Separation of Flavonoids and Naphthopyrones from four Brazilian *Paepalanthus* Species by Droplet Countercurrent Chromatography[1]

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RESUMO: A separação de flavonóides e naftopiranonas presentes em extratos polares dos capítulos de sempre-vivas brasileiras foi obtida através da cromatografia em contracorrente de gotejamento seguida de cromatografias em coluna de PVP e sephadex LH-20. As substâncias isoladas de extratos etanólicos foram identificadas por técnicas espectrométricas e comparadas com dados da literatura. Este procedimento levou ao isolamento e identificação da 9-*O*-β-D-glcopiranosilpaepalantina (**1**), 9-*O*-β-D-glicopiranosil(1→6)alopiranosilpaepalantina (**2**), além dos flavonóides 6-metoxicanferol (**3**), 3-*O*-β-D-glicopiranosil-6-metoxicanferol (**4**), patuletina (**5**), 3-*O*-β-D-rutinosilpatuletina (**6**), 7-*O*-β-D-glicopiranosilquercetagetina (**7**), 5,7,4'-triidroxi-6,3'-dimetoxiflavona (**8**) e 5,7,4'-triidroxi-6,3'-dimetoxiflavonol (**9**).

Palavras-chave: *Paepalanthus*, Eriocaulaceae, DCCC, flavonoids, naphthopyrones.

ABSTRACT: Separation of Flavonoids and Naphthopyrones from four Brazilian *Paepalanthus* Species by Droplet Countercurrent Chromatography. A general procedure was developed for the simultaneous separation of flavonoids and naphthopyrones from the polar extracts of the capitula from Brazilian everlasting plants is described. The ethanolic extracts of several species from the *Paepalanthus* genus (Eriocaulaceae) were fractionated by droplet countercurrent chromatography followed by column chromatography on pvp and sephadex LH-20. The isolated compounds were identified by spectrometric analysis and comparison with literature data. This approach led to the isolation of 9-*O*-β-D-glucopyranosylpaepalantine (1), 9-*O*-β-D-glucopyranosyl (1→6)allopyranosylpaepalantine (2), along with the flavonoids 6-methoxykaempferol (3), 3-*O*-β-D-glucopyranosyl-6-methoxykaempferol (4), patuletin (5), 3-*O*-β-D-rutinosylpatuletin (6), 7-*O*-β-D-glucopyranosylquercetagetin (7), 5,7,4'-trihydroxy-6,3'-dimethoxyflavone (8) and 5,7,4'-trihydroxy-6,3'-dimethoxyflavonol (9)

Keywords: *Paepalanthus*, Eriocaulaceae, DCCC, flavonoids, naphthopyrones.

INTRODUCTION

Paepalanthus ssp belong to the Eriocaulaceae family, in which many species are called "sempre-vivas" (everlasting flowers) (GIULIETTI, 1978). Many occur in the "campos rupestres" vegetation at Serra do Cipó – MG. From the 480 species occurring in the world, Brazil has around 400 (SANO, 1996). The study of this family is important because many species are exported from Brazil to Europe as ornamental plants and their collection and commercialization are a main sources of income for many families in Minas Gerais State (GIULIETTI, 1978). Also, many species contain substances with biological activity, as is the case with paepalantine, found in the capitula of *P.bromelioides* (VILEGAS *et al*, 1990), that presents strong antibiotic, cytotoxic and mutagenic activities (VILEGAS *et al*, 1990; VARANDA *et al*, 1997). However, few species have been chemically investigated (VILEGAS *et al*, 1990; VILEGAS *et al*, 1998).

Separation of the chemical constituents from these plants, especially polar substances has been a major difficulty. For the isolation of polar plant constituents such as glycosides - which are often very difficult to separate under adsorption conditions (HOSTETTMANN, 1980) - Droplet Countercurrent Chromatography (DCCC) is an efficient tool, since the separations are based on the partition of a solute between two imiscible phases and there is no solid support which might cause irreversible adsorption (HOSTETTMANN, 1980).

