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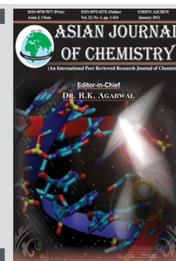

Preparative Isolation and Purification of Phlorizin from Apple Tree Bark by High-Speed Counter-Current Chromatography

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Phlorizin, abundantly existed in apple tree bark, has been found to be highly active in transporter and antioxidant activity. Using high-speed counter-current chromatography, the separation and purification of phlorizin from the crude phlorizin extract of apple tree bark was achieved. It was performed at a preparative scale in one-step separation with a two-phase solvent system composed of chloroform-methanol-*n*-butanol-water (5:4:0.5:3, v/v). The lower phase was used as the mobile phase in the head-to-tail elution mode. By injecting 606 mg of the crude phlorizin extract, 89.5 mg of phlorizin with the purity of 99.0 % were yielded. Its chemical structure was confirmed by UV, FTIR, ESI-MS, ^{1}H and ^{13}C NMR analysis. Furthermore, the optimum extracting conditions of phlorizin from apple tree bark were also investigated by orthogonal test L_{16} (4^3).

Key Words: High-speed counter-current chromatography, Apple tree bark, Phlorizin.

INTRODUCTION

Apple tree bark is bitter and has also been employed in medicine. It is tonic and febrifuge and a decoction of it has been used with advantage in intermittent, remittent and bilious fevers and in convalescence from exhausting diseases. Phlorizin, which abundantly existed in apple tree bark, is glycosylated derivative from phloretin. Its chemical structure is shown in Fig. 1. Phlorizin has good resistance to oxidation, inhibit the formation of pigment in cosmetics. In clinical research, phlorizin is tonic and antiperiodic and has cured cases of intermittent fever. It has unique efficacy on diabetic disease and a good role in lipid-lowering diet, which inhibits fat storage. It is also involved in inhibiting the growth of tumor cells[1-5].

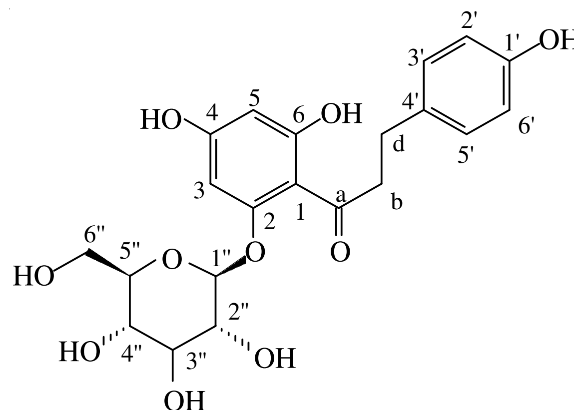

Fig. 1. Chemical structure of phlorizin

The conventional methods of preparative separating and purifying products were to utilize repeated chromatographic steps on silica gel, polyamide and Sephadex LH-20 column, which are easy to enlarge the scale of separation and separate different compounds entirely. However, these methods are tedious and time consuming and the overall yields of these methods are usually poor because the target compounds tend to be irreversibly absorbed onto the solid support during separation. Thus, it is necessary to set up an easy system capable of separating and purifying the bioactive compounds. High-speed counter-current chromatography (HSCCC) is one support-free technique which has gained growing importance in the separation of naturally occurring compounds[6]. Because of the absence of solid stationary phase, adsorption losses are minimized and excellent sample recovery is guaranteed. The crude sample can be directly injected into the column. As a result, the application of HSCCC in purification of natural products is steadily increasing[7-11]. This paper describes a convenient and successful method to prepare phlorizin with high purity from apple tree bark by HSCCC. The extract and isolation conditions were optimized. The chemical structure of the target compound was elucidated by UV, FTIR, ESI-MS, ^{1}H and ^{13}C NMR.

