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Artigo

SEPARATION AND PURIFICATION OF THREE STILBENES FROM THE RADIX OF *Polygonum cillinerve* (Nakai) Ohwl BY MACROPOROUS RESIN COLUMN CHROMATOGRAPHY COMBINED WITH HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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An effective method for the rapid separation and purification of three stilbenes from the radix of *Polygonum cillinerve* (Nakai) Ohwl by macroporous resin column chromatography combined with high-speed counter-current chromatography (HSCCC) was successfully established. In the present study, a two-phase solvent system composed of chloroform-*n*-butanol-methanol-water (4:1:4:2, v/v/v/v) was used for HSCCC separation. A one-step separation in 4 h from 150 mg of crude extract produced 26.3 mg of *trans*-resveratrol-3-O-glucoside, 42.0 mg of pieceid-2″-O-gallate, and 17.9 mg of *trans*-resveratrol with purities of 99.1%, 97.8%, and 99.4%, respectively, as determined by high-performance liquid chromatography (HPLC). The chemical structures of these compounds were identified by nuclear magnetic resonance (NMR) spectroscopy.

Keywords: *Polygonum cillinerve* (Nakai) Ohwl; HSCCC; stilbenes.

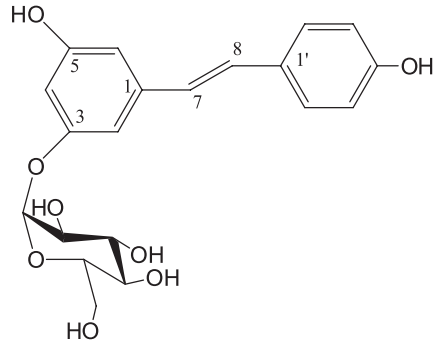
INTRODUCTION

Polygonum cillinerve (Nakai) Ohwl, a perennial herb from the *Polygonum* family, is distributed mainly in Qinghai, Shanxi, Gansu, and Sichuan in China.[1] The radix of the plant is an important traditional herbal medicine that has been shown to be effective in the treatment of inflammation, rheumatism, and bacterial infections.[2] Previous phytochemical studies have demonstrated that stilbenes, anthraquinones, and flavonoids are the major bioactive constituents of this plant.[3-5]

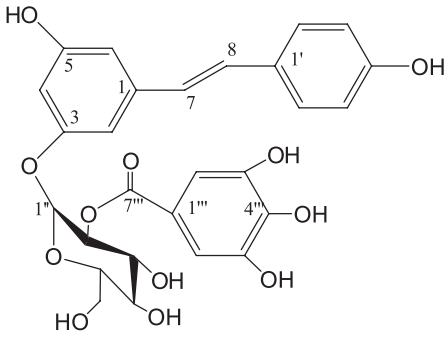
Among these constituents, stilbenes such as *trans*-resveratrol, *trans*-resveratrol-3-O-glucoside, and pieceid-2"-O-gallate have recently attracted much attention due to their pharmacological properties, which include antioxidant,[6-8] anti-influenza viral,[9] anti-fungal,[10] anti-HIV-1, and cytotoxic effects.[11,12] In view of these biological effects, an efficient method for the separation and purification of *trans*-resveratrol-3-O-glucoside, pieceid-2"-O-gallate and *trans*-resveratrol from *Polygonumcillinerve* (Nakai) Ohwl is necessary.

The traditional separation methods for the isolation and purification of stilbenes are mainly silica gel column chromatography and HPLC. However, these separation methods require numerous steps and large quantities of organic solvents. High-speed counter-current chromatography (HSCCC), which was invented by Ito and Conway, provides excellent sample recovery and can be employed for semi-preparative-scale separation in a completely straight forward manner.[13] Therefore, it has been widely used for the separation and purification of the active components of herb plants.[14-23] To the best of our knowledge, no reports have been published on the purification of stilbenes from *Polygonum cillinerve* (Nakai) Ohwl by HSCCC.