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The present paper reports the use of DCCC to fractionate polar extracts of the capitula of *Paepalanthus ssp.*, using the solvent system $CHCl_3$/MeOH/H_2O (43:37:20 and 13:7:4 v:v:v) in the descending mode. The fractionation resulted in the isolation of naphthopyrones and flavonoids, revealing a fast and easy technique for their separation, which is difficult by other techniques. The compounds isolated were identified by spectrometric analysis involving comparison with literature data.

EXPERIMENTAL

Instrumentation: Droplet countercurrent chromatography - Separation was carried out on a 300 DCC chromatograph (Tokyo Rikakikai, Tokyo, Japan) with 300 capillary columns (400x2 mm i.d.) connected in series with a collector (LKB 7000 Ultrorac). NMR spectra in DMSO-d_6, were obtained using a Bruker AC 200 spectrometer operating at 200 MHz for ^1H and 50 MHz for ^{13}C. NMR spectra in CD_3OD were obtained using a Bruker DRX-600 spectrometer, operating at 599 MHz for ^1H and 150.86 MHz for ^{13}C. The DEPT (Distortionless Enhancement by Polarization Transfer) experiments were performed using transfer pulse of 135°. Polarization transfer delays were adjusted to average CH coupling of 135 Hz. ^1H-^1H DFQ-COSY, ^1H-^{13}C HSQC, HMBC and 1D-HOHAHA experiments were obtained using conventional pulse sequences. ESMS spectra were performed in a Platform Micromass spectrometer in the positive mode (100V), the samples were dissolved in MeOH and injected directly

Material : The capitula of *Paepalanthus ssp* were collected in Serra do Cipó, Minas Gerais, Brazil, and subsequently identified by Prof. Paulo Takeo Sano. *Voucher* specimens have been deposited at the herbarium of the Departamento de Botânica do Instituto de Biociências: *P. hilairei* Koern. USP (CFSC 13843) **A**, *P. bromelioides* Silv. (CFSC 13856) **B**, *P. vellozioides* Ruhland (CFSC 13842) **C** and *P. latipes* Silv. CFSC 13846) **D** (Table 1).

TABLE 1. Naphthopyrones and flavonoids isolated by DCCC from four Brazilian *Paepalanthus* species.

Compound	Plants	Mass obtained (mg)	Identification methods	References
1	A, B, C, D	20	NMR(^1H,^{13}C) , UV, IR, ES-MS	I VILEGAS *et al*, 1998
2	A, B, C, D	18	NMR (^1H, ^{13}C, HOHAHA, DQF, HSQC, HMBC), UV, IR, ES-MS	VILEGAS *et al*, 1998
3	A, C	20	NMR(^1H, ^{13}C), UV, IR, ES-MS	WOLLEMBERGER *et al*, 1972;
4	A, B	15	NMR (^1H, ^{13}C), UV, IR	MERFORT & WENDISCH, 1987
5	B, D	8	NMR (^1H), UV	HARBORNE & MABRY, 1982.
6	A	15	NMR (^1H,^{13}C), UV, IR	HARBORNE & MABRY, 1982.
7	B	5	NMR (^1H), UV	HARBORNE & MABRY, 1982.
8	B	20	NMR (^1H , ^{13}C)	HARBORNE & MABRY, 1982.
9	B	5	NMR (^1H, ^{13}C) , UV	HARBORNE & MABRY, 1982.

A – *P.bromelioides*; **B** – *P.hilairei*; **C** – *P. vellozioides*; D – *P. latipes*

Isolation of the constituents from *Paepalanthus ssp.*: The capitula were dried (60º C), powdered and extracted successively with hexane, dichloromethane and ethanol. The extracts were evaporated under vacuum. Ethanolic extract (2.0 g) from each plant were dissolved in 20 ml of a 1:1 mixture of the upper phase and lower phase and injected into the DCCC. 200 fractions of ca. 9 ml each were collected. The system used was chloroform/methanol/water (43:37:20 - system I - for A, C and D) and 13:7:4 (system II) for B in the descending mode as indicated in Table 1.