EXPERIMENTAL

The HSCCC instrument employed in this study is a TBE-300A high-speed counter-current chromatograph (Tauto Biotechnique, Shanghai, China) with three PTFE (polytetrafluoroethylene) multi-layer coil separation column connected in series (i.d. of the tubing = 1.8 mm, total volume = 260 mL) and a 20 mL sample loop. The revolution radius was 5 cm and the β of the multilayer coil varied from 0.6 at internal terminal to 0.8 at the external terminal (β = r/R where r is the distant from the coil to the holder shaft and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An MultiTemp III constant-temperature circulating implement (Amersham Biosciences Inc. USA) was used to control the separation temperature. The effluent was continuously monitored with a UV-Monitor ÄKTAprime plus (Amersham Biosciences Inc. USA) at 280 nm. The data were collected with a PrimeViewTM chromatography workstation (Amersham Biosciences Inc. USA). The two-phase solvent system was pumped into the column by an ÄKTAprime plus pump system (Amersham Biosciences Inc. USA).

Agilent 1100 LC system was used to analyze the crude phlorizin extract and HSCCC fractions, which included a G1310A QuatPump, a G1314A UV-VIS variable wavelength detector, a 20 µL manual-sampler and Agilent HPLC workstation.

Chloroform, *n*-butanol, *n*-hexane, ethyl acetate, ethanol and methanol were of HPLC grade (Institute of Jinan Chemical Industry, China). Water was purified by Cascada Lab Water Systems (18 MΩ cm, Pall Life Sciences, USA). Phlorizin was obtained from Sigma (USA). Fuji apple tree bark was collected from apple tree trunk after apple has matured in apple farm (China).

Selection of two-phase solvent system: Five kinds of solvent systems at different volume ratios were tested (Table-1). Briefly, approximately 3 mg of the crude phlorizin extract were put into a 10 mL test tube, to which 3.0 mL of each phase of the equilibrated two-phase solvent system were added. The tube was shaken vigorously for 5 min to equilibrate the sample thoroughly with the two phases and separated by centrifugation at 3000 × g for 10 min. Then, the same volume of each phase was evaporated to dryness under water bath. The residue was diluted with the mobile phase used in the HPLC analysis and analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of phlorizin in the upper phase divided by that in the lower phase.

Preparation of sample solution: Apple tree bark was dried to constant weight and then ground to powder (*ca.* 30 meshes). 10 g of the powder were extracted with 300 mL of 40 % aqueous ethanol solution under boiling water for 1 min. The mixture was filtered and the filtrate was concentrated to dryness by rotary evaporation at reduced pressure. The residue was extracted by 80 mL of ethyl acetate five times. All the extraction were combined and concentrated to dryness by rotary vaporization. 782 mg of crude phlorizin extract containing 19.2 % of phlorizin were yielded. The crude phlorizin extract was stored in a refrigerator (-4 ºC) for the subsequent HSCCC separation.

The sample solution was prepared by dissolving the crude phlorizin extract in a 20 mL mixture solution of the upper and lower phases (1:1, v/v) of the two-phase solvent system used for HSCCC separation.

Separation procedure: The multilayer coiled column was first entirely filled with the upper phase. Then, the apparatus was rotated at 850 rpm in the head-to-tail elution mode, while the lower phase was pumped into the column at a flow rate of 2 mL min^{-1}. The separation temperature was 10 ºC. After the mobile phase was front emerged and equilibrium was established in the column, 20 mL of the sample solution containing 606 mg of the crude phlorizin extract were injected. The effluent was continuously monitored at 280 nm and the chromatogram was recorded. Peak fractions were collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed by the volume of the stationary phase collected from the column after the separation was completed.