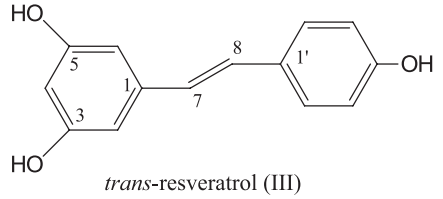
In this paper, a convenient and efficient method has been successfully established for the separation and purification of *trans*-resveratrol-3-O-glucoside, pieceid-2”-O-gallate, and *trans*-resveratrol from the extract of traditional medicinal herb *Polygonum cillinerve* (Nakai) Ohwl using macroporous resin column chromatography combined with HSCCC. The chemical structures of the three stilbenes are shown in Figure 1.



trans-resveratrol-3-O-glucoside (I)



pieceid-2"-O-gallate (II)



trans-resveratrol (III)

Figure 1. *Chemical structures of the three stilbenes isolated from Polygonum cillinerve (Nakai) Ohwl*

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EXPERIMENTAL

Apparatus

HSCCC was performed on a TBE-300A instrument (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation columns connected in series (1.6 mm, total volume = 300 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β-values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range of 0 and 1000 rpm. An HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. An ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent and measure the UV absorbance. This system contains a switch valve and a mixer, which were used for gradient formation. The data were collected with an N-2000 chromatography workstation (Zhejiang university, Zhejiang, China). The HPLC equipment used was a Waters 515 HPLC system including a Waters Quat pump, a 2996 DAD, and an Empower HPLC workstation. Nuclear magnetic resonance (NMR) spectrometry was carried out on a Mercury Plus 400 NMR (Varian Inc., USA) with tetramethylsilane (TMS) as the internal standard.

Reagents and materials

All solvents used for the preparation of crude samples and HSCCC separation were of analytical grade (Tianjin Baishi Reagent Factory, Tianjin, China). Methanol used for HPLC was of chromatographic grade (Shandong Yuwang Chemical Factory, Shandong, China), and the water used was distilled water. Methanol-D4 was used as the solvent for NMR determination.

The radix of *Polygonum cillinerve* (Nakai) Ohwl was collected from Xining, Qinghai, China in 2012 and identified by Professor Shilong Cheng (Northwest Institute of Plateau Biology, Qinghai, China). A voucher specimen was deposited in the herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai, China.

Crude sample preparation

The air-dried and powered radix of *Polygonum cillinerve* (Nakai) Ohwl (2.5 kg) was extracted three times with 75% ethanol (each time for 2 h). After evaporation under vacuum, the extract was suspended in water and then fractionated with petroleum ether (bp. ~60-90 ºC, 2.5 L × 3 times), ethyl acetate (2.5 L × 3 times) and *n*-butanol (2.5 L × 3 times). The ethyl acetate solutions were evaporated to dryness under vacuum at 60 °C to generate 180 g of ethyl acetate extract.

Macroporous resin column chromatography

In order to enrich the target components, the extract of ethyl acetate (180 g) was dissolved in deionized water, loaded into a macroporous resin column (140 × 12 cm containing 10 kg AB-8 MR), and eluted with various proportions of water/ethanol mixture (100:0, 80:20, 70:30, 60:40, 40:60, and 20:80 v/v; about 10 L for each gradient). The flow rate was controlled at approximately 20 mL min^{-1}. The water-ethanol (60:40) fraction was concentrated to yield 8 g of crude sample for subsequent HSCCC isolation and purification.

Preparation of two-phase solvent system and sample solution

The two-phase solvent system used for HSCCC separation was composed of chloroform-*n*-butanol-methanol-water at a ratio of 4:1:4:2 (v/v/v/v). The solvent mixture was prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated at room temperature. The upper and lower phases were then separated and degassed by sonication prior to use. The HSCCC sample solution was prepared by dissolving 150 mg of crude extract sample in 10 mL of the upper phase of the two-phase solvent system.

