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Short communication

Separation of andrographolide and neoandrographolide from the leaves of *Andrographis paniculata* using high-speed counter-current chromatography

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Abstract

The bioactive diterpenes andrographolide and neoandrographolide from the leaves of *Andrographis paniculata* NEES (Acanthaceae) were successfully separated by counter-current chromatography. A single 280-min separation yielded 189 mg of 99.9% andrographolide and 9.5 mg of 98.5% neoandrographolide applying water–methanol–ethyl acetate–*n*-hexane (2.5:2.5:4:1) solvent system. Structure confirmation was done by electrospray MS, one-dimensional NMR experiments, circular dichroism, optical rotation dispersion, and specific optical rotation $[\alpha]_D$.

Keywords: Counter-current chromatography; *Andrographis paniculata*; Plant material; Andrographolide; Neoandrographolide; Diterpenes

1. Introduction

Andrographis paniculata NEES (Acanthaceae) is widely used in the traditional medicine of India, Southeast Asia and China. From the leaves of this plant, andrographolide [1–3] has been isolated as the main bitter substance together with several other diterpenoids, and diterpene glucosides [4,5].

Andrographolide (**1**), deoxyandrographolide, and neoandrographolide (**2**) (Fig. 3) are the main components in the leaves possessing protective effects against galactosamine and paracetamol-induced intoxication in rats [6] and antihepatotoxic effect against carbon tetrachloride-induced hepatotoxicity [7]. They also inhibit human immunodeficiency virus type 1 (HIV)-induced cell cycle dysregulation and lead to a rise in $CD4^+$ lymphocyte levels of HIV-1 infected individuals [8]. Furthermore, andrographolide and some other diterpenes showed significant induction of phagocytosis [9].

As an all-liquid chromatographic technique, high-speed counter-current chromatography (HSCCC) uses no solid support, so adsorbing effects on stationary phase material and artifact formation are eliminated. The technique allows complete recovery of the sample, and is suitable for separations in the gram range [10]. In this paper we report the preparative isolation of the bioactive *ent*-labdane diterpenes

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andrographolide and neoandrographolide in crude extract of leaves of *A. paniculata* using HSCCC. At the same time we confirm the chemical structure of the two compounds by nuclear magnetic resonance spectroscopy (^{1}H-, ^{13}C-, and DEPT-NMR), electrospray ionization (ESI)-MS, values of specific optical rotation $[\alpha]_D$, circular dichroism spectroscopy (CD) and optical rotation dispersion (ORD).

2. Experimental

2.1. Material

The extract from the leaves of *A. paniculata* was purchased from Chashai Kinglong Bioproducts, Changshai, China, containing ~40% of andrographolide. All used solvents were of analytical grade, purchased from Riedel-de Haën, Germany.

2.2. High-speed counter-current chromatography

A multilayer coil counter-current chromatograph manufactured by P.C. (Potomac, MD, USA) was equipped with a 385-ml coil column made of polytetrafluoroethylene tubing (2.6 mm I.D.). The separation was done at a revolution speed of 650 rpm using a Biotronik HPLC pump BT 3020 (Jasco, Gross-Umstadt, Germany) for the delivery of the mobile phase. Fractions were collected with a Superfrac collector model (Pharmacia, Uppsala, Sweden), and elution was continuously monitored with a Knauer UV–Vis variable-wavelength detector (Berlin, Germany) at 350 nm.

2.3. HSCCC solvent system

The two-phase solvent system used for the CCC separation of the diterpenes andrographolide and neoandrographolide was composed of water–methanol–ethyl acetate–*n*-hexane (2.5:2.5:4:1, v/v), which was selected by partition coefficient experiments. The partition coefficients of andrographolide and neoandrographolide in the solvent systems composed of *n*-hexane–ethyl acetate–methanol–water with different volume ratios were calculated by determining the content of andrographolide and neoandrographolide in the two phases of each solvent system with HPLC.

After thorough equilibration in a separatory funnel (at ambient temperature), the two immiscible phases were separated before use. In the CCC experiment, elution mode was head to tail with the upper organic phase as the stationary phase.

