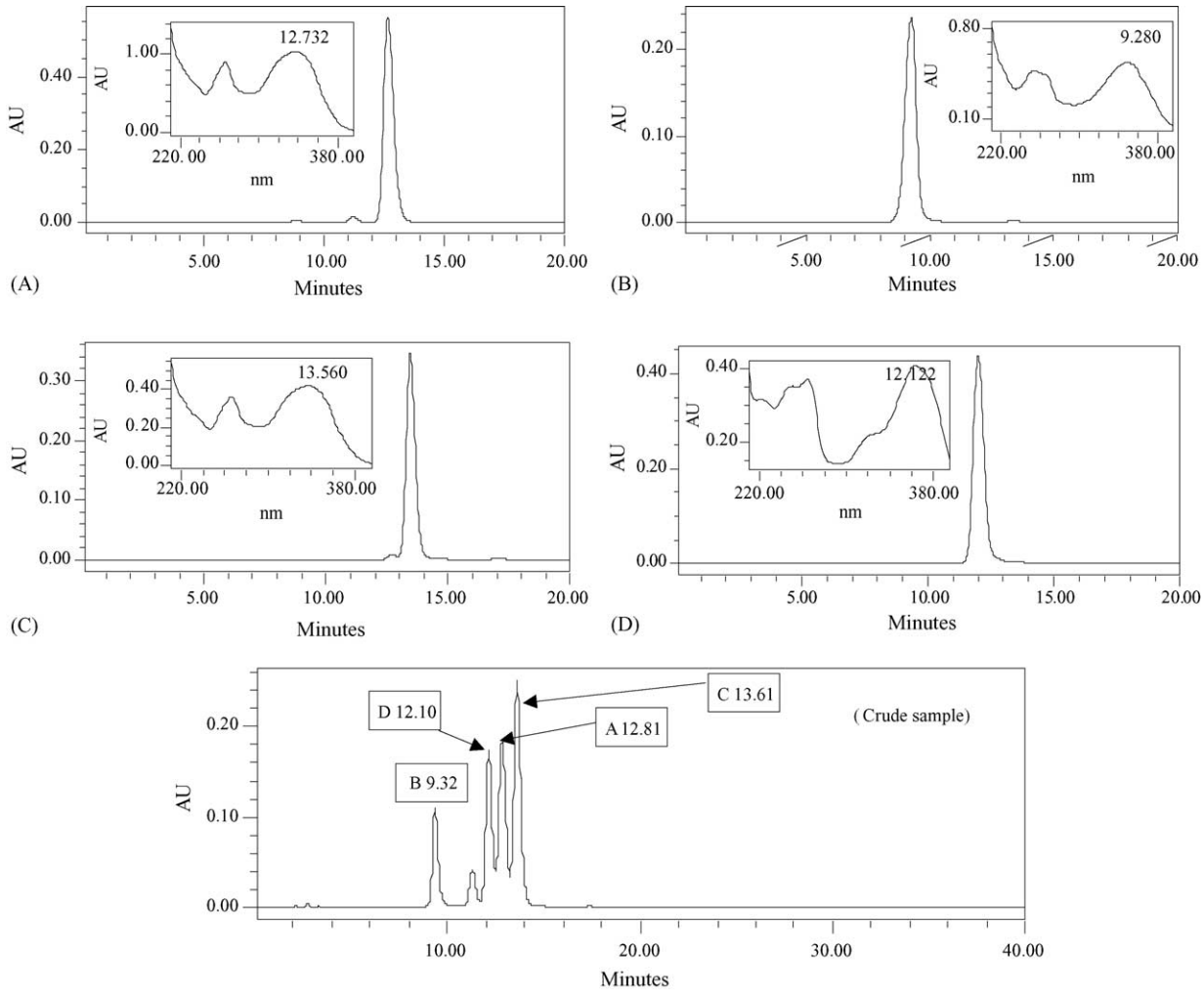

Fig. 2. HPLC chromatogram and UV spectrum of compounds A–D and HPLC chromatogram of the crude extract. *Experimental conditions*: a Shim-pack VP-ODS column (250 mm × 4.6 mm, I.D.); column temperature, 25 °C; mobile phase, methanol–0.05% formic acid (50:50, v/v); flow rate, 1.0 ml/min; detection, 280 nm; injection volume, 10 μl.

