Purification of Rutin and Nicotiflorin from the Flowers of *Edgeworthia chrysantha* Lindl. by High-Speed Counter-Current Chromatography

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Abstract

An ethanol extract of air-dried flowers of *Edgeworthia chrysantha* Lindl. was partitioned between water and petroleum, ethyl acetate, and *n*-butanol. The *n*-butanol extraction was initially purified by silica gel column chromatography to give a partially purified sample. The bioactive compound rutin, along with nicotiflorin, were successfully separated from the partially purified sample by high-speed counter-current chromatography. The two compounds were isolated from the plant of *Edgeworthia* genus for the first time. The two-phase solvent system used was composed of ethyl acetate–*n*-butanol–water at an optimized ratio of 4:1:5 (v/v/v). High-speed counter-current chromatography yielded, from 108 mg of the partially purified extract, 53 mg rutin and 32 mg nicotiflorin with 92.5% and 92.2% recovery, with each at over 96.5% purity by high-performance liquid chromatography analysis. Their structures were identified by ^{1}H NMR and ^{13}C NMR.

Introduction

Edgeworthia chrysantha Lindl *(syn. E. papyrifera S. et Z.; Thymelaceae)* is distributed in eastern Asia. The flowers of *E. chrysantha* Lindl. can be used for treating eye illness, and they have been used as the crude drug "meng hua" in China (1,2). In the course of our studies of the chemical constituents of the plant, we investigated the flavone components of an ethanol extract of the flowers. The flavone glycosides components rutin and nicotiflorin were isolated from the plant of the whole *Edgeworthia* genus for the first time. Rutin (2-phenyl-3,5,7,3',4'-pentahydroxy benzopyrone), also known as vitamin P, has proved to be helpful in preventing cerebral hemorrhage by increasing the resistance of blood vessels (3). In addition, it has been reported that rutin can be used in the treatment of vascular diseases due to its pharmaceutical effect capable of controlling excessive permeation of blood vessels. As recent reports have disclosed, rutin possesses pharmaceutical effects on diabetes and cancers (4). The two compounds, rutin and nicotiflorin, are difficult to purify using conventional methods because they have similar chemical structures (Figure 1). Silica gel column chromatography requires several time- and solvent-consuming steps. However, it is quite easy to purify each compound by high-speed counter-current chromatography (HSCCC).

HSCCC is a support-free liquid–liquid partition chromatographic technique. The method provides an advantage over conventional column chromatography by eliminating the use of a solid support, where an amount of stationary phase is limited, and dangers of irreversible adsorption to the support are inevitably present. It yields a highly efficient separation of multi-gram quantities of samples in several hours (5). In recent years, it has been widely used for the separation and purification of various natural and synthetic products (6–9), and it is considered as a suitable alternative for the separation of flavone glycosides, due to its unique advantages (10). The present paper introduces a method for the purification of rutin and nicotiflorin from the partially purified extract of flowers of *E. chrysantha* Lindl. by HSCCC.

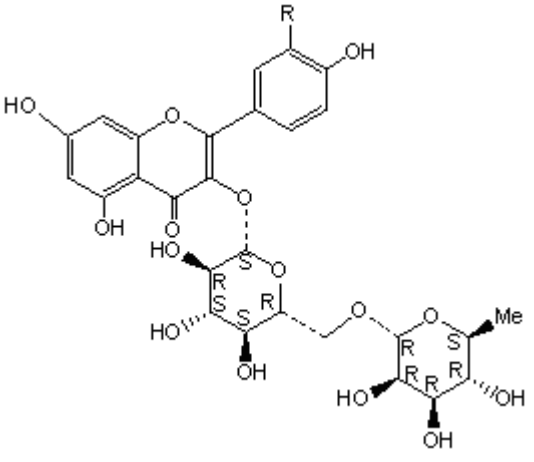

Figure 1. Chemical structures of rutin (R=OH) and nicotiflorin (R=H).

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Experimental

Apparatus

A Model TBE-300A high-speed countercurrent chromatograph (Shanghai Tauto Biotech, Shanghai, China) equipped with three preparative multiplayer coils (270 mL, wound with 2.0 mm i.d. PTFE tubing) was used for the separation and purification of rutin and nicotiflorin. The β values of this preparative column range from 0.46 to 0.73 ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R = 6.5 cm, the revolution radius or the distance between the holder axis and central axis of the centrifuge). The HSCCC columns were installed in a vessel which was maintained at 25°C by a Model HX-1050 constant-temperature controller (Beijing Detianyou Technology, Beijing, China). The two-phase 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 UV-II detector Monitor (Shanghai Institute of Biochemistry of Academy of Science, Shanghai, China). A Sepu3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram.

