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Preparative Separation and Purification of Flavonoids from *Ephedra sinica* Stapf. by High-Speed Counter-Current Chromatography

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Preparative high-speed counter-current chromatography was successfully performed for separation and purification of flavonoids from the stem of *Ephedra sinica* Stapf. with a two-phase solvent system composed of petroleum ether-ethyl acetate-methanol-water (0.5:3.5:1:3, v/v/v/v). This separation process produced four flavonoids, mahuannin A (**1**), mahuannin D (**2**), dihydroquercetin (**3**) and catechin (**4**) from 350 mg of crude sample in a one-step separation with purities of 98.8, 98.6, 98.3 and 98.2 %, respectively. The structures of these flavonoids were confirmed by ESI-MS, ^{1}H NMR and ^{13}C NMR.

Keywords: High-speed counter-current chromatography, Flavonoids, *Ephedra sinica* Stapf.

INTRODUCTION

Ephedra sinica Stapf., called "Ma Huang", is a traditional Chinese medicinal herb and has been used for treatment of autoimmune and inflammatory diseases with safe and efficient function for thousands of years[1,2]. The major pharmacologically active constituents of *E. sinica* are alkaloids, which demonstrated activities of stimulating the central nerve system, dilating bronchial tubes, elevating blood pressure and increasing heart rate[3]. Flavonoids from *E. sinica* were also found to possess antibacterial, immunomodulatory, antimalarial and antitumor activities[4-7]. However, the preparative separation and purification of flavonoids from *E. sinica* by traditional column methods was often difficult because of their structural similarity and adsorption behavior on the stationary phase. Until now, only few flavonoids have been separated from the herbs[8]. Considering such biological activities of flavonoids, it is necessary and beneficial to develop an efficient method to separate and purify large quantities of single flavonoids with high purities for further pharmacological research.

High-speed counter-current chromatography (HSCCC), being as a kind of liquid-liquid partition chromatography, eliminates irreversible adsorption of samples on solid support in conventional column chromatography and offers excellent recovery of target compounds. With a large volume of sample injection, multiform relative pure compounds can be separated and purified at one step. It has been successfully applied for the separation and purification of flavonoids from natural products[9-11]. To the best of our knowledge, there is no report of using HSCCC to separate and purify flavonoids from *E. sinica*. The present paper describes the successful preparative separation and purification of four flavonoids, mahuannin A (**1**), mahuannin D (**2**), dihydroquercetin (**3**) and catechin (**4**) (Fig. 1) from the crude extract of *E. sinica* by HSCCC.

EXPERIMENTAL

Petroleum ether (60-90 °C), methanol, chloroform and ethyl acetate were all of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Acetonitrile used for HPLC analysis was of chromatographic grade (Siyou Special Reagent Factory, Tianjin, China). All aqueous solutions were prepared with pure water produced by Milli-Q system (Millipore, USA).

Preparative HSCCC was carried out using a Model GS10A-2 high-speed counter-current chromatography (Beijing Emilion Science & Technology Co., Beijing, China). Analytical high-performance liquid chromatography (HPLC) system employed throughout this study consisted of a Waters 600 pump, a Waters 600 Multisolvent Delivery, a Waters 996 diode-array detector (DAD), a Waters 600 system controller and a Millennium32 workstation (Waters, Milford, USA).

The stems of *E. sinica* were collected in Gansu, China and identified by Prof. J. Li (College of Pharmacy, Shandong University of Traditional Chinese Medicine, Shandong, China).

Measurement of partition coefficient and separation factor: The two-phase solvent system was selected according to the partition coefficient (K) of each target compound in the sample. The K values were determined according to the literature[12]: 1 mL of each phase of the equilibrated two-phase solvent system was added to a test tube and a suitable amount of sample was placed in it. Then the tube was shaken violently to mix the solution thoroughly. After the equilibration was established, an equal volume of each phase was analyzed by HPLC to obtain the partition coefficients. The K value was calculated by the following equation: $K = A_U/A_L$ (A_U, the peak area of the target compound in the upper phase; A_L, that in the lower phase)[13].

