Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 22 March 2010

To cite this Article Jiang, Xinyu , Shi, Shuyun , Zhang, Yuping and Chen, Xiaoqing(2010) 'Activity-Guided Isolation and Purification of Three Flavonoid Glycosides from *Neo-Taraxacum siphonanthum* by High-Speed Counter-Current Chromatography', Separation Science and Technology, 45: 6, 839 — 843
To link to this Article: DOI: 10.1080/01496390903563116
URL: http://dx.doi.org/10.1080/01496390903563116

Separation Science and Technology, 45: 839–843, 2010

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390903563116

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Activity-Guided Isolation and Purification of Three Flavonoid Glycosides from *Neo-Taraxacum siphonanthum* by High-Speed Counter-Current Chromatography

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DPPH (1,1-diphenyl-2-picryhydrazyl) radical scavenging assay was used to screen different fractions of *Neo-Taraxacum siphonanthum* ethanol extracts. The potent active fraction was isolated and purified by preparative high-speed counter-current chromatography (HSCCC) with a solvent system composed of *n*-hexane-*n*-butanol-water (3:4:7, v/v/v). The flow rate was 1.5 mL/min and resolution speed was 800 rpm. Three flavonoid glycosides with the purity over 99% were obtained and identified as luteolin-3′-*O*-*β*-D-glucopyranoside (I), luteolin-7-*O*-*β*-D-glucopyranoside (II), and luteolin-4′-*O*-*β*-D-glucopyranoside (III) by ESI-MS, ^{1}H NMR and ^{13}C NMR analysis. Antioxidant activity of three flavonoid glycosides was assessed by DPPH assay, all of which showed potent activity.

Keywords activity-guided; flavonoid glycosides; HSCCC; *Neo-Taraxacum siphonanthum*

INTRODUCTION

Neo-Taraxacum (Neo-T.) siphonanthum (Asteraceae) is a species found in Inner Mongolia, China, 1989, which is the only one subjected to a new genus, Neo-Taraxacum genus, because of the different shape of the ligulate flowers compared with those in Taraxacum genus (1,2). This species has commonly been used by Chinese local people for dietary purpose, and especially medicinal purpose for treating inflammatory disorders and viral infectious diseases. However, to the best of our knowledge, there has been only one recently published report on the chemical constituents and antioxidant properties of this species (3). Our previous research showed the crude extract of *Neo-T. siphonanthum* exhibited a high DPPH radical scavenging activity, and therefore it might be a good candidate for further development as antioxidant remedies. Moreover, antioxidants have received a great amount of attention as being primary preventive ingredients against various diseases (4). Therefore, further chemical and pharmacological research of *Neo-T. siphonanthum* is warranted.

Fractionation of plant extracts for active compounds is a time-consuming, labor intensive, and expensive process, and often leads to loss of activity during the isolation and purification procedures due to dilution effects or decomposition (5). Therefore, an activity-guided method was developed for screening complex mixtures for radical scavenging components from complex extracts (6–9). Such techniques allowed for a rapid detection of radical scavenging substances in the presence of many inactive constituents. Meanwhile, HSCCC has been successfully applied for the separation and purification of various natural products as an easy system capable of separating the bioactive compounds (10,11). Wu et al. (12) isolated three flavonoids from *Flos gossypii* by preparative HSCCC. Semi-preparative HSCCC also was used for isolation and purification of three flavonoid glycosides from the leaves of *Nelumbo nucifera* (Lotus) by using a two-phase-solvent system composed of *n*-hexane-ethyl acetate-methanol-water (13).

In this paper, a method composed of an activity-guided antioxidant screening and HSCCC was used to separate and purify three flavonoid glycosides: luteolin-3′-*O*-*β*-D-glucopyranoside (I), luteolin-7-*O*-*β*-D-glucopyranoside (II), and luteolin-4′-*O*-*β*-D-glucopyranoside (III) (Fig. 1) with high purity from *Neo-T. siphonanthum*. The best separation conditions for HSCCC were optimized after the investigation of the effects of the two-phase solvent system, the flow rate, and the resolution speed. The chemical structures of the three active flavonoid glycosides were elucidated by ESI-MS, ^{1}H NMR, and ^{13}C NMR analysis.

