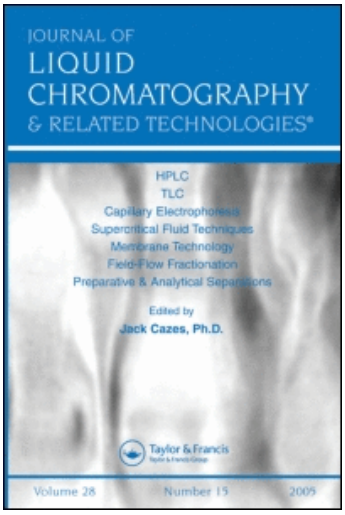

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Preparative Isolation of Kaempferol Pyranosides from a Traditional Chinese Herb using High Speed Countercurrent Chromatography

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Abstract: Preparative countercurrent chromatography has been used successfully for the isolation and purification of kaempferol pyranosides including kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, kaempferol-3-O-β-D-glucopyranoside, and kaempferol-3-O-a-L-rhamnopyranoside from the traditional Chinese herb "Jin qian cao"-*Lysimachia christinae* Hance with a two-phase solvent system composed of ethyl acetate-methanol-water with a volume ratio of 50:1:50, v/v. The composition of the phase system was optimized using analytical high speed countercurrent chromatography (HSCCC). The crude extract in the amount of 924 mg was separated, yielding 69.8 mg of kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, 45.3 mg of kaempferol-3-O-β-D-glucopyranoside, both at a high purity of over 97% and 8.9 mg of kaempferol-3-O-a-L-rhamnopyranoside with the purity of 92%.

Keywords: High speed countercurrent chromatography, Kaempferol-3-O-β-D-glucopyranoside, Kaempferol-3-O-a-L-rhamnopyranoside, Kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, *Lysimachia christinae* Hance

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INTRODUCTION

Lysimachia christinae Hance is a useful traditional Chinese herb, containing flavonoids, which are associated with a reduced risk of developing coronary heart disease.[1,2] This potential health benefit has stemmed a lot of investigations on the inhibitory role of flavonoids in the lipid peroxidation of low density lipoproteins (LD) in relation to the implications of oxidized LDL in the formation of the atherosclerotic plaque.[3–5]

However, the separation of these active compounds from natural sources by using conventional support based chromatography can have the problem of irreversible adsorption to the support, and needs long separation time with several steps.[6,7] High speed countercurrent chromatography (HSCCC), being a support free liquid liquid partition chromatographic technique, eliminates such adsorption problems,[8] and has been widely used in preparative separation of natural products.[9–11] The present paper describes the successful preparative separation and purification of kaempferol-3-O-a-L-rhamnosyl (1-2)-β-D-glucopyranoside, kaempferol-3-O-β-D-glucopyranoside, and kaempferol-3-O-a-L-rhamo-pyranoside from the crude extract of *Lysimachia christinae* Hance on the baseline, by using countercurrent chromatography with a two-phase solvent system composed of ethyl acetate-methanol-water with volume ratio of 50:1:50, v/v.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study was a Model GS 20 analytical high speed countercurrent chromatograph (Beijing Institute of New Technology Application, China). The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 70 m long, 0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the rotational speed of the apparatus could be regulated with a speed controller in the range between 0 to 2000 rpm, an optimum speed of 1600 rpm was used in the present studies. A manual sample injection valve with a 1.0 mL loop was used.

The preparative HSCCC instrument was a Model GS10AB multilayer coil planet centrifuge (Beijing Institute of New Technology Application, China) equipped with a PTFE multilayer coil of 110 m × 1.6 mm I.D. with

a total capacity of 240 mL. The β value of the preparative column ranges from 0.5 to 0.8. A Model NS-1007 constant flow pump (Beijing Institute of New Technology Application, China) was used to elute the mobile phase, while continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor at 254 nm (Beijing Institute of New Technology Application, China). A manual six-port valve with a 20 mL loop was used as the injection valve. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-20A system including two LC-20A solvent delivery units, an SPD-M20A UV-VIS photodiode array detector, a Model 7725 injection valve with a 20 μL loop, an SCL-20A system controller, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

Identification of CCC peak fractions was carried out by MS (Finnigan MAT711), ^{1}H-NMR, and ^{13}C-NMR spectra (av600).

