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PREPARATIVE SEPARATION OF POLYPHENOLS FROM ARTICHOKE BY POLYAMIDE COLUMN CHROMATOGRAPHY AND HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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An efficient method for the rapid separation and purification of polyphenols from artichoke by polyamide column chromatography in combination with high-speed counter-current chromatography (HSCCC) was successfully built. The crude ethanol extracts from dry artichoke were first pre-separated by polyamide column chromatography and divided in two parts as sample 1 and sample 2. Then, the samples were further separated by HSCCC and yielded 7.8 mg of chlorogenic acid (compound I), 24.5 mg of luteolin-7-O-β-D-rutinoside (compound II), 18.4 mg of luteolin-7-O-β-D-glucoside (compound III), and 33.4 mg of cynarin (compound IV) with purity levels of 92.0%, 98.2%, 98.5%, and 98.0%, respectively, as determined by high-performance liquid chromatography (HPLC) method. The chemical structures of these compounds were identified by electrospray ionization-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR).

Keywords: artichoke; HSCCC; polyphenols.

INTRODUCTION

Artichoke (*Cynara scolymus L.*, Compositae family), originated from the southern Mediterranean parts of North Africa, is widely grown in Europe and America.[1] It has a long history of its dual usage as medicine and food, and its safety has been verified by a long-term practice. Artichoke is very popular as a cholagogue and antidiabetic agent[2], and is also used in the European traditional medicine to treat hepatitis and hyperlipidemia.[3] The major chemical components of artichoke include flavonoids, mono- and di-caffeoylquinic acids, and it has been found to be a rich source of polyphenols.[4-6] Polyphenols have many bioactivities, such as hepatoprotection, antioxidation, and antibacterial effect. Chlorogenic acid, luteolin-7-O-β-D-rutinoside, luteolin-7-O-β-D-glucoside, and cynarin are the main active compounds in artichoke.[7-9]

The classical methods used for the preparative separation and purification of compounds from artichoke, such as silica gel and preparative reverse-phase liquid chromatography (RPLC) are tedious, time-consuming, and usually require multiple chromatographic steps.[7,8] High-speed counter-current chromatography (HSCCC), which is a support free, liquid–liquid partition chromatographic technique, eliminates the irreversible adsorption of the sample onto solid supports and is characterized by an excellent sample recovery. HSCCC has been successfully applied to the isolation and purification of compounds from natural materials.[10-13] However, so far no reports have been published on the purification of artichoke components by HSCCC.

The aim of this study was to develop an efficient method for the preparative isolation and purification of polyphenols with high purities from artichoke using polyamide column chromatography combined with HSCCC. The chemical structures of the compounds are shown in Figure 1.

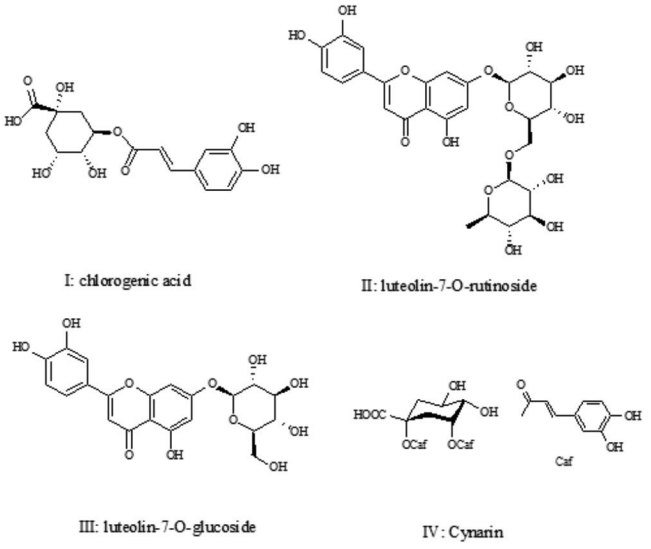

Figure 1. *Chemical structures of compounds I–IV from* Cynara scolymus L.

