PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **13**, 1–3 (2002)
DOI: 10.1002/pca.608

One Step Purification of Corilagin and Ellagic Acid from *Phyllanthus urinaria* using High-Speed Countercurrent Chromatography

Liu Jikai,[1]* Huang Yue,[1] Thomas Henkel[2] and Karlheinz Weber[2]

[1]Department of Phytochemistry, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, People s Republic of China
[2]Bayer AG, Pharma Research, D-42096 Wuppertal, Germany

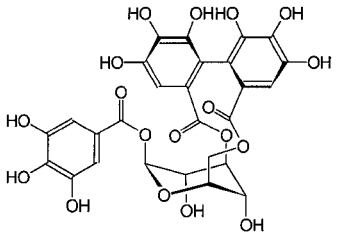
High-speed countercurrent chromatography (HSCCC) has been successfully applied to the preparative separation of corilagin and ellagic acid in one step from the Chinese medicinal plant *Phyllanthus urinaria* L. by use of direct and successive injections of a crude methanolic extract. Some aspects concerning the practical use of this technique in the described application are considered. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: High-speed countercurrent chromatography (HSCCC); one step purification; corilagin; ellagic acid; *Phyllanthus urinaria*.

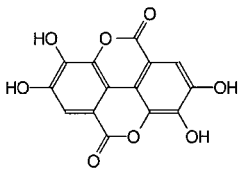
INTRODUCTION

Most separations in the natural product field are performed through chromatography on solid supports, but all-liquid techniques are currently attracting considerable interest. High-speed countercurrent chromatography (HSCCC) is an all-liquid technique that employs no solid support and functions using a multilayer coil rotating at high speed in a device that creates a fluctuating acceleration field which produces successive bands of mixing and settling along a continuous tube (Conway, 1995). As a modern liquid–liquid chromatography without solid sorbent, HSCCC benefits from a number of advantages in comparison with preparative HPLC, for example (i) there is no irreversible adsorption, (ii) the risk of sample denaturation is minimal, (iii) it is possible to use very crude samples, (iv) there is considerable potential for scale-up, and (v) it is the method of choice for the purification of very polar solutes. HSCCC has been used increasingly to solve problems of separation of natural products (Schaufelberger, 1991; Marston and Hostettmann, 1994).

The gallotannin corilagin (**1**), the haemostatic ellagic acid (**2**), as well as seven ellagitannins, which have been shown to be active against Epstein–Barr virus DNA polymerase at the micro-molar level (Liu *et al.*, 1999), have been isolated from *Phyllanthus urinaria* (Euphorbiaceae). Two new phenolic compounds, namely methyl brevifolin carboxylate and trimethyl ester dihydrochebulic acid, have also been isolated from the same source (Yao and Zuo, 1993). Corilagin (**1**) has been reported to show bioactivity in various different therapeutic areas such as cardiovascular disease (anti-hypertensive, Lin *et al.*, 1993; Cheng *et al.*, 1995) and infectious disease (antiviral, Yoon *et al.*, 2000). The present report deals with the one-step isolation of compounds **1** and **2** from the methanolic extract of aerial parts of *P. urinaria*, and demonstrates the rapid and efficient access of the constituents using HSCCC.



Corilagin (**1**)



Ellagic acid (**2**)

EXPERIMENTAL

Plant material. The aerial parts of *Phyllanthus urinaria* were obtained from the Guizhou Province of China and were collected in October 1996. The plant was identified by Dr. Peng Hua (Kunming Institute of Botany) and a voucher specimen is deposited in the herbarium collection of the Institute. Air-dried plant material was powdered and extracted three times with methanol at room temperature for a total of 3 days. The combined methanol extract was concentrated *in vacuo*, and the residue was used to prepare samples for further separation by HSCCC.

High-speed countercurrent chromatographic separation. HSCCC experiments were performed using an Ito multi-layer planet centrifuge chromatograph (P.C. Inc., Potomac, MA, USA). The multi-layer coil was pre-

* Correspondence to: L. Jikai, Department of Phytochemistry, Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, People's Republic of China.
Email: jkl@public.km.yn.cn
Contract/grant sponsor: National Sciences Foundation of Yunnan Province; Contract/grant number: 98C086M; Contract/grant number: 98C008Z.