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China).

Lysimachia christinae Hance was purchased from a local store (Tong Ren Tang Shop, Beijing, China).

Preparation of Crude Extract of *Lysimachia christinae* Hance

About 200 g of dried *Lysimachia christinae* Hance was extracted (refluxed) for 3 h, three times, with 500 mL of petroleum ether (b.p.: 60–90°C), and then extracted (refluxed) for 3 h, three times, with 400 mL of ethyl acetate, passed through a six-layer filter. The residues were re-extracted with 500 mL of 95% ethanol solution, centrifuged, and concentrated to dryness under reduced pressure yielding 11.8 g of a crude sample, which contained kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, kaempferol-3-O-β-D-gluco- pyranoside, and kaempferol-3-O-a-L-rhamnopyranoside.

Preparation of Two-Phase Solvent System and Sample Solutions

The solvent systems utilized in the phase system optimization study were prepared by mixing ethyl acetate-methanol-water (10:1:10, 50:1:50, and 200:1:200, v/v/v). All the mixtures were equilibrated in a separatory funnel at room temperature; the two phases were separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at the appropriate concentrations, according to whether an analytical or preparative protocol was used.

Optimization of Phase System Using Analytical HSCCC

Analytical HSCCC was used for the phase system optimization using a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the appropriate upper phase. The lower phase was then pumped into the head end of the inlet column at a flow-rate of 1.0 mL/min, while the apparatus was running at a revolution speed of 1600 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (9 or 15 or 13 mg in 1 mL of lower phase) 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. Each peak fraction was collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

Preparative HSCCC was performed with a Model GS 10AB HSCCC instrument for preparative isolation of the crude extract as follows: the multilayer coiled column was first entirely filled with the upper phase of the selected two-phase solvent system. The lower phase was then pumped into the head end of the inlet column at flow rate of 2.0 mL/min, while the apparatus was running at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution at the selected concentration 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. Each peak fraction was collected according to the chromatogram.

HPLC Analyses and Identification of HSCCC Peak Fractions

The crude extract of *Lysimachia christinae* Hance and HSCCC peak fractions were each analyzed by HPLC, using a Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 30°C. The mobile phase, composed of methanol and water (including 0.2% H_3PO_4) (1:1, v/v), was isocratically eluted at a flow-rate of 1.0 mL/min and the effluent monitored at 254 nm by a PAD detector.

Identification of the target compounds (kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, kaempferol-3-O-β-D-gluco-pyranoside, and kaempferol-3-O-a-L-rhamnopyranoside) was based on ESI-MS, ^{1}H-NMR, and ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

As shown in Figure 1, based on the HPLC area, the crude extract sample of *Lysimachia christinae* Hance includes a number of compounds: kaempferol-3-O-a-L-rhamnosyl (1-2)-β-D-glucopyranoside, kaempferol-3-O-β-D-glucopyranoside, and kaempferol-3-O-a-L-rhamnopyranoside, which were approximately 23.79%, 23.44%, and 2.09%. Based on the external standard curve, those purities are 10.9%, 9.8%, and 1.0%, respectively.

In order to achieve an efficient resolution of target compounds, a two-phase solvent system, composed of ethyl acetate-methanol-water (x:1:y), was examined by using analytical HSCCC with varying volume ratios of x and y. The results are illustrated in Figure 2A–C.