2.4. Sample solution and separation procedure

After the stationary phase was completely filled, the CCC apparatus was rotated at 650 rpm, and the sample (500 mg of crude extract of *A. paniculata*) was introduced in 15 ml of a mixture of upper and lower phase to the coil-column with an injection loop. The lower aqueous mobile phase was then pumped through the column at a flow rate of 1.5 ml/min.

2.5. Analytical controls

2.5.1. HPLC analysis

The HPLC system was composed of a Knauer HPLC pump 64, a manual injector, an ODS column (Ultrasphere, 5 μm, 150×4.6 mm, Beckman, Germany), a Knauer variable-wavelength monitor, and a data processing system. Methanol–water–acetonitrile (50:50:10) was used as the mobile phase in the isocratic mode.

2.5.2. Physicochemical and spectroscopic analysis

Melting points were determined on a Kofler melting point microscope (Reichert, Austria). Specific optical rotations $[\alpha]_D$ were measured with a Perkin-Elmer polarimeter type-24 (Überlingen, Germany) at 21 °C (λ=589 nm, cell length 10 cm, conc. g/100 ml). Curves of CD and ORD were recorded on a Jasco spectropolarimeter type J-715 at 21 °C (scan range: λ=200–450 nm, cell length: 0.1 cm, c=1 mg/ml). ^{1}H-, ^{13}C- and DEPT 90/135-NMR spectra were recorded in [$^{2}H_5$]pyridine on a Bruker AMX 300 spectrometer (Karlsruhe, Germany) with 300 MHz for ^{1}H and 75.5 MHz for ^{13}C measurements, respectively. Electrospray ionization ion trap tandem mass spectrometry (ESI-MS–MS) experiments were performed on a Bruker Esquire LC–MS–MS system in the positive and negative modes using a syringe pump. Drying gas was nitrogen with a gas

flow of 7 l/min (330 °C), nebulizer pressure was set to 30 p.s.i. (1 p.s.i.=6894.76 Pa), parameters for the negative ESI mode were as follows: capillary, −3500 V; end plate, −3000 V; capillary exit, 90 V; skim 1, 30 V; skim 2, 10 V; and MS–MS fragmentations were done with different fragmentation amplitudes: for positive ESI mode: capillary, 3500 V; end plate, 3000 V; capillary exit, −90 V; skim 1, −30 V; skim 2, −10 V. Thin-layer chromatography was done on normal-phase silica gel plates 60 F_{254} from Merck (Darmstadt, Germany) and reversed-phase plates RP-18W from Machery–Nagel (Düren, Germany). Visualization of the diterpenes was done by spraying anisaldehyde–conc. sulfuric acid–glacial acid (1:2:97), and flash heating up to 110 °C on a hot plate.

3. Results and discussion

3.1. HSCCC separation

The chromatogram of the HSCCC separation of the crude extract from *A. paniculata* NEES (Acanthaceae) is given in Fig. 1 and showed the baseline separation of peak I (127–163 min), peak II (164–194 min), and peak III (195–285 min). Peaks II and III corresponded to peaks 4 and 6 in HPLC chromatogram of the crude extract, respectively, while peak I was still a mixture of several compounds (Fig. 2).

After fractionation and vacuum evaporation of the solvents, peak I gave 2 mg of a mixture of several

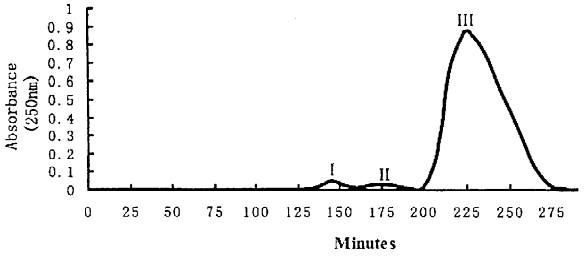

Fig. 1. HSCCC chromatogram of 500 mg of the crude extract from *A. paniculata* NEES (Acanthaceae). Solvent system: water–methanol–ethyl acetate–*n*-hexane (2.5:2.5:4:1, v/v); coil column: 385 ml (2.6 mm I.D. PTFE tubing); rotary speed: 650 rpm; flow rate of mobile phase: 1.5 ml/min; detection wavelength: 250 nm.