HSCCC separation procedure

First, the multilayer coil column was entirely filled with the upper phase (stationary phase). The apparatus was then rotated at 800 rpm while the lower phase (mobile phase) was pumped into the column at a flow rate of 2.0 mL min^{-1}. After hydrodynamic equilibrium was reached, as indicated by the emergence of the mobile phase front, the sample solution (150 mg of crude sample dissolved in 10 mL of the upper phase) was injected into the separation column through the injection valve using an ÄKTA prime system. The separation temperature was controlled at 25 °C. The effluent from the tail end of the column was continuously monitored by a UV detector at 300 nm. Different fractions were collected manually according to the obtained chromatogram and evaporated to dryness under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis. The purity was obtained by HPLC peak area calculation.

HPLC analysis and identification of HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. HPLC analysis was performed on a Merck LiChrospher C_{18} column (250 mm × 4.6 mm; dp = 5 μm) at 25 ºC. The mobile phase was methanol-water (35:75, v/v). The flow-rate and detection wavelength were set at 1.0 mL min^{-1} and 300 nm, respectively. The crude sample and peak fractions separated by HSCCC were analyzed by HPLC under the optimum analytical conditions, and the chromatograms are presented in Figure 2. The identification of HSCCC peak fractions was performed by ^{1}H and ^{13}C NMR spectroscopy.

RESULTS AND DISCUSSION

Optimization of the two-phase solvent system and other HSCCC conditions

Successful HSCCC separation depends on the selection of a suitable two-phase solvent system, which provides an ideal partition coefficient range ($0.2 < K < 2$) for the target compounds.[24] Large K values tend to produce excessive band broadening, while small K values usually result in a poor peak resolution. In the present study, different solvent systems including ethyl acetate-water, ethyl acetate-methanol-water, chloroform-methanol-water, and chloroform-*n*-butanol-methanol-water were tested according to the procedure described in the measurement of partition coefficient section. The K-values of the target compounds in different solvent systems were determined by HPLC, and the results are shown in Table 1. When two-phase solvent systems comprised of ethyl acetate-water (5:5, v/v) and ethyl acetate-methanol-water (5:5:5, 5:3:5, v/v/v) were employed, the K values were too large, resulting in broad peaks and long separation times. When the two-phase solvent system composed of ethyl acetate-methanol-water (3:3:5, v/v/v) was investigated, suitable K values of the target compounds were obtained; however, the separation factor was too small, which would result in poor separation. When two-phase solvent systems composed of chloroform-methanol-water (4:3:3, v/v/v) and chloroform-*n*-butanol-methanol-water (4:0.5:4:2,