As shown in Figure 2A, kaempferol-3-O-β-D-glucopyranoside (peak 5) and kaempferol-3-O-a-L-rhamnopyranoside (peak 6) were not resolved at the volume ratio (10:1:10), while kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside (peak 4) was completely separated. In another experiment, as shown in Figure 2B, kaempferol-3-O-β-D-glucopyranoside and kaempferol-3-O-a-L-rhamnopyranoside resolved well at the volume ratio (50:1:50), while all compounds were completely eluted within two hours. As

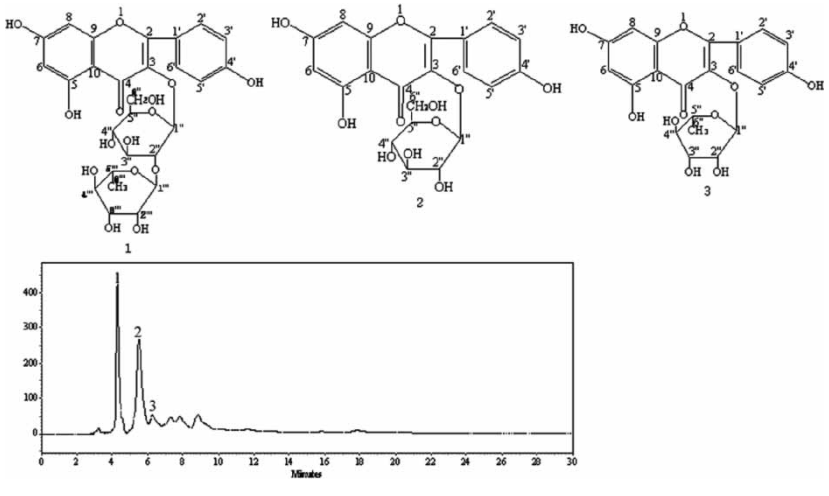

Figure 1. HPLC analyses of the crude extract from *Lysimachia christinae* Hance with the chemical structure of kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, kaempferol-3-O-β-D-glucopyranoside, and kaempferol-3-O-a-L-rhamnopyranoside. HPLC conditions: Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 30°C. The mobile phase, composed of methanol and water (including 0.2% H_3PO_4) (1:1, v/v), was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 254 nm by a PAD detector. Peak 1: kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnohyphen;pyranoside. Peak 2: kaempferol-3-O-β-D-glucopyranoside. Peak 3: kaempferol-3-O-a-L-rhamnopyranoside.