The high-performance liquid chromatography (HPLC) used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver. 6.1 workstation.

Reagents and materials

All organic solvents used for HSCCC were of analytical grade and purchased from Hangzhou HuiPu Chemical Factory (Hangzhou, China). Methanol used for HPLC analysis was of chromatographic grade. Flowers of *E. chrysantha* Lindl. were cultivated and collected in the botanical garden of the university.

Extraction of crude samples

Air-dried flowers of *E. chrysantha* Lindl. (3 kg) were extracted with ethanol (10 1 × 3) under reflux. The combined ethanol extracts were concentrated to dryness under reduced pressure, yielding 190 g of ethanol extract. Then it was redissolved in 2 L water, and, after filtration, the aqueous solution was extracted three times with 2 L of water–saturated petroleum, ethyl acetate, and *n*-butanol successively to yield 45 g of petroleum extract, 29 g of ethyl acetate extract, and 55 g of *n*-butanol extract. The *n*-butanol extract was further subjected to silica gel column chromatography (1500 g of silica gel H, 100~200 mesh, Qingdao Haiyang Chemica, Qingdao, China) eluted successively with chloroform–methanol solvent mixtures of increasing polarity to obtain six fractions. After concentration, the 8% methanol eluate yielded a 108 mg mixture, mainly comprised of rutin and nicotiflorin (see Figure 3A). This partially purified sample was subjected to HSCCC.

Preparation of two-phase solvent system and sample solutions

For the present study, a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (4:1:5, v/v/v) was selected by a partition experiment of the crude extract in a series of solvent systems (see Table I). The experiment of selecting the solvent that gave suitable partition coefficient (K) values was performed as in the literature (5). The solvent mixture was thoroughly equilibrated in a separation funnel and separated shortly before use.

The sample solution was prepared by dissolving the partially purified sample in the mixture of lower phase and upper phases (1:1, v/v) of the solvent system.

Separation procedure

The multilayer coiled column was first entirely filled with the upper phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 2.0 mL/min, while the apparatus was run at a revolution speed of 850 rpm. After hydrodynamic equilibrium was reached, the sample solution [108 mg dissolved in a 10 mL mixture of lower phase and upper phases (1:1, v/v) of the solvent system] was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Eluate was collected with a Model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China), 8 mL for each fraction. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized air.

HPLC analysis and identification of HSCCC peak fractions

The partially purified extract of the flowers of *E. chrysantha* Lindl. and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm i.d.). The mobile phase composed of methanol–water–acetic acid (55:42.5:2.5, v/v) was eluted at a flow-rate of 0.6 mL/min, and the effluent was monitored by a Shimadzu SPD10Avp UV detector at 254 nm.

Identification of HSCCC peak fractions was carried out by ^{1}H NMR and ^{13}C NMR spectra. NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer with tetramethylsilane (TMS) as internal standard.

Results and Discussion

Successful separation of the target compound using HSCCC depends on the selection of suitable solvent systems (5,11). In recent years, various kinds of two-phase solvent systems with different ratios used in the separation of flavone glycosides by HSCCC have been reported, mainly using the following four systems: *n*-hexane–ethyl acetate–methanol–water (12–14), methyl *tert*.-butyl ether–*n*-butanol–acetonitrile–water (15–17), chloroform-methanol–water (18,19) and ethyl acetate–*n*-butanol–water (17,20,21). Among those four solvent systems, the solvent systems *n*-hexane–ethyl acetate–methanol–water and methyl tert.-butyl ether–*n*-butanol–acetonitrile–water are usually used for separation of less polar flavone compounds; thus, they are not suitable for the separation of rutin and nicotiflorin because of their large polar chemical structures. The solvent system chloroform–methanol–water is suitable for the separation of a large number of natural components, including