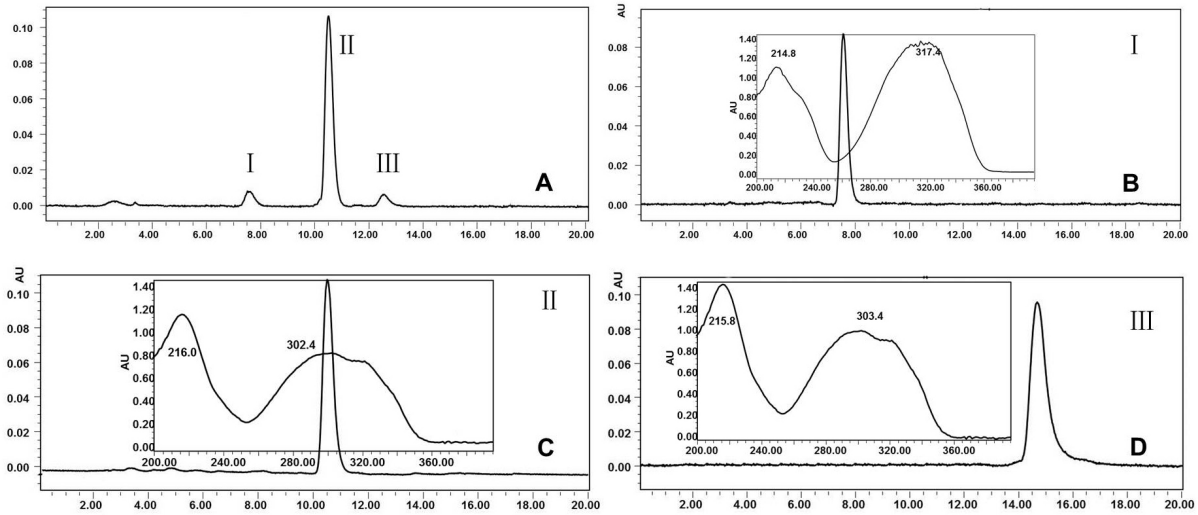

Figure 2. *HPLC chromatograms of the crude extract of Polygonum cillinerve (Nakai) Ohwl (A) after cleaning macroporous resin column chromatography and the three targeted compounds (peak fractions I, II, and III, B-D, respectively) purified by HSCCC. Conditions: column, Kromasil C_{18} column (250 mm×4.6 mm 4 μm); column temperature, 25 °C; mobile phase, methanol–water (35:75, v/v); flow-rate, 1.0 mL min^{-1}, detection wavelength, 300 nm*

v/v/v/v) were used, the K values were large, which might lead to the poor retention of target compounds in the upper phase. Fortunately, an appropriate K value with good resolution could be obtained by using a two-phase solvent system of chloroform-*n*-butanol-methanol-water at the ratio of 4:1:4:2 (v/v/v/v). The K values of the three target compounds were between 0.5 and 2.0, and the separation factors were also large enough.

Table 1. The K values of the target compounds in different solvent systems

Solvent system	Ratio (v/v)	K values		
		I	II	III
ethyl acetate: water	5:5	2.76	5.14	21.16
ethyl acetate:methanol:water	5:5:5	2.08	3.74	17.03
ethyl acetate:methanol:water	5:3:5	1.43	2.79	8.97
ethyl acetate:methanol:water	3:3:5	0.79	1.04	1.51
chloroform: methanol:water	4:3:2	16.34	7.16	2.90
chloroform:*n*-butanol: methanol:water	4:0.5:4:2	4.98	4.32	1.94
chloroform:*n*-butanol: methanol:water	4:1:4:2	1.93	1.14	0.68

The influence of the mobile phase flow rate was also investigated. The results indicated reducing the flow rate could improve the retention of the stationary phase to a certain extent, but the chromatogram peaks were extended at the same time.[25] Thus, a flow rate of 2.0 mL min^{-1} was employed in this study. Additionally, the revolution speed can also impact stationary phase retention; a high revolution speed is likely to cause emulsification.[26] Considering of this, a speed of 800 rpm was used in our isolation procedure.

HSCCC separation

Under the optimized conditions, three fractions (I, II, and III) were obtained in only one round of HSCCC separation in less than 4 h. The HSCCC chromatogram is shown in Figure 3. Three fractions were obtained from 150 mg crude sample by the one-step HSCCC separation: 26.3 mg *trans*-resveratrol-3-O-glucoside (peak I), 42.0 mg pieceid-2”-O-gallate (peak II), and 17.9 mg *trans*-resveratrol (peak III). The purities of *trans*-resveratrol-3-O-glucoside, pieceid-2”-O-gallate, and *trans*-resveratrol were 99.1%, 97.8%, and 99.4%, respectively (Figure 2), as determined by HPLC.