EXPERIMENTAL PART

Apparatus

The HSCCC instrument employed in this study was a Model GS10A-2 high-speed counter-current chromatograph (Beijing Emilion Science & Technology Co., Beijing, China) equipped with two multilayer coil separation columns connected in series (1.6-mm I.D., total volume of 230 mL) and a 20-mL sample loop. The β-value (β = r/R; r is the distance from the coil to the holder shaft and R is the distance between the holder axis and central axis of the centrifuge) of this preparative column ranged from 0.5 to 0.8 for the internal and external parts, respectively. The rotational speed of the apparatus was regulated by a speed controller in the range of 0–1000 rpm. The HSCCC instrument was also equipped with a Model NS-1007 constant-flow pump (Beijing Emilion Science & Technology Co.),

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Model 8823A-UV Monitor (Beijing Emilion Science & Technology Co.) and Model 3057-11 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China).

The high-performance liquid chromatography (HPLC) system was equipped with Waters 600 pump, Waters 600 controller, and Waters 996 photodiode array detector (Waters, USA). Evaluation and quantification of the data were made on an Empower pro data handling system (Waters).

Reagents and materials

All solvents used for the preparation of extracts and HSCCC separation were of analytical grades (Yuwang Chemical Factory, Yucheng, China). Acetonitrile used for HPLC was of chromatographic grade (Yucheng Chemical Factory). Chlorogenic acid was purchased from the National Institute of the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The water used throughout the study was purified with a Milli-Q water purification system (Millipore, USA).

Artichokes were purchased from a local drug store and identified by Dr. Li Jia (Shandong University of Traditional Chinese Medicine, China).

Preparation of crude extracts

Dry artichokes (1 kg) were powdered and extracted by ultrasonic wave extraction for 1 h with 10 L of ethanol/water mixture (60:40 v/v). The ethanol extracts were concentrated in a rotary evaporator at 50 ºC under reduced pressure until the ethanol was completely evaporated. Crude extracts (100 mL) were then subjected to polyamide column chromatography (400 mm × 40 mm I.D.). After the sample had been loaded, the column was eluted by distilled water, ethanol/water (60:40 v/v) with 1% acetic acid and ethanol/water (95:5 v/v) with 1% acetic acid at a flow rate of 15 mL/min. The effluent was monitored at 254 nm.

Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (K_D) of the target compounds. The K_D value was determined by HPLC as follows:[11] a suitable amount of sample (about 1-2 mg) was dissolved with 1 mL of each phase of the equilibrated two-phase solvent system contained in 2-mL test tubes. Then, both upper organic phase and lower water phase were directly analyzed by HPLC. The K_D value was defined as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

Preparation of two-phase solvent system and sample solution

The selected two-phase solvent system was prepared by adding the solvents to a separation funnel and was repeatedly shaken to attain equilibrium. The two phases of the selected solvent system were shortly separated before use and degassed by ultrasounds.

The sample solution used for HSCCC separation was prepared by dissolving the samples in the mixture of 3 mL of upper phase and 3 mL of lower phase.

HSCCC separation

During each separation step, the upper phase was pumped in the multilayer-coiled column as a stationary phase. Further, the apparatus was rotated at 800 rpm with a head-to-tail mode, while the lower phase was pumped through the column as mobile phase at a flow rate of 2 mL/min. After the hydrodynamic equilibrium was reached, the sample solution was injected through the sample port. The effluent was monitored with the UV detector at 254 nm and the chromatogram was recorded. The whole process of separation occurred at room temperature.

Analysis and identification of HSCCC peak fractions

The components pre-purified by polyamide and each purified fraction from the HSCCC separation were analyzed by HPLC with an Amethyst C_{18}-P column (I.D. 4.6 mm × 250 mm, 5 μm) at 25 ºC. The mobile phase [i.e., acetonitrile/water (20:80, v/v) containing 0.2% phosphoric acid] was set at a flow rate of 1 mL/min. The effluent was monitored at 330 nm.

The identification of the HSCCC peak fractions was carried out by electrospray ionization-mass spectrometry (ESI-MS) on an Agilent 1100/MS-G1946. The nuclear magnetic resonance (NMR) spectra were recorded with a Varian-600 spectrometer (Varian, Palo Alto, CA, USA) using tetramethylsilane (TMS) as internal standard.

RESULTS AND DISCUSSION

Separation by polyamide column chromatography

Because polyamide, which can adsorb anions via electrostatic interactions, has an excellent adsorption capacity toward phenols, acids, quinines, and flavonoids, polyamide column chromatography is widely used in the separation of polyphenols.