TABLE-1
PARTITION COEFFICIENT (K) VALUES OF PHLORIZIN IN DIFFERENT TWO-PHASE SOLVENT SYSTEMS

Solvent systems	Volume ratio	Partition coefficient (K)
n-Butanol-ethyl acetate-ethanol-water	1:1:1:1	0.03
n-Butanol-ethyl acetate-ethanol-water	1.5:2:1:1.5	0.22
n-Butanol-ethyl acetate-water	2:3:5	20.4
n-Butanol-ethyl acetate-water	3:2:5	8.24
Ethyl acetate-methanol-water	2:0.5:1	2.44
Ethyl acetate-methanol-water	2:0.5:2	3.08
Ethyl acetate–methanol-water	2:0.5:3	3.74
Chloroform-methanol-water	4:3:2	9.26
Chloroform-methanol-water	8:10:5	2.43
Chloroform-methanol-*n*-butanol-water	4:3:0.5:2	2.95
Chloroform-methanol-*n*-butanol-water	4:3:0.5:3	5.87
Chloroform-methanol-*n*-butanol-water	4:4:0.5:2	3.28
Chloroform-methanol-*n*-butanol-water	4:4:0.5:3	3.22
Chloroform-methanol-*n*-butanol-water	4:3:1:2	1.97
Chloroform-methanol-*n*-butanol-water	5:3:0.5:3	4.97
Chloroform-methanol-*n*-butanol-water	5:3:0.5:2	3.61
Chloroform-methanol-*n*-butanol-water	5:4:0.5:2	2.13
Chloroform-methanol-*n*-butanol-water	5:4:0.5:3	3.60

HPLC analysis and identification of target compound: The crude phlorizin extract and each fraction from HSCCC were analyzed by analytical HPLC, which was performed with an Agilent Eclipse XDB-C_{18} column (150 mm × 4.6 mm i.d., 5 μm) at 30 ºC. The separation was performed with an isocratic elution using methanol-water-acetic acid (30:69:1, v/v) at a flow rate of 1.0 mL min^{-1} and the effluent was monitored at 286 nm with VWD. The components were confirmed from their retention times. Routine sample calculations were made by comparison of the peak area with that of the standard. The analytical curve was c (μg L^{-1}) = $3.17 \times 10^{-5} \times A - 0.08$ where c is the concentration of phlorizin determined and A is the peak area of phlorizin. The linearity of the calibration curve was observed in the concentration range of 18 μg L^{-1} to 30 mg L^{-1} with a correlation coefficient of 0.9997.

The identification of the target compound was carried out by FTIR, electrospray ionization (ESI)-MS, ^{1}H and ^{13}C NMR spectra as well as comparing its HPLC retention time and UV spectra with that of standard sample. IR spectra were recorded as KBr disks on a Nicolet IR 200 spectrometer (USA). ESI-MS spectra were performed with a Mariner API-TOF (ABI, USA). ^{1}H and ^{13}C NMR spectra were recorded on JNM ECP-600 spectrometer (Jeol, Japan) with tetramethylsilane (TMS) as internal standard. All were run at room temperature.

RESULTS AND DISCUSSION

Optimization of sample extraction and HPLC analysis: Using orthogonal test L_{16} $(4^3)^{12}$, phlorizin was extracted from apple tree bark by reflux extraction with ethanol solvent. Total 16 experiments were conducted in present studies. (Table-2). Based on these experiments, the optimum extracting conditions of phlorizin were 40 % of alcoholic concentration, 1:30 ratio of solid/liquid, 1 min of extraction time.

TABLE-2
ORTHOGONAL EXPERIMENT OF REFLUX EXTRACTION

	Alcoholic concentration	Ratio of solid/liquid	Extraction time (min)	Content of phlorizin (mg/g)
1	30%	1:10	Just boiling	15.12
2	30%	1:15	0.5	20.52
3	30%	1:20	1.0	22.44
4	30%	1:30	5.0	24.83
5	40%	1:10	0.5	17.12
6	40%	1:15	Just boiling	22.63
7	40%	1:20	5.0	24.25
8	40%	1:30	1.0	27.12
9	50%	1:10	1.0	19.78
10	50%	1:15	5.0	21.06
11	50%	1:20	Just boiling	21.01
12	50%	1:30	0.5	24.92
13	60%	1:10	5.0	13.02
14	60%	1:15	1.0	23.46
15	60%	1:20	0.5	24.73
16	60%	1:30	Just boiling	24.30
K_1	20.73	16.26	20.76	–
K_2	22.78	21.92	21.82	–
K_3	21.69	23.11	23.20	–
K_4	21.38	25.29	20.79	–
R	2.05	9.03	2.44	–

Based on the good solubility of phlorizin in ethyl acetate, ethyl acetate was used as the extractor to increase the purity of phlorizin, while the amount of impurities was reduced. In the present work, 1997 mg of the residue, which contained 166.9 mg of phlorizin, were extracted by ethyl acetate. 782 mg of the crude phlorizin extract were obtained, which contained 150 mg of phlorizin. The loss of phlorizin was 10.1 %. The result showed that phlorizin could be well extracted by ethyl acetate while the impurities were reduced to facilitate HSCCC analysis.