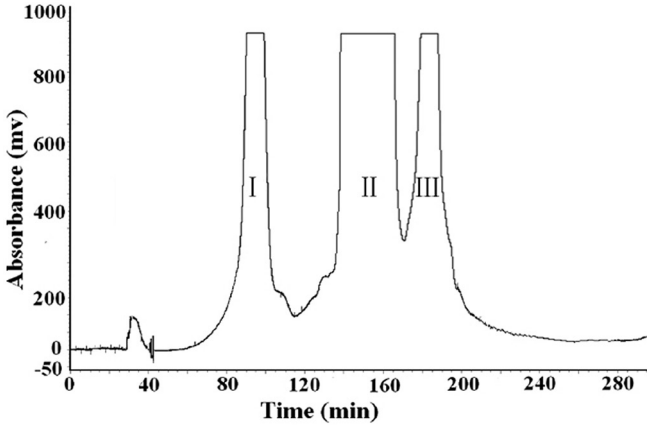

Figure 3. *HSCCC chromatograms of the crude extract from Polygonum cillinerve (Nakai) Ohwl. Conditions: two-phase solvent system, chloroform-n-butanol-methanol-water at the ratio of 4:1:4:2 (v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 2.0 mL min^{-1}; revolution speed: 800 rpm; detection wavelength: 300 nm; sample size: 150 mg of crude sample dissolved in 10 mL of the upper phase; separation temperature: 25 °C*

Structural identification

The chemical structure of each peak fraction separated by HSCCC was identified according to its ^{1}H-NMR and ^{13}C-NMR data. In comparison with reference data, peak I, peak II, and peak III were effectively identified as *trans*-resveratrol-3-O-glucoside, pieceid-2”-O-gallate and *trans*-resveratrol, respectively. The results for each peak fraction were as follows.

Peak I, white powder (methanol): ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.35 (2H, d, *J* = 8.5 Hz, H-2’, H-6’), 7.00 (lH, d, *J* = 16.3 Hz, H-8), 6.84 (lH, d, H-7), 6.78 (1H, s, H-2), 6.76 (2H, d, H-3’, H-5’), 6.61 (1H, s, H-6), 6.44 (1H, s, H-4), 4.88 (1H, d, *J* = 7.1Hz, Glc H-1”), 3.92 (1H, dd, *J* = 1.5 Hz, *J* = 12 Hz, Glc H-6a”), 3.70 (1H, dd, *J* = 5.8 Hz, Glc H-6b”), 3.48-3.38 (4H, m, Glc H-2”, H-3”, H-4”, H-5”). ^{13}C NMR (400 MHz, CD_3OD): δ = 141.4 (C-1), 107.0 (C-2), 160.5 (C-3), 104.1 (C-4), 159.6 (C-5), 108.4 (C-6), 126.7

(C-7), 130.0 (C-8), 130.3 (C-1'), 128.9 (C-2'), 116.5 (C-3'), 158.5 (C-4'), 116.5 (C-5'),128.9 (C-6'), 102.4 (Glc-1), 75.0 (Glc-2), 78.1 (Glc-3), 71.5 (Glc-4),78.3 (Glc-5) and 62.6 (Glc-6). The ^{1}H NMR and ^{13}C NMR data are in agreement with the literature data for *trans*-resveratrol-3-O-glucoside.[27]

Peak II, white powder (methanol): ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.39 (2H, d, J =8.5 Hz, H-2', H-6'), 7.16 (2H, d, *J* = 8.5 Hz, H-2"', H-6"'),7.03 (lH, d, *J* = 16.3 Hz, H-8), 6.85 (lH, d, H-7), 6.82 (2H, d, H-3', H-5'),6.70 (1H, s, H-2), 6.65 (1H, s, H-6), 6.38 (1H, s, H-4), 5.14 (1H, d, *J* = 7.9Hz, Glc H-1"), 4.02 (1H, dd, *J* = 11.4 Hz, Glc H-6a"), 3.70 (1H, m, Glc H-6b"), 5.14-3.45 (4H, m, Glc H-2", H-3", H-4". H-5"). ^{13}C NMR (400 MHz, CD_3OD): δ = 139.9 (C-1), 109.0 (C-2), 160.5 (C-3),104.1 (C-4), 159.6 (C-5), 107.4 (C-6), 126.7 (C-7), 130.0 (C-8),130.3 (C-1'), 128.9 (C-2'), 116.5 (C-3'), 158.5 (C-4'), 116.5 (C-5'),128.9 (C-6'), 102.4 (Glc-1), 78.4 (Glc-2), 76.1(Glc-3), 71.5 (Glc-4), 75.4 (Glc-5), 62.6 (Glc-6), 121.3 (C-1"'), 109.9 (C-2"'), 146.5 (C-3"'), 141.5 (C-4"'), 146.5 (C-5"'), 109.9 (C-6"') and 167.8(C-7"'). The ^{1}H NMR and ^{13}C-NMR data are in agreement with the data for pieceid-2"-O-gallate in the literature.[28]