flavonoids, but chloroform is very toxic and harmful for the environment. So we tried our HSCCC separation with the environmental-friendly solvent system ethyl acetate–*n*-butanol–water. The following systems at different volume ratios were tested: ethyl acetate–methanol–water (10:1:10); ethyl acetate–*n*-butanol–ethanol–water (4:1:0.25:5) and (4:1:0.1:5), and ethyl acetate–*n*-butanol–water (1:4:5), (2:3:5), (1:1:2), (3:2:5), and (4:1:5). Among these two-phase solvent systems, ethyl acetate–methanol–water (10:1:10) and ethyl acetate–*n*-butanol–water (1:4:5), (2:3:5), (1:1:2), and (3:2:5) presented large *K* values. When they were used for the HSCCC separation, the long elution time needed to elute the target compound caused peak broadening. The two-phase solvent systems ethyl acetate–*n*-butanol–ethanol–water (4:1:0.1:5), (4:1:0.25:5) and ethyl acetate–*n*-butanol–water (4:1:5) gave the suitable partition coefficient for both of the two compounds. Though the solvent system ethyl acetate–*n*-butanol–ethanol–water (4:1:0.1:5) presented higher separation factor (α), the partition coefficient for nicotiflorin was large and it needed a long elution time for this component. Finally, the ethyl acetate–*n*-butanol–water (4:1:5) was selected and our experiment showed that it gave the best separation of the major compounds by HSCCC. The *K* values and α value of the two target compounds in the described solvent systems were measured by analytical HPLC according to the literature (5) (Table I).

Table I. The Partition Coefficient (*K*) and Separation Factor (α) of Rutin and Nicotiflorin in Different Solvent Systems*

Solvent system (v/v)†	Rutin (*K*)	Nicotiflorin (*K*)	α
EA–*n*-B–E–W (4:1:0.25:5)	1.25	2.57	2.06
EA–*n*-B–E–W (4:1: 0.1:5)	1.14	3.29	2.89
EA–M–W (10:1:10)	2.40	3.93	1.63
EA–*n*-B–W (1:4:5)	3.63	6.19	1.71
EA–*n*-B–W (2:3:5)	3.65	6.83	1.87
EA–*n*-B–W (3:2:5)	1.96	3.76	1.91
EA–*n*-B–W (4:1:5)	0.94	2.17	2.31

* *K* value is expressed as the solute concentration in the upper phase divided by that in the lower phase.
† EA = Ethyl acetate; *n*-B = *n*-butanol; E = ethanol; W = Water; M = methanol.

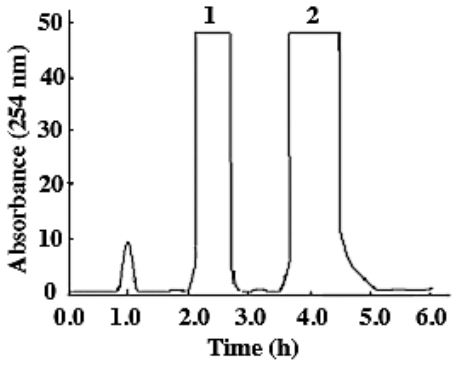

Figure 2. Chromatogram of the partially purified extract from the flowers of *E. chrysantha* Lindl. by HSCCC. Peak 1, rutin; Peak 2, nicotiflorin. Other conditions: solvent system, ethyl acetate–*n*-butanol–water (4:1:5, v/v/v); stationary phase, upper organic phase; mobile phase, lower aqueous phase; flow-rate, 2.0 mL/min; revolution speed, 850 rpm; sample, 108 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1:1, v/v) of the solvent system; retention of the stationary phase, 39.0%.

The partially purified sample of the flowers of *E. chrysantha* Lindl. was analyzed by HPLC. The result indicates that it contained several compounds, including rutin (~ 52%), nicotiflorin (~ 31%), and some unknown compounds (Figure 3A).

A 108 mg quantity of partially purified extract was separated by HSCCC. The retention of the stationary phase was 39.0%, and the separation time was 300 min for a separation run. Figure 2 shows the result obtained from 108 mg of the partially purified extract of the flowers of *E. chrysantha* Lindl. by preparative HSCCC. After this separation, the fractions containing rutin and nicotiflorin, respectively, were collected. The analysis of these fractions indicated that the peak 1 fraction contained rutin, which weighed 53 mg, at over 98.0% purity, and the peak 2 fraction contained nicotiflorin, which weighed 32 mg, at over 96.5% purity, as determined by HPLC (Figures 3B and 3C). The recoveries for rutin and nicotiflorin were 92.5% and 92.2% during a separation run.