The crude extracts of artichoke were pre-separated by polyamide column chromatography. A total of 22 g of sample 1 and 12 g of sample 2 were obtained in 60% ethanol (1% acetic acid) and 95% ethanol (1% acetic acid) fractions. All the samples were stored in the refrigerator until subsequent HSCCC separation. The chromatogram of polyamide column chromatography is shown in Figure 2, while the HPLC chromatogram of the crude extracts from artichoke is shown in Figure 3(a).

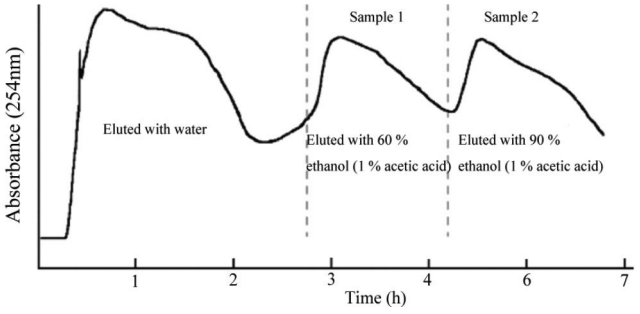

Figure 2. Chromatogram of polyamide column chromatography column separation

HSCCC separation

In general, the selection of a solvent system is the first and critical step for a successful HSCCC separation. The partition coefficient (K_D) is the most important parameter to consider when selecting the solvent system. In order to obtain an efficient separation and a suitable run time, K_D should range from 0.5 to 3. If K_D is much smaller than 0.5, the solutes would elute close to each other near the solvent front, resulting in loss of peak resolution; if K_D is much greater than 3, the solutes would elute in excessively broad peaks, leading to extended elution times.[12] A series of solvent systems were tested according to the physicochemical properties of the target compounds

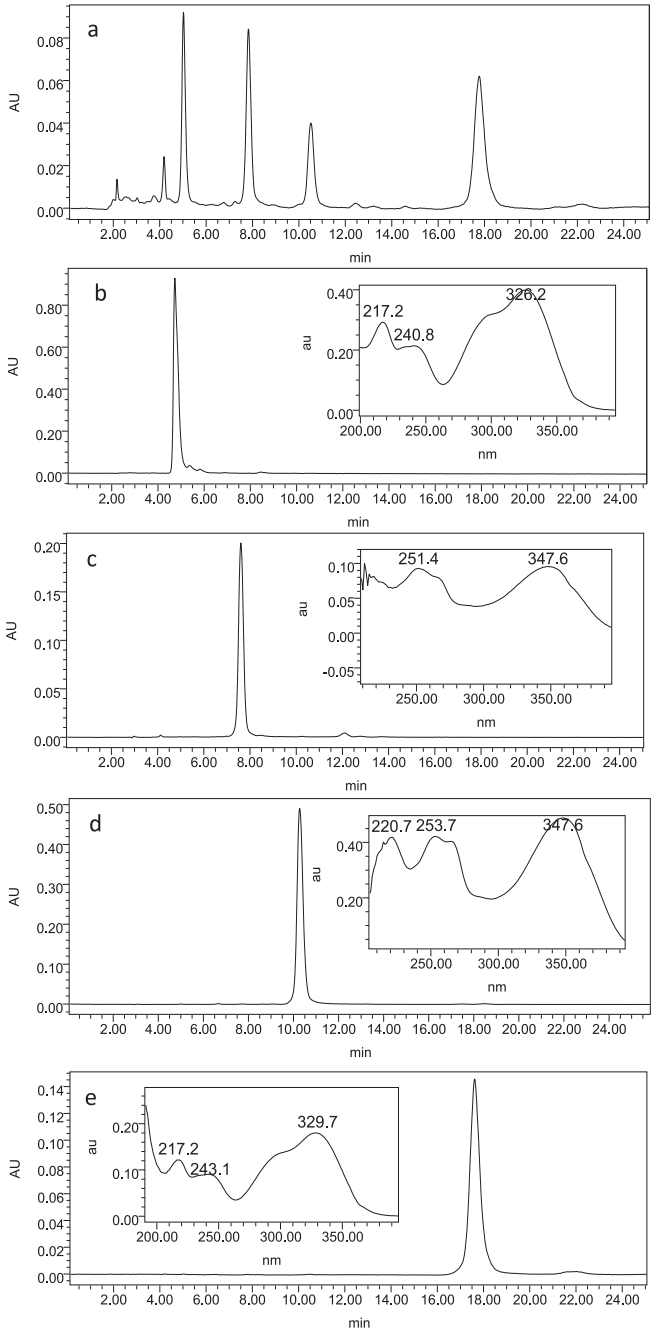

Figure 3. *HPLC chromatograms of (a) crude extracts from the pericarps of artichoke; (b) chlorogenic acid (I); (e) luteolin-7-O-β-D-rutinoside (II); (f) luteolin-7-O-β-D-glucoside (III); (g) cynarin (IV)*