Peak III, white powder (methanol): ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.33 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.96 (lH, d, *J* = 16.3 Hz, H-8), 6.81 (lH, d, H-7), 6.75 (2H, d, H-3', H-5') , 6.45 (2H, s, H-2, H-6), 6.17 (1H, s, H-4). ^{13}C NMR (CD_3OD, 400 MHz): δ = 141.3 (C-1), 105.7 (C-2), 159.6 (C-3),102.6 (C-4), 158.3 (C-5), 105.7 (C-6), 126.9 (C-7), 129.4 (C-8), 130.4 (C-1'), 128.8 (C-2'), 116.5 (C-3'), 158.5 (C-4'), 116.5 (C-5'), 128.8 (C-6'), The ^{1}H NMR and ^{13}C NMR data are in agreement with the data for *trans*-resveratrol in the literature.[29]

CONCLUSION

In the present study, A convenient and efficient method for the preparative separation and purification of three active components (*trans*-resveratrol-3-O-glucoside, pieceid-2"-O-gallate and *trans*-resveratrol) from the Chinese traditional herb *Polygonum cillinerve* (Nakai) Ohwl was successfully developed using macroporous resin column chromatography combined with high-speed counter-current chromatography. The compounds obtained may 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 stilbenes from natural products.

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REFERENCES

1. Gang, A. S.; Cai, X.; *J. Syst. Evol.* **2008**, *46*, 742.
2. Editorial Committee of Chinese Bencao of State Administration of Chinese Materia Medica of State Administration of Traditional Chinese Medicine; *Chinese Bencao*; Shanghai Science and Technology Publishing House: Shanghai, 2000.
3. Yoshizaki, M.; Fujino, H.; Arise, A.; Ohmura, K.; Arisawa, M.; Morita, N.; *Planta Med.* **1987**, 273.
4. Iliya, I.; Tanaka, T.; Iinuma, M.; Ali, Z.; Furasawa, M.; Nakaya, K.; Shirataki, Y.; Murata, J.; Darnaedi, D.; *Chem. Pharm. Bull.* **2002**, *50*, 796.
5. Chu, X.; Sun, A. L.; Liu, R. M.; *J. Chromatogr. A* **2005**, *1097*, 33.
6. Teguo, P. W.; Fauconneau, B.; Deffieux, G.; Huguet, F.; Vercauteren, J.; Merillon, J.M.; *J. Nat. Prod.* **1998**, *61*, 655.
7. Merillon, J. M.; Fauconneau, B.; Teguo, P. W.; Barrier, L.; Vercauteren, J.; Huguet, F.; *Clin. Chem.* **1997**, *43*, 1092.
8. Fauconneau, B.; WaffoTeguo, P.; Huguet, F.; Barrier, L.; Decendit, A.; Merillon, J. M.; *Life Sci.* **1997**, *61*, 2103.
9. Liu, A.L.; Yang, F.; Zhu, M. A.; Zhou, D.; Lin, M.; Lee, S. M. Y.; Wang, T.; Du, G. H.; *Planta Med.* **2010**, *76*, 1874.