The structural identification of the two components was carried out by ^{1}H NMR and ^{13}C NMR spectra. After comparing the data with spectral information from literature (22), the first component was confirmed as rutin. Comparing with the reported data, the spectra data of the second component was in agreement with those of nicotiflorin (23).

Rutin is widely distributed in many traditional Chinese medicines, such as *Sophora japonica* L., *Carthamus tinctorius*

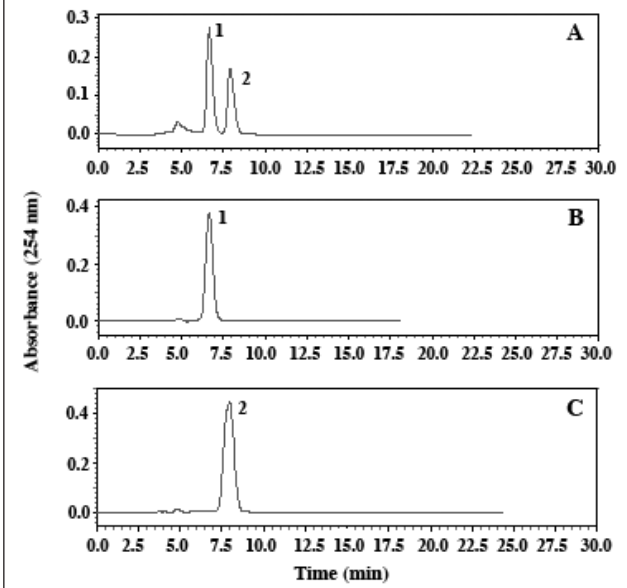

Figure 3. Results of HPLC analyses of the crude sample of the flowers of *E. chrysantha* lindl. and its HSCCC fractions. Conditions: column, Shim-Pack CLC-ODS C18 column (250 mm × 6 mm i.d.); mobile phase, methanol–water–acetic acid (55:42.5:2.5, v/v); flow-rate, 0.6 mL/min; detector, 254 nm. The partially purified sample (A); HSCCC fraction from peak 1 (Figure 2) (B); HSCCC fraction from peak 2 (Figure 2) (C).

L., and *Ruta graveolens* L. (24). The content of rutin in the traditional Chinese medicine herb *Sophora japonica* L. is over 22%. Various kinds of methods have been used to extract rutin from *Sophora japonica* L. Nicotiflorin can also be found in *Hedyotis chrysotricha* and *Nymphaea candida* Presl (~ 3%) (23,25). Compared with those herbs, the contents of rutin and nicotiflorin in *E. chrysantha* Lindl. are very low. However, the two flavone glycosides were separated from the plant of the whole *Edgeworthia* genus for the first time. An efficient HSCCC method for the separation and purification of rutin and nicotiflorin from the partially purified sample was developed by ethyl acetate–*n*-butanol–water at an optimized volume ratio of 4:1:5 (v/v) as the two-phase solvent system.