such as sample polarity, solubility, ionic form, and ability to form complexes (Table 1). Because the compounds I, II, III, and IV could not be properly separated using a single step, they were separated by different solvent systems.

The K_D values of compounds I, II, and III are shown in Table 1. Because the K_D value of compound III in the two-phase solvent system constituted by ethyl acetate/n-butanol/water (2:1:3, v/v/v) was too large, the separation of compound III needed a long experimental time to elute and resulted in poor resolution. The K_D values of compound II and compound I in the solvent system of ethyl acetate/ethanol/water/acetic acid (4:1:5:0.2, v/v/v/v) were small and similar, thereby resulting in low purity. The K_D values of compounds I, II, and III in the solvent systems of ethyl acetate/n-butanol/ethanol/water/acetic acid (4:0.5:0.5:5:0.2, v/v/v/v/v) and ethyl acetate/n-propyl alcohol/ethanol/water/acetic acid (4:0.5:0.5:5:0.2, v/v/v/v/v) were suitable for achieving an appropriate separation of compounds I, II, and III from sample 2. However, when the solvent system composed of ethyl acetate/n-propyl alcohol/ethanol/water/acetic acid (4:0.5:0.5:5:0.2, v/v/v/v/v) was used, compounds I and II did not separate, thereby resulting in low purity. When the solvent system composed of ethyl acetate/n-butanol/ethanol/water/acetic acid (4:0.5:0.5:5:0.2, v/v/v/v/v) was used, the three compounds were separated with an acceptable resolution. The HSCCC chromatogram of sample 1 is shown in Figure 4(A).

As shown in Table 1, the two-phase solvent systems n-hexane/ethyl acetate/methanol/water (2:8:1:9, v/v/v/v), n-hexane/ethyl acetate/methanol/water (0.2% acetic acid) (2:8:1:9, v/v/v/v), and n-hexane/ethyl acetate/methanol/water (0.2% HCl) (2:8:1:9, v/v/v/v) were suitable for achieving an appropriate separation. When the solvent systems n-hexane/ethyl acetate/methanol/water (2:8:1:9, v/v/v/v) and n-hexane/ethyl acetate/methanol/water (0.2% acetic acid) (2:8:1:9, v/v/v/v) were used, baseline separation could not be achieved. When n-hexane/ethyl acetate/methanol/water (0.2% HCI) (2:8:1:9, v/v/v/v) was used, the baseline separation was achieved and the experimental time was acceptable. The HSCCC chromatogram of sample 2 was shown in Figure 4(B).

Both sample 1 and 2 were successfully separated and purified under optimum HSCCC conditions. A total of 7.8 mg of chlorogenic acid (compound I), 24.5 mg of luteolin-7-O-β-D-rutinoside (compound II) and 18.4 mg of luteolin-7-O-β-D-glucoside (compound III) were obtained from 150 mg of sample 1, and a total of 33.4 mg of cynarin (compound IV) from 150 mg was obtained from sample 2 with purities of 92.0%, 98.2%, 98.5%, and 98.0%, respectively as determined by HPLC. The HPLC chromatograms of compound I–IV are shown in Figure 3(b–e).

Structural identification

The chemical structure of each peak fraction of HSCCC was

Table 1. The K_D value of compound I-IV in different two-phase solvent systems

Solvent system (v/v)	K_{DI}	K_{DII}	K_{DIII}	K_{DIV}
ethyl acetate-n-butanol-water (2:1:3)	1.4	2.6	4.2	14.0
ethyl acetate-ethanol-water-acetic acid (4:1:5:0.2)	0.35	0.68	1.24	7.83
ethyl acetate-n-propyl alcohole-ethanol-water-acetic acid (4:0.5:0.5:5:0.2)	0.56	0.84	1.84	8.20
ethyl acetate-n-butanol-ethanol-water-acetic acid (4:0.5:0.5:5:0.2)	0.78	1.40	2.32	8.90
n-hexane-ethyl acetate-methanol-water (2:8:2:8)	-	-	-	0.56
n-hexane-ethyl acetate-methanol-water (2:8:1:9)	-	-	0.11	0.72
n-hexane-ethyl acetate-methanol-water (0.2% acetic acid)(2:8:1:9)	0.18	0.24	0.31	1.80
n-hexane-ethyl acetate-methanol-water (0.2% HCl)(2:8:1:9)	0.23	0.38	0.57	2.30