Table 1
Partition coefficients (*K*) of these compounds

Solvent systems	Compound 1	Compound 2	Compound 3	Compound 4
n-Butanol–ethanol–water 4:1:5	13.10	16.01	11.20	25.50
Ethyl acetate–*n*-butanol–ethanol–water 3:0.6:1:5	3.72	7.65	8.91	14.56
Ethyl acetate–methanol–acetone–water 5:1:0.5:5	0.61	1.16	1.95	4.08
Ethyl acetate–methanol–water 5:1.5:5	0.50	1.50	2.48	4.21
Ethyl acetate–ethanol–acetic acid–water 4:1:0.25:5	0.96	1.73	2.69	4.56

Experimental procedure: 2 ml of each phase of the equilibrated two-phase solvent system was added to approximately 1 mg of crude sample placed in a 10 ml test tube. The test tube was caped, and was shaken vigorously for 1 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficients (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

acid–water at ratio 4:1:0.25: 5, appropriate peak resolution and the retention of the stationary phase (about 34%) were obtained. Fig. 1 shows the preparative HSCCC separation of 40 mg of the crude sample using the optimized solvent system. Based on the HPLC analysis and the elution curve of the preparative HSCCC, all collected fractions were combined into different pooled fractions. Five milligrams compound A, 4 mg compound B, 9 mg compound C and 2.5 mg compound D were obtained, with the purity of 94, 97, 97 and 96%, respectively (Fig. 2). The crude sample was also analyzed by HPLC and four intense peaks could be detected with retention times 9.32, 12.10, 12.81, 13.61 min, corresponding to the isolated compounds A–D. The UV spectra of the peaks A–D show the characteristics spectra of flavonoids, with bands at 250–280 and 310–350 nm (Fig. 2).

3.2. Structural identification of the compounds

Apigenin-7-*O*-neohesperidoside (compound A): negative ESI-MS, *m*/*z* 577 (*M* − H); positive ESI-MS, *m*/*z* 579 (*M* + H), 601 (*M* + Na). EI-MS *m*/*z* (%): 270 (aglycone, 100), 242, 213, 153, 152, 121, 118. The ^{1}H and ^{13}C NMR (data not shown) of the compound A matched with the reported NMR data for apigenin-7-*O*-neohesperidoside (Fig. 3) from literature [10].

Luteolin-7-*O*-D-glucoside (compound B): Negative ESI-MS, *m*/*z* 447 (*M* − H); positive ESI-MS, *m*/*z* 449 (*M* + H), 471 (*M* + Na). EI-MS *m*/*z* (%): 286 (aglycone, 100), 258, 153, 134, 124, 73. The ^{1}H and ^{13}C NMR (data not shown) of the compound B matched with the reported NMR data for luteolin-7-*O*-D-glucoside (Fig. 3) from literature [11,12].

Apigenin-7-*O*-D-glucoside (compound C): Negative ESI-MS, *m*/*z* 431 (*M* − H); positive ESI-MS, *m*/*z* 433 (*M* + H), 455 (*M* + Na). EI-MS *m*/*z* (%): 270 (aglycone, 100), 242, 153, 152, 124, 121, 118. The ^{1}H and ^{13}C NMR (data not shown) of the compound C matched with the reported NMR data for apigenin-7-*O*-D-glucoside (Fig. 3) from literature [11,13].