Preparation of the crude extract from *E. sinica* Stapf: The dried stems of *E. sinica* (2 Kg) were powdered and extracted by reflux with 90 % ethanol for three times (1.5 h each time). The ethanol solutions were combined and evaporated to dryness under reduced pressure. This sample was dissolved in distilled water and extracted successively with petroleum ether (60-90 °C), chloroform and ethyl acetate. The ethyl acetate extract (20 g) was subjected to silica gel column chromatography using a gradient system of $CHCl_3$-CH_3OH at the ratios of 10:1, 5:1, 2:1 and 1:1. The fractions (4.6 g) eluted by $CHCl_3$-CH_3OH (5:1, v/v) were chosen for further separation and purification by HSCCC.

High-speed counter-current chromatography separation procedure: In HSCCC separation, the coil column was first entirely filled with the upper phase. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 2 mL/min. After the establishment of hydrodynamic equilibrium, indicated by a clear mobile phase eluting from the tail outlet, the sample solution (350 mg in 20 mL) was injected through the sample port. The effluent of the column was continuously monitored with a UV detector at 254 nm and each peak fraction was manually collected according to the elution profile.

HPLC analysis, ESI-MS, ^{1}H NMR and ^{13}C NMR identification of the fractions: The crude sample and each purified fraction from the preparative HSCCC separation were analyzed by HPLC on an Amethyst C_{18}-P column (250 × 4.6 mm, I.D., 5 μm) at 25 °C. The mobile phase was composed of acetonitrile and water in gradient elution as follows: 0-40 min, linear change from 15 % acetonitrile to 50 % acetonitrile. The flow rate and detection were set at 1 mL/min and 254 nm, respectively. The purified compounds were identified by electrospray ionization mass spectrometry (ESI-MS) on an Agilent 1100/MSG1946 (Agilent, CA, USA) and ^{1}H and ^{13}C NMR spectra on a Varian INOVA 600 spectrometer (Varian, Palo Alto, USA) with DMSO as solvent and tetramethysilane (TMS) as internal standard.

RESULTS AND DISCUSSION

Optimization of HPLC conditions: The measurement of K values and the fractions obtained by HSCCC were all analyzed by HPLC. So it is of great significance to choose a good HPLC separation condition. A series of elution systems were tested in HPLC separation of crude sample, such as isocratic and gradient elution of CH_3OH-H_2O, CH_3CN-H_2O and CH_3OH-CH_3CN-H_2O. The results indicated that the baseline separation of the target compounds could be achieved by using CH_3CN-H_2O in gradient elution as follows: 0-40 min, linear change from 15 % CH_3CN to 50 % CH_3CN. As shown in Fig. 3, the four target peaks could achieve baseline separation under the optimum HPLC condition.

