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Short communication

Preparative separation of the naphthopyranone glycosides by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was applied to the preparative separation and purification of naphthopyranone glycosides from a crude 70% ethanolic extract of the capitula of *Paepalanthus microphyllus*. The solvent system used was composed of water–ethanol–ethyl acetate–hexane (10:4:10:4, v/v). This technique led to the separation of four different naphthopyranone glycosides in pure form in only 7 h. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Counter-current chromatography; Preparative chromatography; *Paepalanthus microphyllus*; Glycosides; Naphthopyranones; Carbohydrates

1. Introduction

The fractionation of polar extracts from plant origin are often a difficult task. The separation and purification of secondary metabolites using the conventional methods such column chromatography and high-performance liquid chromatography (HPLC) require several steps resulting in low recoveries of products. Adsorption chromatographic methods almost always led to irreversible adsorption or decomposition of labile substances. To overcome these problems, we have been using droplet counter-current chromatography (DCCC) [1,2]. Inspite of its good resolution and reproducibility, it is a time-consuming method. As an alternative method we have now been using high-speed counter-current chromatography (HSCCC). The separation is based on a liquid–liquid partition chromatograph method which do not employ a solid supporting matrix [3,4]. This technique has been successfully applied to analysis and separation of apolar as well as polar natural products [5–8].

The *Paepalanthus* species (Eriocaulaceae) are popularly known as ''sempre-vivas'' (everlasting plants) and have ornamental value. They are found commonly in regions of Minas Gerais State in Brazil. This genus produces mainly naphthopyranones and their glycosides. Naphthopyranones are a relatively rare class of natural compounds, mostly isolated from fungi [9]. The naphthopyranones isolated from *Paepalanthus* species have shown potent antibiotic, citotoxic and mutagenic activities [10–13]. Therefore, it is important to separate these compounds fast and efficiently.

Previous screening of *Paepalanthus microphyllus*

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(Giull.) Kunth. by thin-layer chromatography (TLC) have shown a mixture of four naphthopyranone glycosides. Attempts to isolate these compounds by silica-gel open column chromatography or by gel permeation column chromatography followed by purification of the compounds by HPLC led to the decomposition of a large part of the compounds. The present paper introduces a method for the separation and purification of naphthopyranone glycosides from a crude extract of *Paepalanthus microphyllus* using HSCCC.

2. Experimental

2.1. Chemicals

All solvents used for HSCCC were of p.a. grade from Merck. The solvents used for HPLC were of analytical grade from Mallinckrodt Baker S.A., Brazil.

2.2. Preparation of crude sample and sample solution

Capitula (39 g) of *Paepalanthus microphyllus* were collected at Serra do Cipó, Minas Gerais State, Brazil. The plant material was dried in an oven at 60°C during 1 week and powdered. The resulting material was macerated at room temperature sequentially with hexane, methylene chloride, ethanol and 70% ethanol during 1 week with each solvent. After filtration and evaporation of the solvents under reduced pressure we obtained approximatelly 1.5 g of each extract. A portion of the 70% ethanolic extract (0.9 g) was dissolved in 20 ml of a mixture consisting of 10 ml lower phase+10 ml upper phase of the solvent system water–ethanol–ethyl acetate–hexane (10:4:10:4, v/v).

2.3. High-speed counter-current chromatography (HSCCC)

The preparative HSCCC instrument employed in the present study was from P.C. Inc., Potomac, USA. It was equipped with a multilayer with two coils of 1.68 mm i.d polytetrafluoroethylene (PTFE) tubing of approximately 80 and 240 ml with a total capacity of 320 ml. The β value varied from 0.50 at the internal terminal to 0.85 at the external terminal and the revolution radius was 10 cm ($\beta = r/R$, where r is the distance from the coil to the holder, and R, the revolution radius or the distance between the holder axis and the central shaft). The speed (varying between 0 and 1200 rpm) was adjusted with a controller to an optimum speed of a 800 rpm. The flow-rate was controlled with an FMI-50 QD-O SSY, BS/BS constant-flow pump. The sample was injected with a P.C. Inc. Injection Module with a 20-ml sample injection loop.