Compound I with a trivial name such as dracocephaloside was first isolated from *Dracocephalum thymiflorum* (14), and was also found in plants such as *Carduncellus mareoticus* (Del.) hanelt (15), and *Callicarpa nudiflora* (16). Compound III with a trivial name as juncein was first isolated from *Spartium junceum* (17), and was also found in *Callicarpa nudiflora* (16). Compound II with a trivial name as cynaroside, which can be found in dandelion coffee,

Received 1 June 2009; accepted 29 November 2009.
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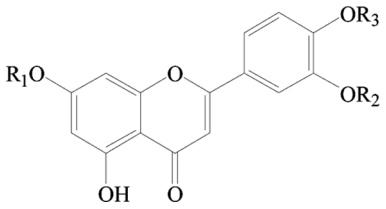

(I) $R_1 = R_3 = H$; R_2 =β-D-glucopyranoside
(II) R_1 =β-D-glucopyranoside; $R_2 = R_3 = H$
(III) $R_1 = R_2 = H$; R_3 =β-D-glucopyranoside

FIG. 1. The chemical compounds of luteolin-3′-*O*-*β*-D-glucopyranoside (I), luteolin-7-*O*-*β*-D-glucopyranoside (II) and luteolin-4′-*O*-*β*-D-glucopyranoside (III).

Nelumbo nucifera Gaertn (18), and *Ixeris chinensis* Nakai (19), has significant antimicrobial, anti-inflammatory, and analgesic activites (18). As far as we know, this is the first report to isolate and purify flavonoid glycosides from the plant of genus Neo-Taraxacum.

EXPERIMENTAL

Chemicals and Reagents

Ethanol, *n*-hexane, *n*-butanol, and methanol for the preparation of active fraction and HSCCC separation were analytical grade and purchased from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Methanol used for analytical HPLC was of chromatographic grade (Merk, Darmatadt, Germany). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Bedford, MA, USA). D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China), which was a cross-linked polystyrene copolymer. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·, 95%) was bought from Sigma-Aldrich (Steinheim, Germany), and DPPH radical solutions were freshly prepared in methanol every day and kept protected from light. Multi-well plates (Greiner) and multi-well plates readers (Bio-Tek Instruments, USA) were used in the antioxidant activity experiments. *Neo-Taraxacum siphonanthum* was purchased from Bozhou, Anhui province in November, 2006, and identified by Prof. Juanhua Xu, College of Pharmaceutical Sciences, Zhejiang University.

Apparatus

The preparative HSCCC was performed on a seal-free high-speed counter-current chromatography (Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China). The apparatus was equipped with a polytetrafluoroethylene (PTFE) multilayer coiled column with an average I.D. of 2.6 mm and a total volume of 420 mL. The column revolves around its own axis at the angular velocity in the same direction (20). The revolution speed of the apparatus could be regulated between 0 and 1000 rpm. The revolution radius or the distance between the holder axis and central axis of the centrifuge was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79. $\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge. The solvent was pumped into the tubing with a FMI pump (Zhejiang Instrument Factory, Hangzhou, China). The effluent was continuously monitored with a variable wavelength PC300 detector at 254 nm and the chromatogram with a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China). A manual sample injection valve with a 20 mL loop was connected to the system.

The analytical HPLC used consisted of two LC-8A pumps, a Prominence SPD-M20A diode array detector performing the wavelength scanning from 190 to 950 nm, a manual injection valve with a 20 μL loop, and an LC Solution workstation (Shimadzu, Japan).

Preparation of Extracts

Air-dried and pulverized whole plant of *Neo-T. siphonanthum* (2.0 Kg) was extracted with 95% ethanol under reflux for 3 h and concentrated under reduced pressure to give a brown syrup (217 g). A portion of this syrup (200 g) was then subjected to column chromatography (25.0 cm × 200 cm, contained 3.0 kg D101 macroporous resin) and eluted with MeOH-H_2O step gradients to yield five main fractions: F_1 (10% methanol aqueous solution, 23 g), F_2 (30% methanol aqueous solution, 27 g), F_3 (50% methanol aqueous solution, 12 g), F_4 (70% methanol aqueous solution, 15 g), and F_5 (90% methanol aqueous solution, 9 g).

HPLC Analysis

Samples were analyzed by using a reversed phase Symmetry® C_{18} (250 mm × 4.6 mm i.d., 5 μm, Milford, MA, USA) column and a security guard C_{18} ODS (4.0 mm × 3.0 mm i.d.) from Phenomenex (Torrance, California, USA). The mobile phase was consisted of A (0.1% aqueous acetic acid) and B (methanol), which was programmed as follows: from 0 to 8 min, 20% B (80% A), 8–25 min, linear increase from 20% to 40% B (80% to 60% A). The flow rate was 0.8 mL/min while the ambient temperature was controlled at 25°C by air conditioner. Spectra were recorded from 200 to 500 nm (peak width

0.2 min and data rate 1.25 s^{-1}) while the chromatogram was acquired at 254 nm.