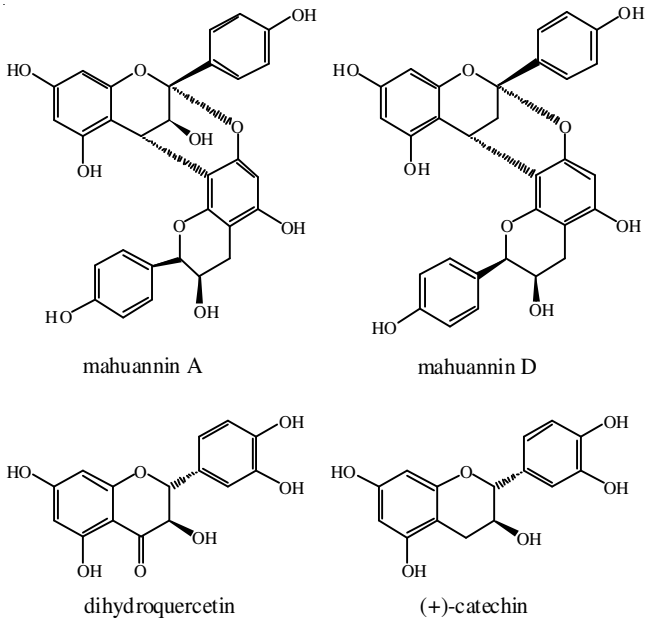

Fig. 1. Chemical structures of the four compounds

Selection of two-phase solvent system: The selection of a suitable two-phase solvent system is the critical step for a successful HSCCC separation. The key of solvent optimization is first to find a solvent system in which the sample could be freely soluble, then to optimize this solvent system to provide an ideal range of partition coefficient (K_D, 0.5-2)[14,15]. Too large K_D values ($K_D > 2$) usually tend to produce excessive broad peaks and lead to extended elution time, while too small K_D values ($K_D < 0.5$) often result in loss of peak resolution. In our research, the solvent system of petroleum ether-ethyl acetate-methanol-water was chosen and the K_D values in systems at different volume ratios were summarized in Table-1. The results indicated that good separation results could be obtained when petroleum ether-ethyl acetate-ethanol-water with the volume ratio of 0.5:3.5:1:3 was used as the two-phase solvent system.

TABLE-1
PARTITION COEFFICIENTS (K_D) OF FOUR TARGET COMPOUNDS

Solvent system (petroleum ether-ethyl acetate-methanol-water)	K_I	K_{II}	K_{III}	K_{IV}
1:1:1:1	0.05	0.12	0.25	0.37
1:4:1:4	0.87	1.75	3.39	5.14
1:3:1:3	0.29	0.43	0.97	1.68
0.5:3.5:1:3	0.63	0.98	1.42	1.85

Purification of flavonoids by HSCCC: Fig. 2 showed the separation of HSCCC using this solvent system. 350 mg crude sample of *E. sinica* Stapf. was separated and purified under the optimum HSCCC conditions. The retention of the stationary phase was about 65 % and the total separation time was less than 5 h. The HSCCC fractions were analyzed by HPLC and the HPLC chromatograms of the collected fractions were shown in Fig. 3, respectively. The separation produced 28 mg of compound **1**, 31 mg of compound **2**, 52 mg of compound **3** and 39 mg of compound **4** with the purity of 98.8, 98.6, 98.3 and 98.2 %.

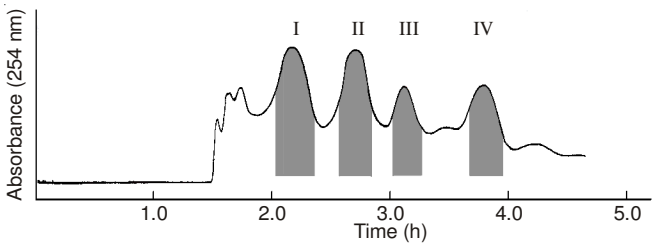

Fig. 2. High-speed counter-current chromatography chromatogram of the crude sample of *Ephedra sinica* Stapf.; solvent system: petroleum ether-ethyl acetate-methanol-water (0.5:3.5:1:3, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2 mL/min; revolution speed: 800 rpm; retention of stationary phase: 65 %; sample size: 250 mg crude sample; detection: 254 nm; zone I: dihydroquercetin, zone II: catechin, zone III: mahuannin D, zone IV: mahuannin A

Identification of separated peaks

Compound 1: Positive ESI-MS, *m/z* 327 $[M + Na]^+$, $C_{15}H_{12}O_7$. ^{1}H NMR (600 MHz, pyridine-d_6) δ ppm: 7 (1H, brs, H-12), 6.96 (1H, d, *J* = 8.2 Hz, H-16), 6.81 (1H, d, *J* = 8.2 Hz, H-15), 5.90 (1H, d, *J* = 1.9 Hz, H-8), 5.87 (1H, d, *J* = 1.9 Hz, H-6), 4.90 (1H, d, *J* = 11.8 Hz, H-2), 4.50 (1H, d, *J* = 11.8 Hz, H-3). ^{13}C NMR (125 MHz, Pyridine-d_6) δ ppm: 198 (C-4), 168.5 (C-7), 165.1 (C-5), 164.5 (C-9), 146.3 (C-14), 146 (C-13), 129.5 (C-11), 121 (C-16), 116 (C-15), 115.6 (C-12), 101.1 (C-10), 97.2 (C-6), 96.3 (C-8), 84.7 (C-2), 73.4 (C-3). Compared with the data given in the literature[16], compound **1** was identified as dihydroquercetin.