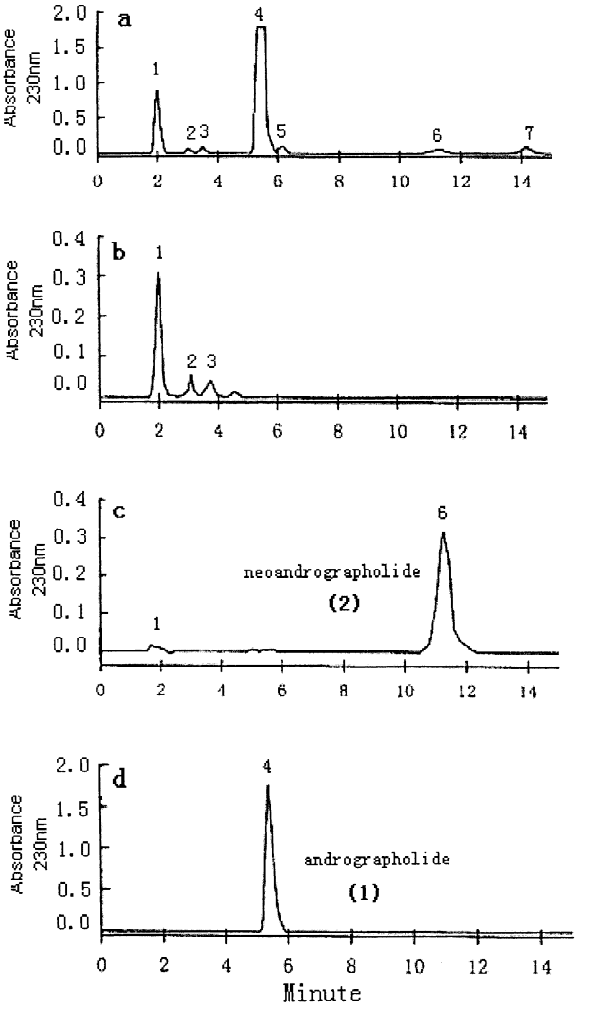

Fig. 2. HPLC analysis of the crude extract and the components from HSCCC separation. Separation column: ODS column (Ultrasphere, 5 μm, 150×4.6 mm, Beckman, Germany); detection wavelength: 230 nm; elution: 1 ml/min of methanol–water–acetonitrile (50:50:10) in the isocratic mode. (a) Crude extract, 10 mg in 10 ml methanol, injection 10 μl; (b) component corresponding to peak I in the HSCCC chromatogram, 1.0 mg in 100 ml methanol, injection 10 μl; (c) component corresponding to peak II in HSCCC chromatogram, 1.0 mg in 10 ml methanol, injection 10 μl; (d) component corresponding to peak III in the HSCCC chromatogram, 1.0 mg in 10 ml methanol, injection 10 μl.

co-eluting compounds (Fig. 2b), peaks II and III yielded 9.5 mg of 98.5% neoandrographolide (Fig. 2c) and 189 mg of 99.9% andrographolide (Fig. 2d) determined by HPLC. Each was a colorless powder.

The products were directly used for confirmation of their chemical structure.

3.2. Confirmation of chemical structure

The compounds corresponding to peaks II and III were andrographolide (**1**) and neoandrographolide (**2**) (Fig. 3) based on the information from ^{1}H-, ^{13}C-, and DEPT-NMR, LC–ESI-MS, $[\alpha]_D$ values, CD and ORD. The detailed data are given below.