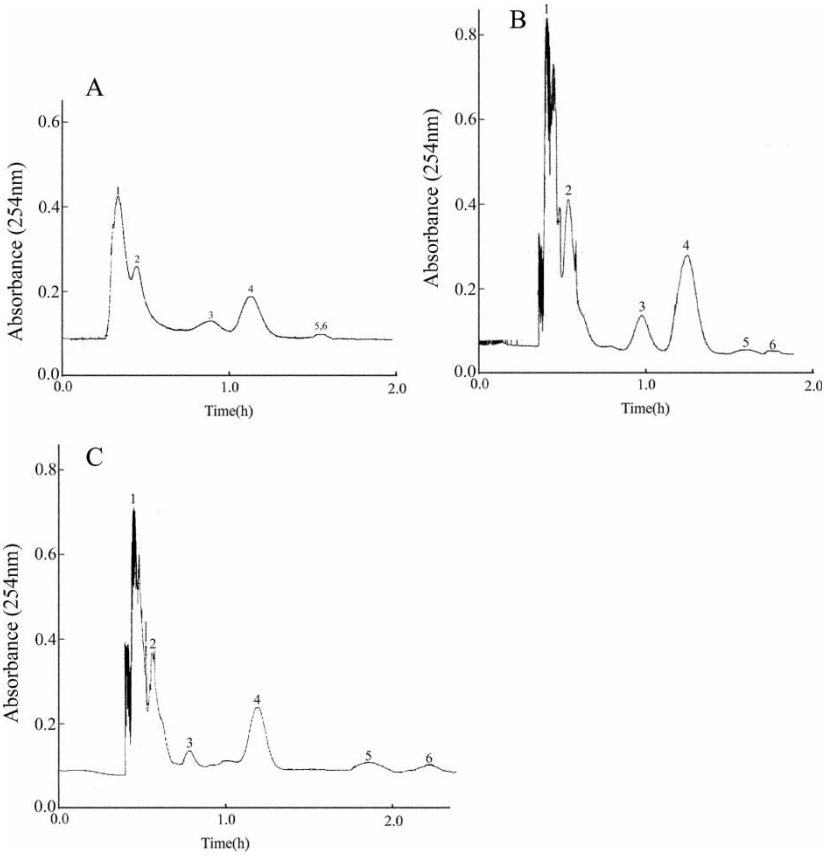

Figure 2. Chromatogram of the crude extract from *Lysimachia christinae* Hance by analytical HSCCC. Solvent system A: ethyl acetate-methanol-water (10:1:10, v/v), solvent system B: ethyl acetate-methanol-water (50:1:50, v/v), solvent system C: ethyl acetate-methanol-water (200:1:200, v/v), stationary phase: upper organic phase, mobile phase: lower aqueous phase; flow rate: 1.0 mL/min; revolution speed: 1600 rpm, sample: 9 mg or 15 mg or 13 mg dissolved in 1 mL lower phase of solvent system. Peak 4: kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside. Peak 5: kaempferol-3-O-β-D-glucopyranoside. Peak 6: kaempferol-3-O-a-L- rhamnopyranoside.

shown in Figure 2C, kaempferol-3-O-β-D-glucopyranoside and kaempferol-3-O-a-L-rhamnopyranoside were resolved at the volume ratio (200:1:200), while all compounds were completely eluted in two and half hours.

As suggested by these results of the analytical HSCCC experiment, if the crude extract of *Lysimachia christinae* Hance was separated by preparative HSCCC using the above solvent system at a volume ratio of (200:1:200), according to my previous study it would take a long time for elution. The volume ratio of 50:1:50 was most suitable for the HSCCC run for purification

of those three compounds because of separation on the baseline and little time consuming.

Figure 3 shows the result obtained from 924 g of the crude extract of *Lysimachia christinae* Hance by preparative HSCCC yielding 69.8 mg of kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, 45.3 mg of kaempferol-3-O-β-D-glucopyranoside both at a high purity of over 97%, and 8.9 mg of kaempferol-3-O-a-L-rhamnopyranoside with the purity of 92% based on HPLC analysis.

The structural identification of kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside, kaempferol-3-O-β-D-glucopyranoside, and kaempferol-3-O-a-L-rhamnopyranoside was carried out by ESI-MS, ^{1}H-NMR, and ^{13}C-NMR Spectra as follows: the ESI-MS: m/z 595$[(M+1)]^+$, 449$[(M+1)-146]^+$, 287$[(M+1)-146-162]^+$ showed the molecular ion at m/z 594, which is in agreement with the molecular formula $C_{27}H_{30}O_{15}$ of kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside. The ESI-MS: m/z 449$[(M+1)]^+$, 287$[(M+1)-162]^+$ showed the molecular ion at m/z 448, which is in agreement with the