The coiled column was first entirely filled with the stationary phase (lower phase). Then the apparatus was rotated forward at 800 rpm, while the mobile phase (upper phase) was pumped into the column in a head to tail (H→T) direction at a flow-rate of 3.0 ml/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 20 ml of the sample solution containing 0.9 g of the crude extract was injected through the injection module at a flow-rate 3.0 ml/min. We collected 250 fractions of 5 ml each with a Redifrac automated fraction collector (Pharmacia, Sweden), in approximately 7 h.

2.4. Preparation of two-phase solvent system

The solvent system composed of water–ethanol–ethyl acetate–hexane (10:4:10:4, v/v) was throughly equilibrated overnight in a separatory funnel at room temperature and the two phases separated shortly before use.

2.5. Analyses of the compounds by TLC and HPLC

The crude sample and fractions were analyzed using silica gel TLC plates on glass (20×20 cm, Aldrich) developed with a solvent mixture composed of water–ethanol–ethyl acetate–hexane (10:4:10:4, v/v, upper layer). The spots on the TLC plates were observed under ultraviolet lamp (254 nm). Fractions of similar retention factors (R_F) were joined and weighed.

Representative fractions were analysed using a Varian, ProStar HPLC system equipped with a RP-18 column (250×4.6 mm I.D., 8 μm, Microsorb-MV).

The mobile phase used a linear gradient of water–methanol (50–100% methanol) over 30 min was eluted at a flow-rate of 1.0 ml/min, and the effluent was monitored using a ProStar 330 photodiode-array ultraviolet detection system at 273 nm.

2.6. Structural identification of the compounds

Nuclear magnetic resonance (NMR) spectra in CDOD (compounds **1–4**) were obtained using a Bruker spectrometer, operating at 200 MHz for ^{1}H and 50 MHz for ^{13}C.

Electrospray mass spectometry (ESI–MS) was performed in a Fisons VG Platform instrument in the positive mode (100 V) to compounds **1–4**. The samples were dissolved in methanol and injected directly into the mass spectrometer through a Rheodyne injector. Acetonitrile was used as the carrier solvent and nitrogen gas was used both as a drying gas and for nebulization.

3. Results

Fig. 1 shows the HPLC–DAD analysis of the crude 70% ethanolic extract of *P. microphyllus*. The UV spectra of the compounds **1–4** with retention times 9.4 min, 11.5 min, 12.7 min and 14.5 min presented bands at 280 and 390 nm (Fig. 2), typical of naphthopyranone derivatives [10–14]. Identification of each peak arose from the co-injection of the extract with each isolated compound.

The solvent system employed for the HSCCC

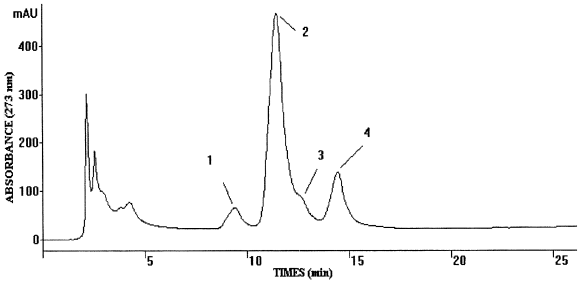


Fig. 1. HPLC separation of the crude extract from *P. microphyllus*. Experimental conditions: apparatus: Varian ProStar HPLC system equipped with a RP-18 column (250×4.6 mm I.D., 8 μm); mobile phase: gradient water–methanol (50–100% methanol) over 30 min; flow-rate: 1.0 ml/min; DAD detection: 273 nm.