Received 9 October 2000
Revised 7 August 2001
Accepted 7 August 2001

pared from polytetrafluoroethylene (PTFE) tubing (107.2m × 1.68mm i.d.; total capacity 238 mL). The two-phase solvent system was *n*-butanol:acetic acid:-water (4:1:5), which had been thoroughly equilibrated in a separating funnel by repeated vigorous shaking and degassing at room temperature. The column was initially filled completely with the upper (stationary) phase of the solvent system, and then the centrifuge was rotated at 700 rpm (forward) while the aqueous lower (mobile) phase of the solvent system was pumped from the head of the column towards the tail (the head–tail relationship of the rotating coil is conventionally defined in terms of an Archimedes screw where all objects of different densities are driven towards the head of the coil) at a flow rate of 2 mL/min using a Knauer (Berlin, Germany) HPLC pump model 64. After the stationary phase had been displaced and preliminary stabilisation of the mobile phase flow had been reached, 2 g of crude methanolic extract dissolved in 2 mL of both phases was loaded onto the column. The effluent from the outlet of the column was collected in test tubes in 6 mL fractions using an LKB Bromma (Uppsala, Sweden) 2211 Superrac fraction collector and detected in the UV at 254 nm. HSCCC separation consisted of two successive injections of the sample at 2.5 h intervals. When separation was completed, 70 mg of corilagin (**1**) and 40 mg of ellagic acid (**2**) were obtained. The retention of the stationary phase was measured by collecting the column contents after forcing them out of the column with pressurised nitrogen gas.

Conventional isolation of 1 and 2. Crude methanolic extract (1 g) was dissolved in 10 mL methanol and centrifuged. The supernatant was subjected to gel-filtration over Sephadex LH-20 (100 × 5 cm i.d. column; Pharmacia, Uppsala, Sweden) eluted with a gradient of methanol (20–100%) in water. Further purification of the 100% methanol fractions was performed by preparative HPLC to yield 25 mg of corilagin (**1**). Preparative HPLC was performed on a Gilson (Villiers Le Bel, France) model 305/306 chromatograph equipped with a Nucleosil 100 C-18 (Knauer) column (250 × 16 mm i.d.; 7 μm) eluted with a gradient of 0.05% trifluoroacetic acid in water:acetonitrile at a flow-rate of 12 mL/min.

RESULTS AND DISCUSSION

Separation of a crude methanolic extract of the aerial parts of *Phyllanthus urinaria* employing a sequence of three conventional steps including liquid-liquid distribution, gel permeation over Sephadex LH-20 and repeated reversed-phase HPLC chromatography gave, after optimisation, a yield of corilagin (**1**) of ca. 25 mg/g raw extract material and required 2–3 days of labour. In comparison with this, HSCCC separation was performed in just one step in a time of 3.5 h. In addition, the separation could be accelerated by successive injections of the crude sample at 2.5 h intervals, enabling rapid access to significant sample amounts. The HSCCC elution chromatogram detected at 254 nm is shown in Fig. 1 and exhibited just two peaks; the corresponding components were analysed by HPLC and shown to be corilagin (peak 1, yield 35 mg/g crude methanolic extract) and ellagic acid (peak 2), both compounds being

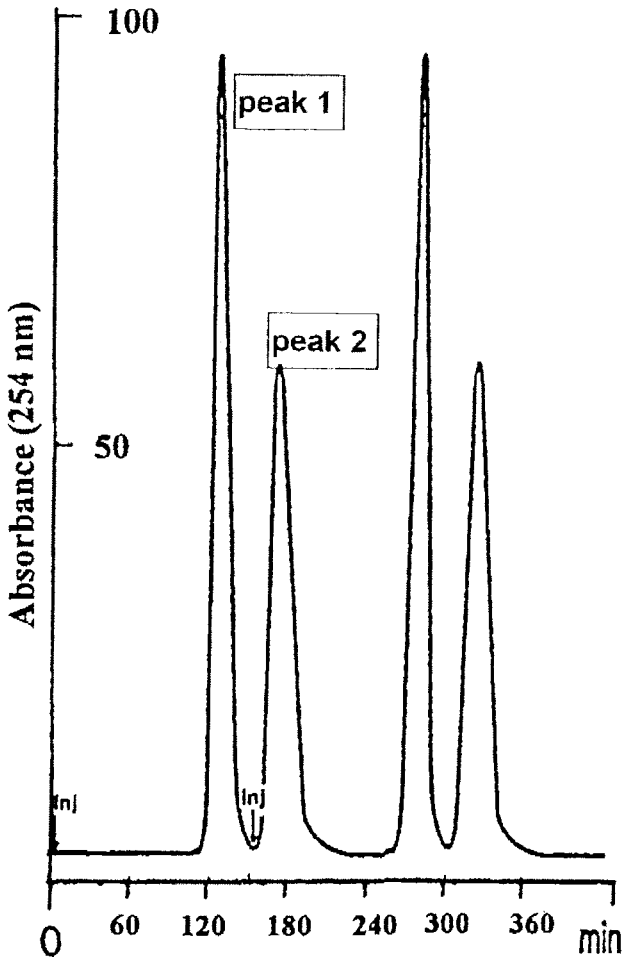


Figure 1. High-speed countercurrent chromatogram (detected at 254 nm) following two separate injections (at the points "Inj", indicted) of a crude methanolic extract of *Phyllanthus urinaria*. Key to peak identity: **1**, corilagin; **2**, ellagic acid. Note that components which remained on the stationary phase during elution have not been investigated further (for chromatographic protocols see Experimental section)

present in a purity >95% compared to the respective standard. The remainder of the injected raw material remained in the tube of the coil and has not been further investigated.