Kaempferol-7-*O*-D-glucoside (compound D): negative ESI-MS, *m*/*z* 431 (*M* − H); positive ESI-MS, *m*/*z* 433 (*M* + H), 455 (*M* + Na). EI-MS *m*/*z* (%): 286 (aglycone, 100), 258, 257, 229, 213, 184, 121, 93. The ^{1}H and ^{13}C NMR (data not shown) of the compound D matched with the reported NMR data for kaempferol-7-*O*-D-glucoside (Fig. 3) from literature [12].

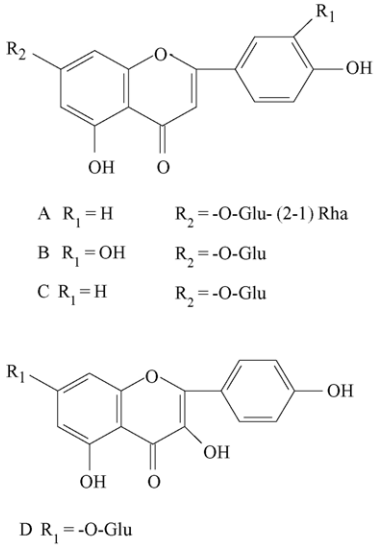

Fig. 3. Structure of the isolated flavonoids: (A) apigenin-7-*O*-neohesperidoside; (B) luteolin-7-*O*-glucoside; (C) apigenin-7-*O*-glucoside; (D) kaempferol-7-*O*-glucoside.

4. Conclusion

The results of our studies clearly demonstrate the potential of HSCCC for the preparative isolation of apigenin-7-*O*-neohesperidoside (A), luteolin-7-*O*-glucoside (B), apigenin-7-*O*-glucoside (C) and kaempferol-7-*O*-glucoside (D) from the flowers of *P. suffruticosa Andr*. In particular, preparative HSCCC with its speedy separation and minimum solvent consumption offers a very efficient method for the separation and purification of natural products.

Acknowledgements

Financial support from Natural Science Foundation of Shandong (Q2002b04) is gratefully acknowledged. We also thank Dr. Xining Li of Beijing Institute of New Technology Application for his excellent technical assistance.

References

[1] T.K. Huang (Ed.), A Handbook of the Composition and Pharmacology of Common Chinese Drugs, Press of Chinese Medicine Technology, Beijing, 1994, p. 1040.
[2] T.K. Huang, Z.Z. Ding, S.X. Zhao (Eds.), Xin Ben Cao Gang Mu, Press of Chinese Medicine Technology, Beijing, 2001, p. 448.
[3] X. Wang, X. Shi, C. Zheng, Food Ferment. Ind. 30 (2004) 55.
[4] X. Wang, Y. Wang, Y. Geng, F. Li, C. Zheng, J. Chromatogr. A 1038 (2004) 171.
[5] H.T. Lu, Y. Jiang, F. Chen, J. Chromatogr. A 1017 (2003) 117.
[6] Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography Chemical Analysis, 132, Wiley/Interscience, New York, 1996, p. 36 (Chapter 1).
[7] X. Wang, Y. Wang, J. Yuan, L. Sun, C. Zheng, J. Chromatogr. A 1055 (2004) 135.
[8] H.T. Lu, Y. Jiang, F. Chen, J. Chromatogr. A 1023 (2004) 159.
[9] Y. Ito, Countercurrent chromatography, in: N.B. Mandava, Y. Ito (Eds.), Theory and Practice, Marcel Dekker, New York, 1988, p. 443.
[10] R.J. Tang, West China J., Pharmacology 11 (1996) 5.
[11] Y.L. Ren, J.S. Yang, Chin. Pharm. J. 36 (2001) 590.
[12] X.D. Yang, J.F. Zhao, J. Guo, S.X. Mei, L. Li, Chin. Tradit. Herb. Drugs 25 (2004) 257.
[13] X.M. Du, N.Y. Sun, Y. Shoyama, Phytochemistry 53 (2000) 997.