Compound 2: Positive ESI-MS, *m/z* 313 $[M + Na]^+$, $C_{15}H_{14}O_6$. ^{1}H NMR (600 MHz, pyridine-d_6) δ ppm: 6.85 (1H, d, *J* = 1.8 Hz, H-12), 6.76 (1H, d, *J* = 8 Hz, H-15), 6.72 (1H, dd, *J* = 8 Hz, 1.8 Hz, H-16), 5.93 (1H, d, *J* = 2.2 Hz, H-8), 5.86 (1H, d, *J* = 2.2 Hz, H-6), 4.55 (1H, d, *J* = 7.5 Hz, H-2), 4 (1H, m, H-3), 2.87 (1H, dd, *J* = 16 Hz, 5.5 Hz, H-4), 2.50 (1H, dd, *J* = 16 Hz, 5.5 Hz, H-4). ^{13}C NMR (125 MHz, pyridine-d_6) δ ppm: 157.4 (C-9), 157 (C-7), 156.7 (C-5), 146.2 (C-13), 146.1 (C-14), 132.2 (C-11), 120 (C-16), 116.1 (C-15), 115.1 (C-12), 100.6 (C-10), 96.2 (C-6), 95.5 (C-8), 82.7 (C-2), 68.8 (C-3), 28.4 (C-4). Compared with the data given in the literature[16], compound **2** was identified as catechin.

Compound 3: Positive ESI-MS, *m/z* 551 $[M + Na]^+$, $C_{30}H_{24}O_9$. ^{1}H NMR (600 MHz, pyridine-d_6) δ ppm: 7.49 (1H, d, *J* = 8.6 Hz, H-12'), 7.49 (1H, d, *J* = 8.6 Hz, H-16'), 7.47 (1H, d, *J* = 8.5 Hz, H-12), 7.47 (1H, d, *J* = 8.5 Hz, H-16), 6.86 (1H, d, *J* = 8.6 Hz, H-13'), 6.86 (1H, d, *J* = 8.6 Hz, H-15'), 6.85 (1H, d, *J* = 8.5 Hz, H-13), 6.85 (1H, d, *J* = 8.5 Hz, H-15), 6.15 (1H, s, H-6'), 6 (1H, d, *J* = 2.3 Hz, H-8), 5.85 (1H, d, *J* = 2.3 Hz, H-6), 5.06 (1H, s, H-2'), 4.45 (1H, t, *J* = 2.9 Hz, H-4), 4.25 (1H, brs, H-3'), 2.90 (2H, ddd, *J* = 2.1 Hz, 4.2 Hz, 16.8