The structure of **1** was confirmed on the basis of its mass and NMR spectra in comparison with data of the known compounds in the literature. The HPLC-ESI MS showed a molecular ion at *m/z* 669 ($[M - H + 2H_2O]^+$) corresponding with a molecular weight of 634 ($C_{27}H_{22}O_{18}$). The ^{1}H-NMR spectrum exhibited 11 protons including four aromatic (singlet) protons and seven aliphatic protons which referred to a sugar-like moiety. From the ^{13}C-NMR spectrum, 27 carbons could be identified, namely, six aliphatic carbons (60–95 ppm), 3 carbonyl carbons and 18 aromatic carbons (100–150 ppm). According to these data it was deduced that **1** contained three aromatic rings, one glucose moiety and an highly oxygenated skeleton. Taking the connectivity pattern of the HMBC/HMQC spectra into account, a gallic acid, a biphenylic and a pyranose moiety become evident. By searching for known natural products in the literature with similar substructure elements the identity of **1** with corilagin was proven (Haddock *et al.*, 1982; Yoshida *et al.*, 1992). Ellagic acid (**2**) was identified from

its UV spectrum and HPLC retention time using an in-house reference database.

For rapid and efficient isolation of corilagin (**1**) from a crude extract, high-speed countercurrent chromatography was demonstrated to be a very powerful tool. This all-liquid technique is an attractive proposition for the separation of bioactive natural products due to its ability to handle crude material directly with no binding to, or deactivation by, a solid sorbent. In addition its potential for accelerated isolation was successfully applied in the above example. The key to successful separation using HSCCC depends on the choice of an appropriate solvent system. Selection of the solvent system can be guided and optimised by determination of the appropriate partition coefficients using analytical HPLC. For convenience and efficiency, a system in which the compounds of interest display partition coefficients between 0.67 and 1.50 is sought. Typically, chloroform:methanol:water may be chosen as a starting point and through modification of the relative proportions of each individual solvent, or through successive solvent substitution, it is possible finally to obtain the required distribution of sample between the two phases of the solvent system.

Acknowledgements

This work was carried out under contract with grant sponsorship from the Natural Sciences Foundation of Yunnan Province (98C086M, 98C008Z).

REFERENCES

Cheng JT, Lin TC, Hsu FL. 1995. Anti-hypertensive effect of corilagin in the rat. *J Physiol Pharmac* **73:** 1425–1429.

Conway WD. 1995. *Modern Countercurrent Chromatography*, Conway WD, Petroski RJ (eds). American Chemical Society: Washington, DC; 1–14.

Haddock EA, Gupta RK, Haslam E. 1982. The metabolism of gallic acid and hexahydroxydiphenic acid plants. *J Chem Soc Perkin Trans I* 2535–2545.

Lin TC, Hsu FL, Cheng JT. 1993. Anti-hypertensive activity of corilagin and chebulinic acid, tannins from *Lumnitzera racemosa*. *J Nat Prod* **56**: 629–632.

Liu KC, Lin MT, Lee SS, Chiou JF, Ren S, Lien EJ. 1999. Anti-viral tannins from two *Phyllanthus* species. *Planta Med* **65**: 43–46.

Marston A, Hostettmann K. 1994. Countercurrent chromatography as a preparative tool. Applications and perspectives. *J Chromatogr A* **658**: 315–341.

Schaufelberger DE. 1991. Application of analytical high-speed countercurrent chromatography in natural products chemistry. *J Chromatogr* **538**: 45–57.

Yao QQ, Zuo CX. 1993. Chemical studies on the constituents of *Phyllanthus urinaria*. *Acta Pharm Sin* **28**: 829–835.

Yoon S, Chung TH, Chung CC. 2000. Pharmaceutical composition for the treatment of Hepatitis B comprising corilagin or brevifolin. Patent WO0027413.

Yoshida T, Itoh H, Matsunaga S, Tanaka R, Okuda T. 1992. Tannins and related polyphenols of Euphorbiaceous plants. *Chem Pharm Bull* **40**: 53–60.