Reversed-phase C_{18} column was used in HPLC analysis. Phlorizin tended to appear as a tailing peak due to the residual silanols on the stationary phase. In order to avoid its adsorption, methanol was used as the mobile phase while acetic acid was added to restrain ionization. It was found that an isocratic elution consisted of methanol-water-acetic acid (30:69:1, v/v) achieved excellent separation at 30 ºC. UV detection was set at 286 nm. Under these conditions, the crude phlorizin extract was separated and determined (Fig. 2). Although there was much interference present in the crude phlorizin extract, they did not affect the separation and determination of phlorizin.

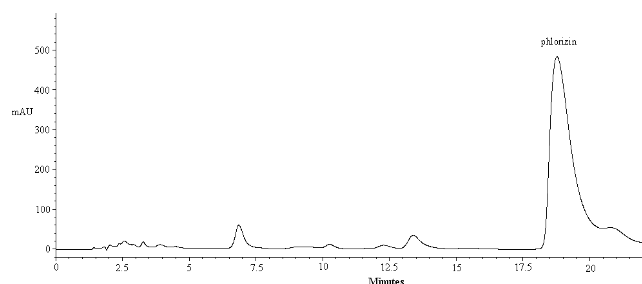

Fig. 2. HPLC chromatogram of the crude phlorizin extract

Selection of two-phase solvent system: The first step in a HSCCC experiment is to select a good solvent system, which can provide an ideal partition coefficient (K). A much smaller K value elutes the solute closer to the solvent front with lower resolution, while a much larger K value tends to give better resolution but broader, more peaks due to a longer elution time. In this paper, a series of solvent systems with a broad range of hydrophobicities were tested. Their K-values of phlorizin in these solvent systems were summarized in Table-1. When *n*-butanol-ethyl acetate-ethanol-water (1:1:1:1, 1.5:2:1:1.5, v/v) were used as the two-phase solvent systems, their K-values were small. It was difficult to separate phlorizin from the other compounds. When *n*-butanol-ethyl acetate-water (2:3:5, 3:2:5, v/v) were used as the two-phase solvent systems, their K-values were too large which phlorizin was eluted in an excessively broad peak with a long elution time. When ethyl acetate-methanol-water (2:0.5:1, 2:0.5:2, 2:0.5:3, v/v) were used as the two-phase solvent systems, their K-values were suitable. But phlorizin could not be separated from the other compounds. When chloroform-methanol-water (4:3:2, 8:10:5, v/v) and chloroform-methanol-*n*-butanol-water (4:3:0.5:2, 4:3:0.5:3, 4:4:0.5:2, 4:4:0.5:3, 4:3:1:2, 5:3:0.5:3, 5:3:0.5:2, 5:4:0.5:2, 5:4:0.5:3, v/v) were used with bigger K-values, phlorizin could be separated from the other compounds. Among them, the two-phase solvent system composed of chloroform-methanol-*n*-butanol-water (5:4:0.5:3, v/v) was found to be the best. Although the K-value was 3.6, phlorizin could be well separated with the impurities and the

retention percentage of the stationary phase was of a high level.

Other factors, such as the flow rate of the mobile phase, the temperature and the revolution speed of the separation column, were also investigated. The result showed that the optimum separating conditions were 2.0 mL min^{-1} of the flow rate, 850 rpm of revolution speed and 10 ºC of column temperature.

Separation of phlorizin by HSCCC: A 606-mg quantity of the crude phlorizin extract was dissolved in 20 mL of both phases. The sample solution was separated and purified by HSCCC using chloroform-methanol-*n*-butanol-water (5:4:0.5:3, v/v) as the two-phase solvent system. The separation was performed at a preparative scale in one-step separation. The total separation time was 420 min (Fig. 3a). Based on the HPLC analysis and the chromatogram of the preparative HSCCC, peak 1 was corresponded to phlorizin. 89.5 mg of phlorizin at 99.0 % purity were yielded. Its HPLC chromatogram and UV spectrum were shown in Fig. 3b. The recovery of phlorizin was 76.9 %. The retention percentage of the stationary phase was 73 %.