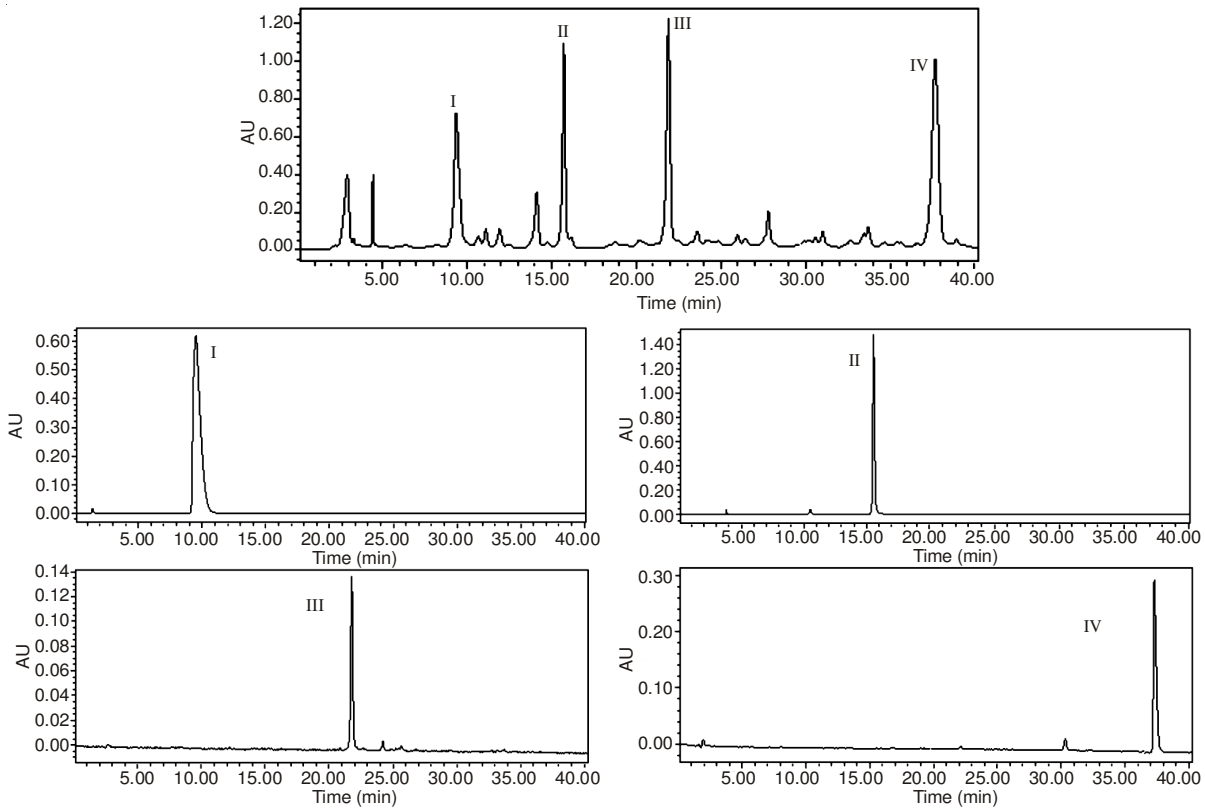

Fig. 3. HPLC chromatograms of the crude sample from *Ephedra sinica* Stapf. and four purified fractions from HSCCC separation. Experimental conditions: column, Amethyst C18-P (250 × 4.6 mm, I.D., 5 μm); column temperature, 25 °C; mobile phase: acetonitrile-water (0-40 min, 15-50 % acetonitrile, v/v); flow rate, 1 mL/min; UV detection wavelength, 254 nm; peak I, dihydroquercetin; peak II, catechin; peak III, mahuannin D; peak IV, mahuannin A

Hz, H-4'), 2.21 (2H, dq, *J* = 3.2 Hz, 13.2 Hz, H-3). ^{13}C NMR (125 MHz, pyridine-d_6) δ ppm: 158.7 (C-14), 158.4 (C-14'), 158 (C-7), 156.3 (C-5'), 155.1 (C-5), 154.6 (C-9), 152.5 (C-7'), 151.3 (C-9'), 134.3 (C-11), 130.5 (C-11'), 129.2 (C-12'), 129.2 (C-16'), 128.1 (C-12), 128.1 (C-16), 116 (C-13'), 116 (C-15'), 115.7 (C-13), 115.7 (C-15), 107.4 (C-8'), 107.4 (C-10), 101.7 (C-10'), 99.8 (C-2), 97.5 (C-6), 97 (C-6'), 96.6 (C-8), 81 (C-2'), 67.1 (C-3'), 35.0 (C-3), 29.5 (C-4'), 21.6 (C-4). Compared with the data given in the literature[17], compound **3** was identified as mahuannin D.