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References

1. P.G. Xiao. *Modern Chinese Material Medica* (II), 1st ed. S.R. Yu, R.Z. Feng, and Y.X. Feng, Eds. Chemical Industry Press, Beijing, 2002, pp. 800–801.
2. J.X. Cao, Y.L. Chen, C.X. Fu, and P. Wu. GC-MS analysis of essential oil components from flowers of *Edgeworthia chrysantha* Lindl. *Chin. J. Pharm. Anal.* **25:** 1211–14 (2005).
3. The Committee of Chinese Herb Information. *Handbook of Effective Component in Traditional Chinese Medicine*, 1st ed. J.W. Jiang, Q.X. Xiao, Eds. People's Medical Publishing House, Beijing, 1986, pp. 902–903.
4. Y.C. Park, T.K. Kim, M.N. Lee and B.R. Kim. U.S. Patent 20050204619 (2005).
5. Y. Ito. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr. A* 1065: 145–68 (2005).
6. Y. Shibusawa, Y. Yamakawa, R. Noji, A.Yanagida, H. Shindo, and Y. Ito. Three-phase solvent systems for comprehensive separation of a wide variety of compounds by high-speed counter-current chromatography. *J. Chromatogr. A* **1133:** 119–25 (2006).
7. S.Q. Tong, J.Z. Yan, and J.Z. Lou. Preparative isolation and purification of harpagoside from *Scrophularia ningpoensis* Hemsley by high-speed countercurrent chromatography. *Phytochem. Anal.* **17:** 406–408 (2006).
8. X.L. Cao, Y.T. Xu, G.M. Zhang, S.M. Xie, Y.M. Dong, and Y. Ito. Purification of coenzyme Q10 from fermentation extract: High-speed counter-current chromatography versus silica gel column chromatography. *J. Chromatogr. A* **1127:** 92–96 (2006).
9. Y. Ma, L. Lang, D.O. Kiesewetter, B.K. Vuong, M. Channing, Y. Ito, and W.C. Eckelman. Purification of the precursor for the automated radiosynthesis of [^{18}F]FCWAY by counter-current chromatography. *J. Chromatogr. A* **1034:** 149–53 (2004).
10. L.M. Yuan, R.N. Fu, and T.Y. Zhang. Application of high-speed countercurrent chromatography in separation of effective component of plants. *Chin. J. Pharm. Anal.* **18:** 60–64 (1998).
11. N.B. Mandava and Y. Ito. *Countercurrent Chromatography: Theory and Practice*, 1st ed. J. Cazes, Eds. Marcel Dekker, Inc., New York and Basel, 1988, pp. 387–89.
12. X. Wang, F.W. Li, H.X. Zhang, Y.L. Geng, J.P. Yuan, and T. Jiang. Preparative isolation and purification of polymethoxylated flavones from Tangerine peel using high-speed counter-current chromatography. *J. Chromatogr. A* **1090:** 188–92 (2005).
13. C.J. Ma, G.S. Li, D.L. Zhang, K. Liu, and X. Fan. One step isolation and purification of liquiritigenin and isoliquiritigenin from *Glycyrrhiza uralensis* Risch. using high-speed counter-current chromatography. *J. Chromatogr. A* **1078:** 188–92 (2005).
14. Q.Z. Du, P. Chen, G. Jerz, and P. Winterhalter. Preparative separation of flavonoid glycosides in leaves extract of *Ampelopsis grossedentata* using high-speed counter-current chromatography. *J. Chromatogr. A* **1040:** 147–49 (2004).
15. A. Yanagida, A. Shoji, Y. Shibusawa, H. Shindo, M. Tagashira, M. Ikeda, and Y. Ito. Analytical separation of tea catechins and food-related polyphenols by high-speed counter-current chromatography. *J. Chromatogr. A* **1112:** 195–201 (2006).
16. Q.Z. Du, G. Jerz, and P. Winterhalter. Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography. *J. Chromatogr. A* **1045:** 59–63 (2004).
17. A. Degenhardt, S. Hofmann, H. Knapp, and P. Winterhalter. Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *J. Agric. Food Chem.* **48:** 5812–18 (2000).
18. R. Slimestad, A. Marston, and K. Hostettmann. Preparative separation of phenolic compounds from *Picea abies* by high-speed counter-current chromatography. *J. Chromatogr. A* **719:** 438–43 (1996).
19. I. Slacanin, A. Marston, K. Hostettmann, N. Delabays, and C. Darbellay. Isolation and determination of flavone glycosides from epilobium species. *J. Chromatogr. A* **557:** 391–98 (1991).
20. X.L. Cao, Y. Tian, T.Y. Zhang, X. Li, and Y. Ito. Separation and purification of isoflavones from *Pueraria lobata* by high-speed counter-current chromatography. *J. Chromatogr. A* **855:** 709–13 (1999).
21. J.Y. Peng, G.R. Fan, Z.Y. Hong, Y.F. Chai, and Y.T. Wu. Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography. *J. Chromatogr. A* **1074:** 111–15 (2005).
22. B.C. Li, K.W. Wen, Y.L. Dong, J.Y. Zhou, G.Y. Sheng, and J.M. Fu. Separation and identification of rutin in leaves of *Kandelia Candel* from Danzhou, Hainan province. *Rdai Haiyang Xuebao* **21:** 90–94 (2002).
23. J.N. Peng, X.Z. Feng, and X.T. Liang. Chemical constituents of medicinal plant *Hedyotis chrysotricha*. *Zhong Cao Yao* **30:** 170–72 (1999).
24. L.H. Zhao. *Application of HPLC in Traditional Chinese Medicine*, 1st ed. Zhongguo Yiyao Keji Publishing House, Beijing, 2005, pp. 1062–66.
25. P. Sheng, A. Pa, Zhang, Z.C. Zhou, and N.S. Du. Determination of nicotiflorin in *Nymphaea candida* Presl. by HPLC. *Zhong Yao Cai* **29:** 1313–14 (2006).

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