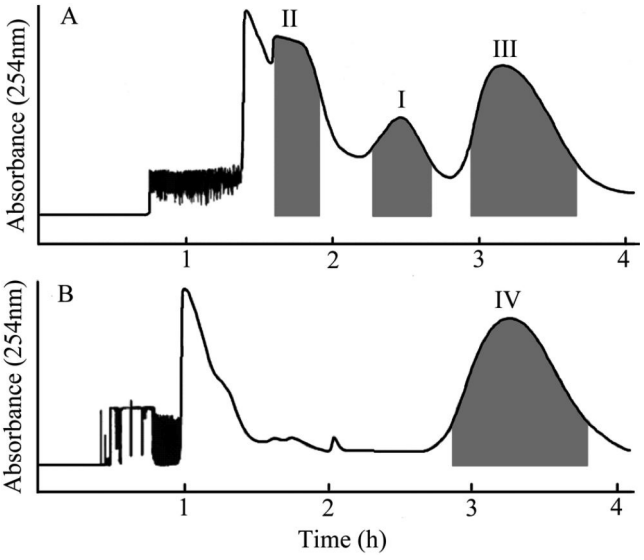

Figure 4. *HSCCC chromatograms of sample 1 (A) and sample 2 (B). The two-phase solvent system for A was ethyl acetate/n-butanol/ethanol/water/acetic acid (4:0.5:0.5:5:0.2, v/v/v/v/v), while for B it was n-hexane/ethyl acetate/methanol/water (0.2 % HCl) (2:8:1:9, v/v/v/v). Flow rate: 2.0 mL/min, revolution speed: 800 rpm; detection wavelength: 254 nm; retention of the stationary phase: A 42 %, B 65 %; Peak I: chlorogenic acid, Peak II: luteolin-7-O-β-D-rutinoside, Peak III: luteolin-7-O-β-D-glucoside, Peak IV: cynarin*

solved by ESI-MS and ^{1}H- and ^{13}C-NMR.

Compound I [peak I in Fig. 4(A)]: Positive ESI-MS, m/z 354.2 $[M+H]^+$. ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 7.42 (1H, d, J = 15 Hz, H-7), 6.15(1H, d, J = 15 Hz, H-8), 7.05 (1H, d, J = 21 Hz, H-2), 6.99 (1H, d, J = 8.2 Hz, H-5), 6.77 (1H, d, J = 8.2 Hz, H-5), 5.07 (1H, m, H-3), 3.93 (1H, m, H-4), 3.42(1H, m, H-5), 2.02~1.77 (2H, H-2, 6). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 175.0 (C-7), 165.8 (C-9›), 148.4 (C-4›), 145.6 (C-3›), 145.0 (C-7›), 125.6 (C-1›), 121.4 (C-6›), 115.8 (C-5›), 114.8 (C-2›), 114.3 (C-8›), 73.5 (C-1), 70.9 (C-5), 70.3 (C-4), 68.0 (C-3), 37.2 (C-2), 36.2 (C-6). Comparing the results with reference data,[14] compound I was identified as chlorogenic acid, which was confirmed by HPLC using a reference standard material.