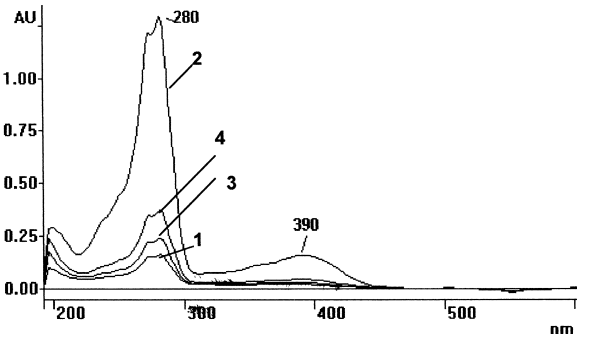


Fig. 2. UV spectrum from the the photodiodo-array signals of the naphthpyranones **1**–**4** from *P. microphyllus*; DAD with absorbance monitored in the range 190–600 nm.

separation was chosen based on the results of the TLC analyses and also by the distribution of a small amount of sample between the two imiscible phases [3,4]. The R_F values of compounds **1–4** were between 0.3 and 0.7, indicating a suitable separation using the organic layer as mobile phase.

Table 1 shows the results of separations of the compounds **1–4** from *P. microphyllus*. The structures of these naphthopyranone glycosides are shown in Fig. 3.

The elution of the four compounds was accomplished during 7 h. As the total number of phenolic hydroxyls is the same for all studied compounds, the separation of these naphthopyranone glycosides seems to be dependent not only on the number of sugar attached to the aglycone, but also on the total number of the alcoholic hydroxyls.

The first compound to be eluted was the relatively less polar monoglucoside **4** (paepalantine-9-*O*-β-D-glucopyranoside) after 2.5 h, that has only one sugar moiety. The disaccharide **1** (paepalantine-9-*O*-β-glucopyranosyl-(1→6)-β-D-glucopyranoside) was the most polar compound from this mixture of naphthopyranones and has seven alcoholic OH-groups. It eluted after 6 h and was also isolated in a pure form. The most critical separation was between compounds **2** and **3**, that occurred between 3.5 and 6 h. These two disaccharides **3** (paepalantine-9-*O*-α-rhamnopyranosyl-(1→6)-β-D-glucopyranoside) and **2** (paepalantine-9-*O*-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside) differ only by the terminal sugar. Both arabinose and rhamnose present three free OH-groups, α-configuration and in both of these sugars

Table 1
Compounds isolated from *P. microphyllus* by HSCCC. (%) Yield based on the crude extract

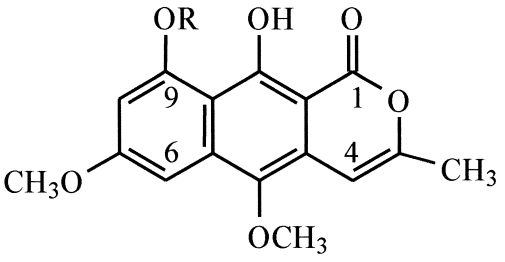
Compound	Fractions	Mass obtained (mg)	Purity (%)	Yield (%)
1	217–228	10	92	1.1
2	165–212	80	97	8.8
3	120–150	9	92	1.0
4	97–110	25	98	2.7

one of the OH-group is in the axial position. Despite they presented almost co-eluted peaks in the RP-HPLC system, a good separation was obtained using the preparative HSCCC system, as shown by the HPLC, TLC, NMR and ESI–MS analyses of the eluted substances.

The four compounds were previously isolated by gel permeation chromatography (GPC). But, as GPC separation is based on the molecular size of the compounds, a further purification by RP-HPLC was necessary, because compounds **1–3** present almost identical molecular size [15]. Using the GPC–RP-HPLC approach, the minor compounds **1** and **3** were only hardly isolated, because of their partial decomposition. On the other hand, as the separation mechanism of the HSCCC is based mainly on the differential solubility of the compounds between the two miscible phases, HSCCC allowed the fast and efficient isolation of these compounds without decomposition.

All naphthopyranone glycosides were obtained in good yields from the injected crude extract (Table 1).

Therefore, the results of our studies clearly demonstrated the advantages of preparative HSCCC. It has proven to be an efficient method for the isolation of naphthopyranone glycosides **1–4** from *P. microphylus* in only one step without need of time-consuming clean-up and minimizing the loss of material due to decomposition.



Compounds	R
1	glc(1→6)glc
2	glc(1→6)ara
3	glc(1→6)rha
4	glc

Fig. 3. Structures of compounds isolated from *P. microphyllus* by HSCCC.

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