Andrographolide (**1**): colorless plates, m.p. 205–210 °C (from MeOH), TLC-SiO_2 (CH_2Cl_2–MeOH, 9:1) R_F=0.55, TLC-RP18W: (MeOH–water 1:1): R_F=0.21, anisaldehyde–H_2SO_4–glacial acid reagent: grey-blue, $[\alpha]_D^{20}=-97.2°$ (c=0.61 g/100 ml, MeOH), UV (MeOH): λ_{max} nm (ϵ)=207 (sh, 18 095), 224 (22 447), CD (MeOH): λ_{max} nm (θ)= 200 (+29 890), 224 (−31 124), 280 (+1132), ORD (MeOH): λ_{max} nm (ϕ)=205 (+33 775), 245 (−19 100), ESI-MS (pos) m/z: 373 $[M+Na]^+$, 389 $[M+K]^+$, 723 $[2M+Na]^+$, ESI-MS (neg) m/z: 349 $[M-H]^-$, 331 $[M-H-H_2O]^-$, $C_{20}H_{31}O_5$, ^{1}H-NMR ($C_5^2H_5N$): δ 0.70 (3H, s, CH_3-20), 1.51 (3H, s, CH_3-18), 1.9 (signal overlapped) (H-9), 2.73 (br t, J=7.0 Hz, H-11), 3.60–3.64 (signal overlapped) (2H, br m, H-19$_A$, H-3), 4.43 (1H, d, J=10.5 Hz, H-19$_B$), 4.50 (1H, dd, J_1=10.5 Hz, J_2=2.5 Hz, H-15$_A$), 4.60 (1H, dd, J_1=10.0 Hz, J_2=6.0 Hz, H-15$_B$), 4.85 (1H, br d, J=1.0 Hz, H-17$_A$), 4.88 (1H, br d, J=1.0 Hz, H-17$_B$), 5.37 (br m, H-14), 7.18 (1H, td, J_1=7.0 Hz, J_2=1.5 Hz, H-12); ^{13}C-NMR ($C_5^2H_5N$) (Table 1).

Neoandrographolide (**2**): colorless amorphous material, m.p. ~100 °C (from MeOH), TLC-SiO_2 (CH_2Cl_2–MeOH–water 9:1) R_F=0.33, anisaldehyde–H_2SO_4–glacial acid reagent: purple, $[\alpha]_D^{20}=-20.2°$ (c=0.61 g/100 ml, MeOH), UV

Table 1
^{13}C-NMR chemical shift assignments of andrographolide and neoandrographolide (ppm measured in $C_5^2H_5N$)

Carbon	Andrographolide (**1**)		Neoandrographolide (**2**)	
	DEPT[a]	^{13}C	DEPT	^{13}C
1	CH_2	37.4	CH_2	39. 2
2	CH_2	29.1	CH_2	19.5
3	CH	80.0	CH_2	36.6
4	q[b]	43.3	q	39.9
5	CH	55.5	CH	56.3
6	CH_2	24.5	CH_2	25.1
7	CH_2	38.3	CH_2	38.9
8	q	148.0	q	148.3
9	CH	56.5	CH	56.8
10	q	39.3	q	38.7
11	CH_2	25.1	CH_2	22.2
12	CH	147.0	CH_2	24.8
13	q	130.3	CH	134.3
14	CH	66.1	CH	145.3
15	CH_2	75.4	CH_2	70.6
16	q	170.7	q	174.0
17	CH_2	108.8	CH_2	107.0
18	CH_3	23.8	CH_3	28.2
19	CH_2	64.2	CH_2	72.7
20	CH_3	15.3	CH_3	15.5
1′			CH	105.5
2′			CH	75.4
3′			CH	78.8
4′			CH	72.0
5′			CH	78.4
6′			CH_2	63.0

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

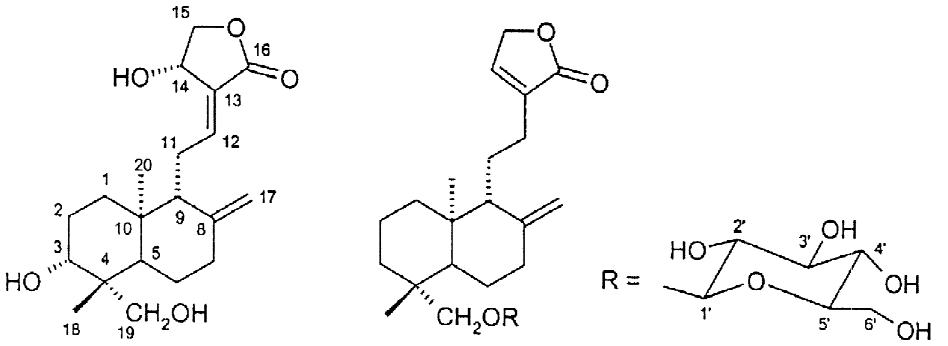

Fig. 3. Chemical structures of andrographolide (**1**) and neoandrographolide (**2**).