Compound II [peak II in Fig. 4(A)]: Positive ESI-MS, m/z 594.5 $[M+H]^+$. ^{1}H-NMR(600 MHz, DMSO) δ: 12.98 (1H, OH), 7.45 (1H, d, J = 2.0 Hz, H-2'), 7.41 (1H, d, J = 8.0, 2.0 Hz, H-6'), 6.92 (1H, d, H-5'), 6.73 (1H, d, J = 1.8 Hz, H-8), 6.45 (1H, d, J = 2.0 Hz, H-6), 5.07 (1H, d, J = 7.2 Hz, anomeric H of Glc), 4.65 (1H, s, anomeric H of Rha), 1.07 (3H, d, J = 6.2 Hz, Rha-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 181.8 (C-4), 164.3 (C-2), 162.7 (C-7), 161.3 (C-5), 156.8 (C-9), 149.7 (C-4›), 145.6 (C-3›), 121.3 (C-1›), 119.1 (C-6›), 116.2 (C-5›), 113.4 (C-2›), 105.3 (C-10), 103.2 (C-3), 99.8 (C-6), 94.7 (C-8), Glc-99.8; (glc C-1), 77.1 (glc C-5), 76.4 (glc C-3), 73.1 (glc C-2), 69.5 (glc C-4), 60.6 (glc C-6); Rha-99.9 (rha-1), 72.0 (rha-4), 70.7 (rha-3), 70.3 (rha-2), 68.3 (rha-5), 17.8 (rha-6). Comparing the results with reference data,[15] compound II was identified as luteolin-7-O-β-D-rutinoside.

Compound III [peak III in Fig. 4(A)]: Positive ESI-MS, m/z 448.2 $[M+H]^+$. ^{1}H-NMR (600 MHz, DMSO-d_6) δ: 5.10 (1H, d, J = 7.0 Hz, H-1''), 6.45 (1H, d, J = 2.1 Hz, H-6), 6.77 (1H, s, H-3), 6.80 (1H, d, J = 8.2Hz, H-8), 6.92 (1H, d, J = 8.2 Hz, H-5'), 7.43–7.45 (2H, m, H-2', 6'), 9.43 (1H, brs, H-3'),10.03 (1H, s, H-4'), 13.00 (1H, s, H-5). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 181.9 (C-4), 164.4 (C-2), 162.9 (C-7), 161.1 (C-5), 156.9 (C-9), 149.9 (C-4›), 145.8 (C-3›), 121.4 (C-1›), 119.2 (C-6›), 116.0 (C-5›), 113.6 (C-2›), 105.3 (C-10), 103.2 (C-3), 99.8 (C-6), 99.8 (glc C-1), 94.7 (C-8), 77.1 (glc C-5), 76.4 (glc C-3), 73.1 (glc C-2), 69.5 (glc C-4), 60.6 (glc C-6). Comparing the results with reference data,[4] compound III was identified as luteolin-7-O-β-D-glucoside.

Compound IV [peak IV in Fig. 4(B)]: Positive ESI-MS, m/z 516.3 $[M+H]^+$. ^{1}H-NMR (600MHz, CD_3OD) δ: 7.48 (1H, d, J = 16.0 Hz), 7.44 (1H, d, J = 16.0 Hz), 7.01 (2H, d, J = 1.6Hz), 6.95 (2H, m), 6.76 (2H, d, J = 8.0 Hz), 6.25 (1H, d, J = 16.0 Hz), 6.19 (1H, d, J = 16.0 Hz), 5.20 (1H, m), 4.17 (1H, m), 3.75 (1H, dd, J = 9.6, 3.2 Hz), 2.43 (2H, m), 2.24 (1H, dd, J = 15.6, 3.6 Hz). ^{13}C-NMR (125 MHz, CD_3OD) δ: 34.9 (C-2), 40.3 (C-6), 68.6 (C-5), 72.1 (C-3), 75.4 (C-4), 81.2 (C-1), 173.8 (C-7), 114.8, 115.3 (2 × C-8›), 115.4, 115.8 (2 × C-2›), 116.4, 116.4 (2 × C-5›), 121.3, 121.7 (2 × C-6›), 125.9, 126.1 (2 × C-1›), 144.8, 145.4 (2 × C-3›), 146.1, 146.2 (2 × C-7›), 7.4, 149.0 (2 × C-4›), 165.5, 166.6 (2 × C-9›), Comparing the results with reference data,[16] compound IV was identified as cynarin.

CONCLUSION

The results of this study clearly demonstrate that polyamide column chromatography combined with HSCCC is an efficient method for the separation and purification of polyphenols from the crude extracts of artichoke. Four bioactive compounds found in artichoke, such as chlorogenic acid (I), luteolin-7-O-β-D-rutinoside (II), luteolin-7-O-β-D-glucoside (III) and cynarin (IV), were successfully separated. The obtained compounds could be used as reference substances for chromatographic purposes as well as for further pharmaceutical studies. The method might also be successfully applied to the separation of various other polyphenols from natural products.

ACKNOWLEDGEMENTS

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