The pooled fraction was concentrated by a rotary evaporator and each fraction was analyzed by analytical HPLC to check the purity prior to characterization. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks.

Preparation of Two-Phase Solvent System for HSCCC

The solvent system for HSCCC separation was selected according to the difference of partition coefficients (K) of three active compounds in various solvent systems. The partition coefficient was calculated by HPLC peak area of each active compound in the upper phase and lower phase when a crude sample was added into a two-phase solvent system. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use. The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

HSCCC Separation Procedure

HSCCC was performed as follows: the multiplayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase was pumped into the inlet of the column at the flow rate of 1.5 mL/min, while the apparatus was rotated at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, a sample (500 mg) dissolved in 20 mL of the upper phase was injected into the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm and the peak fractions were collected manually according to the chromatographic profile. After the target compounds were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase. The effluent was collected for purity analysis.

Identification of the Fractions

Identification of the target compounds was accomplished by their mass data and NMR spectra. ESI-MS data were measured on an Apex III instrument (Bruker Daltonics Corporation, USA). The ^{1}H NMR and ^{13}C NMR experiments were performed on a VARIAN INOVA-400 (Varian Corporation, USA) NMR spectrometer using DMSO-d_6 as solvent. The reference compound TMS was used as internal standard for the determination of chemical shifts.

DPPH Radical Scavenging Assay

The DPPH radical scavenging assay was performed as described (21). The free radical scavenging efficiency of the compounds was determined by decoloration of the DPPH radical. In brief, 25 μL of diluted sample (4 mg/mL dissolved in DMSO) mixed with 40 μL DPPH· methanol solution (0.4 mg/mL) and made up with methanol to a final volume of 250 μL. The methanol solution of the DPPH was served as a control. The absorbance was measured at 517 nm after the mixture was incubated at 37°C for 30 min. The antiradical activity is expressed as a percentage of the DPPH radical elimination calculated according to the following formula: $[(A_{blank}\text{-}A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the DPPH radical solution and A_{sample} is the absorbance of the DPPH radical solution after the addition of the sample. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage. All tests were run in triplicate, and the average value was calculated.

RESULTS AND DISCUSSION

Antioxidant Activity of Different Fractions of *Neo-T. siphonanthum*

The ethanol extract of *Neo-T. siphonanthum* was separated by D101 column chromatography to yield five fractions, and the radical scavenging activity of each fraction was evaluated using a DPPH radical scavenging assay. The F_3 fraction (50% ethanol aqueous solution) showed potent capacity to scavenge DPPH radical compared with other fractions (Table 1), then HSCCC was applied to isolate the active compounds from this active fraction.

Compounds Isolated by HSCCC

HSCCC, first invented by Ito (22), is a support-free liquid-liquid chromatographic technique with no solid support matrix, and separation is based on fast partitioning effects of the substances between two immiscible liquid phases. Therefore, the selection of an appropriate solvent system is the most important, and is also the most difficult

TABLE 1
DPPH radical scavenging capacity of fractions recovered from the ethanol extract of *Neo-T. siphonanthum*

Components	Concentration (mg/mL)	DPPH radical inhibition (%)[a]
F_1	4	5.7 ± 0.8
F_2	4	18.2 ± 1.1
F_3	4	43.1 ± 3.5
F_4	4	27.6 ± 1.2
F_5	4	20.4 ± 2.4

[a]Each value is the mean of triplicate measurements and the standard deviation (SD).

step in HSCCC separation. A suitable two-phase solvent system requires the partition coefficient (K) of the target compound should be close to 1 to get an efficient separation and a suitable run time. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in the loss of peak resolution; if the K value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time (23).

Since *n*-butanol is a good solvent for dissolving compounds with high polarity, a series of solvent systems based on *n*-butanol and water were made up for the partition coefficient experiments. Table 2 shows K values for three target compounds in different systems. Among them, *n*-hexane-*n*-butanol-water at ratio of 3:4:7 (v/v/v) was found to be satisfactory for the separation of the three target compounds from the enriched extract with a short retention time.

Other factors such as the flow rate of the mobile phase and the resolution speed of the separation column were also investigated. The results showed that a stationary phase of 52.4% could be obtained and a good separation could be achieved using a 420 mL capacity column when the flow rate was 1.5 mL/min and the rotation speed was 800 rpm.

Under the optimum conditions, three fractions (I-III) were obtained in one-step elution and less then 6 h as shown in Fig. 2a, which is 26.3 mg of fraction I (collected during 145–180 min), 7.8 mg of fraction II (collected during 200–225 min), and 49.5 mg of fraction III (collected during 238–280 min), and the HPLC analysis of each HSCCC fraction revealed that pure compound could be obtained from the enriched extract. The purity of these compounds was 99.5%, 99.2%, and 99.4%, respectively (Fig. 2b).