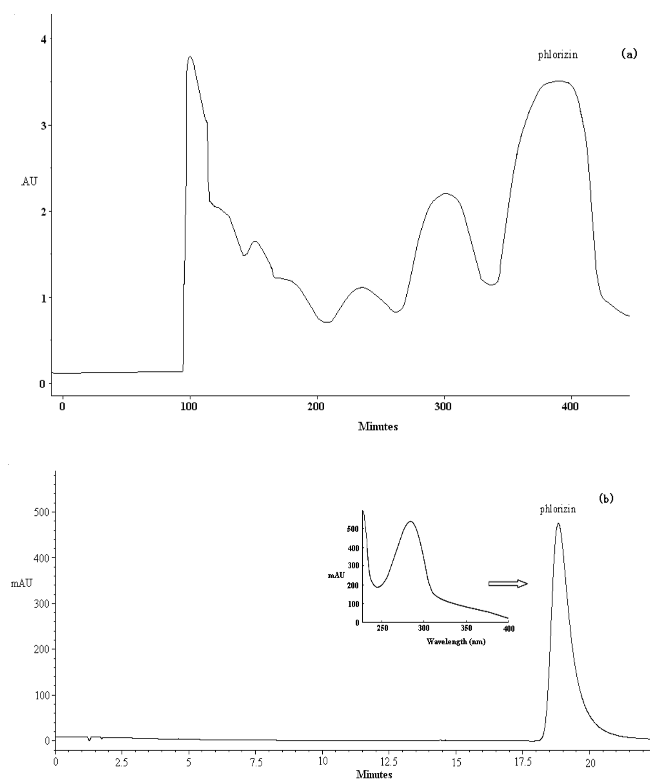

Fig. 3. HSCCC chromatogram of the crude phlorizin extract from apple tree bark (a) and HPLC chromatogram of the purified product (b)

Structural identification: The identification of target compound was carried out by UV, FTIR, MS, ^{1}H and ^{13}C NMR analysis as follows: UV: λ_{max} (MeOH): 286 nm; FTIR (cm^{-1}): 3383(OH), 1640 (C=O), 1580, 1450 (Ar), 1200 (Ar-O-C), 1087 (C-OC); ESI-MS m/z: 437.1 [M + H]$^{+}$; ^{1}H NMR (600 MHz, DMSO-d_6): 13.52 (s, 1H, 6-OH), 10.59 (s, 1H, 4-OH), 9.12 (s, 1H, 1'-OH), 7.03 (d, 2H, 4', 5'-H), 6.64 (d, 2H, 2',6'-H), 6.12 (d, 1H, 3-H), 5.92 (d, 1H, 5-H), 5.31 (d, 1H, 1''-H), 5.17 (d, 1H, 2''-OH), 5.07 (d, 1H, 4''-OH), 4.93 (d, 1H, 3''-OH), 4.61 (t, 1H, 6''-OH), 3.39 (m, 1H, 5''-H), 3.32 (m, 1H, 4''-H), 3.27 (m, 2H, b-H), 3.19 (m, 1H, 3''-H), 2.78 (t, 2H, d-H); ^{13}C NMR: 204.72, 165.38, 164.45, 160.85, 155.27, 131.53, 129.18, 114.97, 105.13, 100.79, 96.79, 94.29, 77.25, 76.66, 73.18, 69.39, 60.37, 44.97, 34.67. On basis of all these results, the structure of target compound was identified as phlorizin[13].

Conclusion

An efficient high-speed counter-current chromatography method was developed for the purification and separation of phlorizin from apple tree bark. In present study, using chloroform-methanol-*n*-butanol-water (5:4:0.5:3, v/v) as the two-phase solvent system, 89.5 mg of phlorizin was yielded from 606 mg of the crude phlorizin extract in a one-step separation. The purity of phlorizin was 99.0 %. Its structural identification was carried out by FTIR, ESI-MS, ^{1}H and ^{13}C NMR analysis.

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