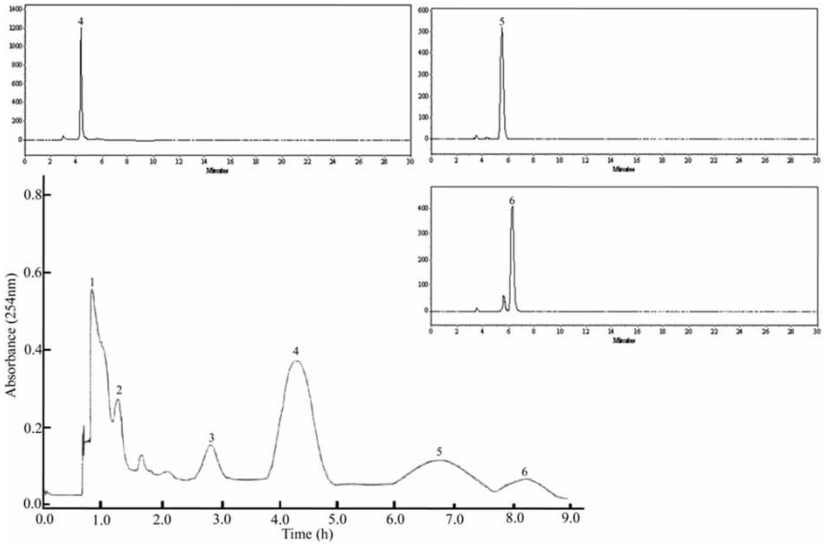

Figure 3. Chromatogram of the crude extract from *Lysimachia christinae* Hance by high speed countercurrent chromatography. Solvent system: ethyl acetate-methanol-water (50:1:50, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm. HPLC conditions: Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 30°C. The mobile phase, composed of methanol and water (including 0.2% H_3PO_4) (1:1, v/v), was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 254 nm by a PAD detector. Peak 4: kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside. Peak 5: kaempferol-3-O-β-D-glucopyranoside. Peak 6: kaempferol-3-O-a-L- rhamnopyranoside.

molecular formula $C_{21}H_{20}O_{11}$ of kaempferol-3-O-β-D-glucopyranoside. The ESI-MS: m/z 433$[(M+1)]^+$, 287$[(M+1)-146]^+$ showed the molecular ion at m/z 432, which is in agreement with the molecular formula $C_{21}H_{20}O_{10}$ of kaempferol-3-O-a-L- rhamnopyranoside.

Kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside: ^{1}H-NMR (600 MHz, DMSO) δ ppm: 12.671 (s, 1H, 5C-OH), 8.101 (d, 2H, 2′C-H, 6′C-H), 6.887 (d, 2H, 3′C-H, 5′C-H), 6.367 (s, 1H, 8C-H), 6.200 (s, 1H, 6C-H), 5.201 (2H, 1″C-H, 1‴C-H), 3.157–4.885 (8H, 2″C–H, 3″C-H, 4″ C-H, 5″C-H, 6″C-H, 2‴C-H, 3‴C-H, 4‴C-H), 2.501 (1H, 5‴C-H), 0.841 (3H, 6‴C-H). Kaempferol-3-O-β-D-glucopyranosyl (2-1)-a-L-rhamnopyranoside: ^{13}C-NMR (600 MHz, DMSO) δ ppm: 178.25 (4-C), 164.67 (7-C), 161.78 (5-C), 160.01 (4′-C), 157.95 (9-C), 156.88 (2-C), 136.94 (3-C), 134.76 (2′-C, 6′-C), 120.09 (1′-C), 108.40 (3′-C, 5′-C), 104.50 (10-C), 102.42 (1″-C), 101.02 (1‴-C), 99.13 (6-C), 93.98 (8-C), 77.60 (2″-C), 77.45 (3″-C), 77.44 (5″-C), 71.77 (4‴-C), 71.01 (3‴-C), 70.88 (2‴-C), 70.49 (5‴-C), 70.31 (4″-C), 60.82 (6″-C), 17.98 (6‴-C), the results were similar to those in reference.[7,12,13]

Kaempferol-3-O-β-D-glucopyranoside: ^{1}H-NMR (600 MHz, DMSO) δ ppm: 12.642 (s, 1H, 5C-OH), 7.932 (d, 2H, 2′C-H, 6′C-H), 6.780 (d, 2H, 3′C-H, 5′C-H), 6.386 (s, 1H, 8C-H), 6.204 (s, 1H, 6C-H), 5.255 (2H, 1″C-H), 3.308-3.744 (5H, 2″C-H, 3″C-H, 4″C-H, 5″C-H, 6″C-H). Kaempferol-3-O-β-D-glucopyranoside: ^{13}C-NMR (600 MHz, DMSO) δ ppm: ^{13}C-NMR (600 MHz, DMSO) δ ppm: 177.90 (4-C), 166.60 (7-C), 162.04 (5-C), 160.26 (4′-C), 157.22 (9-C), 157.59 (2-C), 134.07 (3-C), 130.84 (2′-C, 6′-C), 121.33 (1′-C), 114.71 (3′-C, 5′-C), 103.81 (10-C), 103.05 (1″-C), 98.95 (6-C), 93.83 (8-C), 76.61 (3″-C), 76.93 (5″-C), 74.21 (4″-C), 70.57 (2″-C), 61.14 (6″-C), the results were similar to those in reference.[12–14]