Compound 4: Positive ESI-MS, *m/z* 567 $[M + Na]^+$, $C_{30}H_{24}O_{10}$. ^{1}H NMR (600 MHz, pyridine-d_6) d ppm: 7.52 (1H, d, *J* = 8.5 Hz, H-12'), 7.52 (1H, d, *J* = 8.5 Hz, H-16'), 7.48 (1H, d, *J* = 8.5 Hz, H-12), 7.48 (1H, d, *J* = 8.5 Hz, H-16), 6.86 (1H, d, *J* = 8.5 Hz, H-13'), 6.86 (1H, d, *J* = 8.5 Hz, H-15'), 6.84 (1H, d, *J* = 8.5 Hz, H-13), 6.84 (1H, d, *J* = 8.5 Hz, H-15), 6.10 (1H, s, H-6'), 6.07 (1H, d, J = 2.3 Hz, H-8), 5.91 (1H, d, *J* = 2.3 Hz, H-6), 5.10 (1H, s, H-2'), 4.41 (1H,d, *J* = 3.5 Hz, H-3), 4.25 (1H, brs, H-3'), 4.17 (1H, d, *J* = 3.5 Hz, H-4), 2.91 (2H, dd, *J* = 2.1 Hz, 17 Hz, H-4'). ^{13}C NMR (125 MHz, pyridine-d_6) d ppm: 159 (C-7'), 158.3 (C-9), 158.1 (C-14'), 156.6 (C-7), 156.7 (C-5), 154.1 (C-5'), 152.1 (C-14), 151.2 (C-9'), 131.7 (C-11), 130.5 (C-11'), 129.5 (C-12), 129.4 (C-16), 129.2 (C-12'), 129.2 (C-16'), 116.1 (C-13), 116.1 (C-15), 115.4 (C-13'), 115.4 (C-15'), 107 (C-10'), 104.1 (C-10), 102.0 (C-8'), 100.5 (C-2), 98 (C-6), 96.4 (C-8), 96 (C-6'), 81 (C-2'), 67.7 (C-3'), 67 (C-3), 29.5 (C-4'), 29.3 (C-4). Compared with the data given in the literature[18], compound **4** was identified as mahuannin A.

Conclusion

A HSCCC method has been developed and successfully applied to the separation and purification of flavonoids and flavones from the stem of *E. sinica* Stapf. Four flavonoids, mahuannin A, mahuannin D, dihydroquercetin and catechin were obtained from 350 mg of crude sample in a one-step separation. This research demonstrates that HSCCC is a fast and valuable technique in separating and purifying flavonoids from this plant.

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REFERENCES

1. Editorial Committee of Chinese Materia Medica, Chinese Materia Medica, Shanghai Science & Technology Press, Shanghai, Vol. 6, p. 213 (2000).
2. The State Pharmacopoeia Commission of China, Pharmacopoeia of China, Chemical Industry Press, Beijing, p. 300 (2010).
3. P. Zheng, G.D. Dai and H.Q. Li, *Ningxia Med. J.*, **24**, 126 (2002).
4. Z.H. Xu, G.W. Qin, X.Y. Li and R.S. Xu, *Acta Pharmacol. Sin.*, **36**, 668 (2001).
5. Z. Xu, S.J. Jia, G.S. Tan and Y.J. Li, *China J. Modern Med.*, **14**, 88 (2004).
6. L. Xu and M.H.Yin, *J. Medical Sci. Yanbian Univ.*, **32**, 246 (2009).
7. X.K. Zheng, T.L. Ning, X.L. Wang, C.X. Liu, Y.Y. Liu and W.S. Feng, *Chin. Pharm. J.*, **46**, 998 (2011).
8. H.Z. Wu, Y. Lu, L.Q. Ai, X.W. Wu and Y.F. Yang, *Asia-Pacific Tradit. Med.*, **4**, 144 (2008).
9. L.J. Chen, D.E. Games and J. Jones, *J. Chromatogr. A*, **988**, 95 (2003).
10. R.M. Liu, A.F. Li, A.L. Sun, J.C. Cui and L.Y. Kong, *J. Chromatogr. A*, **1064**, 53 (2005).
11. H.B. Li and F. Chen, *J. Chromatogr. A*, **1074**, 107 (2005).
12. X. Wang, Y.Q. Wang, Y.L. Geng, F.W. Li and C.C. Zheng, *J. Chromatogr. A*, **1036**, 171 (2004).
13. J.Y. Peng, G.R. Fan and Y.T. Wu, *J. Chromatogr. A*, **1091**, 89 (2005).
14. H. Oka, K.I. Harada, Y. Ito and Y. Ito, *J. Chromatogr. A*, **812**, 35 (1998).
15. H. Oka, K.I. Harada, M. Suzuki and Y. Ito, *J. Chromatogr. A*, **903**, 93 (2000).
16. H. Hikino, N. Shimoyama, Y. Kasahara, M. Takahashi and C. Konno, *Heterocycles*, **19**, 1381 (1982).
17. H. Hikino and Y. Kasahara, *Heterocycles*, **20**, 1953 (1983).
18. C.C. Shen, Y.S. Chang and L.K. Hott, *Phytochemistry*, **34**, 843 (1993).