10. Pacher, T.; Seger, C.; Engelmeier, D.; Vajrodaya, S.; Hofer, O.; Greger, H.; *J. Nat. Prod.* **2002**, *65*, 820.
11. Pflieger, A.; Teguo, P. W.; Papastamoulis, Y.; Chaignepain, S.; Subra, F.; Munir, S.; Delelis, O.; Lesbats, P.; Calmels, C.; Andreola, M. L.; Merillon, J. M.; Auge-Gouillou, C.; Parissi, V.; *PLoS One* **2013**, *8*, e81184.
12. Dai, J. R.; Hallock, Y. F.; Cardellina, J. H.; Boyd, M. R.; *J. Nat. Prod.* **1998**, *61*, 351.
13. Ito, Y; Conway, W. D.; *Anal. Chem.* **1984**, *56*, A534.
14. Fang, L.; Liu, Y. Q.; Yang, B.; Wang, X.; Huang, L. Q.; *J. Sep. Sci.* **2011**, *34*, 2545.
15. Sun, Y. S.; Lin, H. M.; Wang, J. H.; Hu, J. N.; Liu, Z. B; Gao, A. D.; *Phytochem. Anal.* **2011**, *22*, 526.
16. Lu, H. T.; Liu, J.; Deng, R.; Song, J. Y.; *Phytochem. Anal.* **2012**, *23*, 637.
17. Wang, D. J.; Lin, Y. L.; Lin, X. J.; Geng, Y. L.; Wang, X.; Zhang, J. J.; Qiu, J. Y.; *Quim. Nova* **2012**, *35*, 337.
18. Xu, P. P.; Guan, S. H.; Feng, R. H.; Tang, R. N.; Guo, D. A.; *Phytochem. Anal.* **2012**, *23*, 228.
19. Chu, C.; Zhang, S. D.; Tong, S. Q.; Li, X. N.; Yan, J. Z.; *J. Sep. Sci.* **2013**, *36*, 3958.
20. Li, J. L.; Zhang, Y. Q.; Lin, Y. L.; Wang, X.; Fang, L.; Geng, Y. L.; Zhang, Q. D.; *Quim. Nova* **2013**, *36*, 686.
21. Liang, J. R.; Ito, Y.; Zhang, X. X.; He, J.; Sun, W. J.; *J. Sep. Sci.* **2013**, *36*, 3934.
22. Shu, X. K.; Wang, M.; Liu, D. C.; Wang, D. J.; Lin, X. J.; Liu, J. H.; Wang, X.; Huang, L.Q.; *Quim. Nova* **2013**, *36*, 836.
23. Liu, F.; Xi, X. J.; Wang, M.; Fan, L.; Geng, Y. L.; Wang, X.; *J. Sep. Sci.* **2014**, *37*, 376.
24. Oka, F.; Oka, H.; Ito, Y.; *J. Chromatogr.* **1991**, *538*, 99.
25. Yue, H. L.; Zhao, X. H.; Mei, L. J.; Shao, Y.; *J. Sep. Sci.* **2013**, *36*, 3123.
26. Wang, P.; Liu, Y. L.; Chen, T.; Xu, W. H.; You, J. M.; Liu, Y. J.; Li, Y. L.; *Phytochem. Anal.* **2013**, *24*, 603.
27. Teguo, P. W.; Decendit, A.; Vercauteren, J.; Deffieux, G.; Merillon, J. M.; *Phytochemistry* **1996**, *42*, 1591.
28. Lee, J. P.; Min, B. S.; An, R. B.; Na, M. K.; Lee, S. M.; Lee, H. K.; Kim, J. G.; Bae, K. H.; Kang, S. S.; *Phytochemistry* **2003**, *64*, 759.
29. Chen, L.; Han, Y. S.; Yang, F. Q.; Zhang, T. Y.; *J. Chromatogr. A* **2001**, *907*, 343.