Kaempferol-3-O-a-L-rhamnopyranoside: ^{1}H-NMR (600 MHz, DMSO) δ ppm: 12.651 (s, 1H, 5C-OH), 7.791 (d, 2H, 2′C-H, 6′C-H), 6.864 (d, 2H, 3′C-H, 5′C-H), 6.365 (s, 1H, 8C-H), 6.182 (s, 1H, 6C-H), 5.255 (2H, 1″C-H), 3.146-3.788 (4H, 2″C-H, 3″C-H, 4″C-H, 5″C-H), 0.825 (3H, 6″C-H). Kaempferol-3-O-a-L-rhamnopyranoside: ^{13}C-NMR (600 MHz, DMSO) δ ppm: 177.62 (4-C), 164.32 (7-C), 161.91 (5-C), 159.88 (4′-C), 156.61 (9-C), 156.40 (2–C), 133.50 (3-C), 130.58 (2′-C, 6′-C), 121.11 (1′-C), 115.07 (3′-C, 5′-C), 104.12 (10-C), 101.93 (1″-C), 98.23 (6-C), 93.53 (8-C), 71.22 (4″-C), 70.71 (3″-C), 70.48 (2″-C), 70.17 (5″-C), 16.87 (6″-C), the results were similar to those in reference.[12–13,15]

CONCLUSIONS

The results of this study demonstrate that high speed countercurrent chromatography is a good method for the preparative separation of kaempferol-3-O-a-L-

rhamnosyl (1-2)-β-D-glucopyranoside, kaempferol-3-O-β-D-glucopyranoside and kaempferol-3-O-a-L-rhamnopyranoside from *Lysimachia christinae* Hance.

ACKNOWLEDGMENTS

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REFERENCES

1. Hollman, P.C.H.; Katan, M.B. Arch. Toxicol., Suppl. **1998**, *20*, 237–248.
2. Mennen, L.I.; Sapinho, D.; Bree, A.; de Arnault, N.; Bertrais, S.; Galan, P.; Hercberg, S.J. Nutr. **2004**, *134*, 923–926.
3. Janisch, K.M.; Williamson, G.; Needs, P.; Plumb, G.W. Free Rad. Res. **2004**, *38*, 877–884.
4. Whalley, C.V.; Rankin, S.M.; Hoult, J.R.S.; Jessup, W.; Leake, D.S. Biochem. Pharmacol. **1990**, *39*, 1743–1750.
5. Dufour, C.; Loonis, M. Biochim. Biophys. Acta **2007**, *1770*, 958–965.
6. Cui, D.B.; Wang, S.Q.; Yan, M.M. Acta Pharm. Sinica **2003**, *38*, 196–198.
7. Wang, Y.J.; Sun, Q.S. Chinese J. Med. Chem. **2005**, *15*, 357–359.
8. Ito, Y. CRC Crit. Rev. Anal. Chem. **1986**, *17*, 65–143.
9. Kohler, N.; Winterhalter, P.J. Chromatogr. A **2005**, *1072*, 217–222.
10. Wei, Y.; Ito, Y.J. Chromatogr. A **2006**, *1115*, 112–117.
11. Baldermann, S.; Reinhard, A.; Kohler, N.; Fleischmann, P. J. Chromatogr. A **2007**, *1151*, 183–186.
12. Yu, D.Q.; Yang, J.S. *Analytical Chemistry Manual VII Fascicle: NMR Analyses*; Chemical Industry Press: Beijing, 1999; 301–305.
13. Yu, D.Q.; Yang, J.S. *Analytical Chemistry Manual VII Fascicle: NMR Analyses*; Chemical Industry Press: Beijing, 1999; 836–839.
14. Finkl, T.; Holbrook, N.J. Nature **2000**, *408*, 239–247.
15. Wang, K.J.; Chen, L.Z.; Li, N.; Yu, X.P. J. Chin. Pharm. **2006**, *41*, 493–496.

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