(MeOH): λ_{max} nm (ϵ) = 208 (1088), CD (MeOH): λ_{max} nm (θ) = 202 (−10 494), 205 (+17 764), 221 (−3500), ORD (MeOH): λ_{max} nm (ϕ) = 203 (−17 230), 212 (+9032), 230 (−520), ESI-MS (pos) m/z: 503 $[M+Na]^+$, ESI-MS (neg) m/z: 479 $[M-H]^-$, 539 $[M+Na+K-H]^-$, 959 $[2M-H]^-$, MS/MS (479): 317 $[M-glucose-H]^-$, $C_{26}H_{41}O_8$, ^{1}H-NMR ($C_5^2H_5N$): δ aglycone: 0.63 (3H, s, CH_3-20), 0.9 (obscured by signal overlapping) (2H, CH_2-3), 1.17 (3H, s, CH_3-18), 2.2 (obscured by signal overlapping) (1H, m, H-12_A), 2.50 (1H, m, H-12_B), 3.52 (1H, d, J = 9.5 Hz, H-19_A), 4.32 (1H, d, J = 9.5 Hz, H-19_B), 4.72 (obscured by signal overlapping) (s, H-17_A), 4.72–4.73 (obscured by signal overlapping) (2H, CH_2-15), 4.91 (1H, br s, H-17_B), 7.15 (1H, t, J = 1.5 Hz, H-14), β-D-glucose-moiety: 3.95 (1H, m, H-5′), 4.03 (1H, m, H-2′), 4.19–4.24 (obscured by signal overlapping) (2H, m, H-3′, H-4′), 4.38 (1H, dd, J_1 = 12.0 Hz, J_2 = 5.5 Hz, H-$6'_A$), 4.54 (1H, dd, J_1 = 12.0 Hz, J_2 = 3.0 Hz, H-$6'_B$), 4.82 (1H, d, J = 7.5 Hz, H-1′), ^{13}C-NMR ($C_5^2H_5N$) (Table 1).

Our study demonstrates that high-speed counter-current chromatography (HSCCC) is a fast and efficient technique to prepare pure andrographolide and neoandrographolide from crude extracts of leaves of *A. paniculata* which is used as a medicinal plant.

References

[1] M.P. Cava, W.R. Chan, L.J. Haynes, L.F. Johnson, B. Weinstein, Tetrahedron 18 (1962) 397.
[2] M.P. Cava, W.R. Chan, R.P. Stein, C.R. Willis, Tetrahedron 21 (1965) 2617.
[3] T. Fujita, R. Fujitani, Y. Takeda, Y. Takaishi, T. Yamada, I. Miura, Chem. Pharm. Bull. 32 (1984) 2117.
[4] A.D. Balmain, J. Connolly, J. Chem. Soc. Perkin Trans. I (1973) 1247.
[5] W. Chen, X. Liang, Planta Med. 45 (1982) 245.
[6] P.K.S. Visen, B. Shukla, G.K. Patnaik, J. Ethnopharmacol. 40 (1993) 131.
[7] S.S. Handa, A. Sharma, Indian J. Med. Res. 92 (1990) 276.
[8] C. Calabrese, S.H. Berman, J.G. Babish, X.F. Ma, L. Shinto, M. Dorr, K. Wells, C.A. Wenner, L.J. Standish, Phytother. Res. 14 (2000) 333.
[9] T. Matsuda, M. Kuroyanagi, S. Sugiyama, K. Umehara, A. Ueno, K. Nishi, Chem. Pharm. Bull. 42 (1994) 1216.
[10] Q. Du, Z. Li, Y. Ito, J. Chromatogr. A 923 (2001) 271.