TABLE 2
The partition coefficients (K) of the target components in different ratio of volume in *n*-hexane-*n*-butanol-water solvent system (component I, luteolin-3′-*O*-*β*-D-glucopyranoside; component II, luteolin-7-*O*-*β*-D-glucopyranoside; component III, luteolin-4′-*O*-*β*-D-glucopyranoside)

n-Hexane-*n*-butanol-water (v/v/v)	K		
	Component I	Component II	Component III
1:4:5	2.02	2.75	3.91
2:4:6	1.31	1.82	2.89
3:4:7	0.48	0.98	1.62
4:4:8	0.26	0.58	0.85
5:4:9	0.17	0.36	0.51

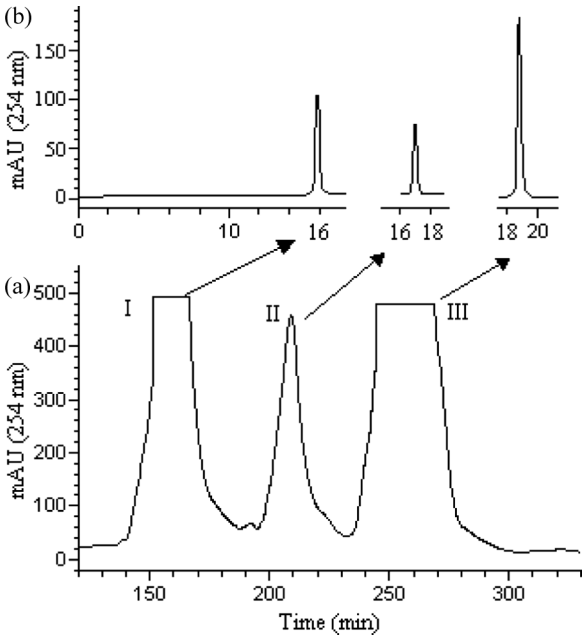

FIG. 2. (a) Preparative HSCCC separation of the enriched sample from *Neo-T. siphonanthum*. (b) HPLC chromatogram of HSCCC fractions. Fractions I, II, and III correspond to luteolin-3′-*O*-*β*-D-glucopyranoside, luteolin-7-*O*-*β*-D-glucopyranoside and luteolin-4′-*O*-*β*-D-glucopyranoside.

Identification and Antioxidant Activity of Target Compounds

Identification of each HSCCC fraction was carried out by ESI-MS, ^{1}H NMR and ^{13}C NMR. Three target compounds were identified as luteolin-3′-*O*-*β*-D-glucopyranoside (I), luteolin-7-*O*-*β*-D-glucopyranoside (II), and luteolin-4′-*O*-*β*-D-glucopyranoside (III), which were in agreement with published data (24–26). All the three flavonoid glycosides were isolated from this plant for the first time.

Flavonoids are well known to occur in plant extracts and to possess many different biological activities besides antioxidant activity (27–29). The DPPH radical scavenging activity of the three target compounds was evaluated. The result showed that the compounds I-III possessed potent-free radical scavenging capacities with IC_{50} values of 13.12, 9.58, and 13.65 μM, respectively. Flavonoids with free hydroxyl groups act as free radical-scavengers, and multiple hydroxyl groups, especially on the B-ring, enhance their antioxidant activity, and the glycosylation of 3' or 4′-hydroxyl group tended to reduce the activity (30–32). In the three compounds, compound II has two hydroxyl groups at the B-ring, while compounds I and III have only one hydroxyl group at the B-ring. Therefore, compound II showed the highest degree of free radical scavenging activity.

CONCLUSIONS

Activity-guided HSCCC was used for screening and purification of radical scavengers from active fraction of *Neo-T. siphonanthum* with a solvent system composed of *n*-hexane–*n*-butanol–water (3:4:7, v/v/v). The results obtained revealed the presence of three active flavonoid glycosides in the active extract of *Neo-T. siphonanthum*: luteolin-3′-*O*-*β*-D-glucopyranoside, luteolin-7-*O*-*β*-D-glucopyranoside and luteolin-4′-*O*-*β*-D-glucopyranoside. All of them were isolated from *Neo-T. siphonanthum* for the first time, and may be used as reference substances for chromatographic purpose without additional cleanup. The described method has a broad applicability and is rapid and robust for screening and preparing antioxidants from crude plant extracts.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 20775092) and the China Postdoctoral Science Foundation (No. 2080440991).

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