The fractions were analysed by TLC on silicagel 60 (Merck) using $CHCl_3$: MeOH : H_2O

(43:37:20 and 13:7:4 v:v:v) as mobile phase, sprayed with NP/PEG reagent (WAGNER *et al*, 1984) and viewed under UV light (254-363 nm). They were screened and grouped (Figures 2 and 3).

In the system I, the mixtures **1+3** and **2+4** were also obtained. However, **1, 3, 2, 4** and **5** were also isolated in the pure form (Figures 2).

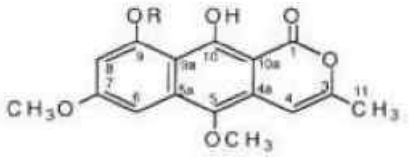
On the other hand, in the system II, there were some mixtures: **1 + 8 + 9** and **2 + 6**; substance **7** was obtained pure (Figure 3). The mixture **2 + 6** was dissolved in methanol, centrifuged and separated by gel permeation chromatography on Sephadex LH-20 (20 X 1.5 cm) eluted with methanol. The mixture **1 + 8 + 9** was dissolved in methanol, centrifuged and further purified on a PVP CC of 10 x 1.5 cm eluted with methanol.

Table 1 lists the spectrometric methods used for the identification of each substance: 9-*O*-β-D-glucopyranosylpaepalantine (**1**), 9-*O*-β-D-glucopyranosyl (1→6)allopyranosylpaepalantine (**2**), along with the flavonoids 6-methoxykaempferol (**3**), 3-*O*-β-D-glucopyranosyl-6-methoxykaempferol (**4**), patuletin (**5**), 3-*O*-β-D-rutinosylpatuletin (**6**), 7-*O*-β-D-glucopyranosylquercetagetin (**7**), 5,7,4'-trihydroxy-6,3'-dimethoxyflavone (**8**) and 5,7,4'-trihydroxy-6,3'-dimethoxyflavonol (**9**)

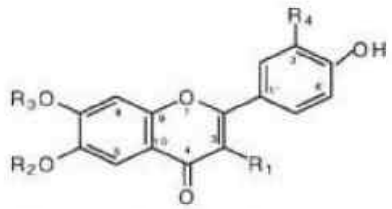
RESULT AND DISCUSSION

Compounds **1-9** are common in many *Paepalanthus* species, as screened by TLC. However, previous attempts to isolate them by adsorption CC on silica gel led to strong adsorption and loss of material. The main purpose of this study was to apply a method based on DCCC appropriate for the separation of such compounds (Figure 1).

Figures 2 and 3 show that substances **1** and **2** were easily separated in both solvent systems, system **I** leading to a faster separation than **II**. In system **I** (Figure 2) the monoglucoside **1** eluted in fractions 30-50, and the diglucoside **2** in fractions 90-110. Inspite some overlapping of the substances, the flavonoids could also be separated from the naphthopyrones by using the system **I**, in which the aglycone **3** was obtained pure in fractions 55-65, the monoglucoside **4** was obtained pure in fractions 105-115, and the diglucoside eluted in fractions 170-210.



(**1**) R = glc
(**2**) R = glc(1→6)allo



(**3**) R_1 = OH, R_2 = CH_3, R_3 = H, R_4 = H
(**4**) R_1 = Oglc, R_2 = CH_3, R_3 = H, R_4 = H
(**5**) R_1 = OH, R_2 = CH_3, R_3 = H, R_4 = OH
(**6**) R_1 = Oglc(1→6)rha, R_2 = CH_3, R_3 = H, R_4 = H
(**7**) R_1 = OH, R_2 = H, R_3 = glc, R_4 = OH
(**8**) R_1 = H, R_2 = CH_3, R_3 = H, R_4 = OCH_3
(**9**) R_1 = OH, R_2 = CH_3, R_3 = H, R_4 = OCH_3

FIGURE 1 - Compounds isolated and identified from capitula of *Paepalanthus* ssp. glc = glucose, rha = rhamnose, allo = allose

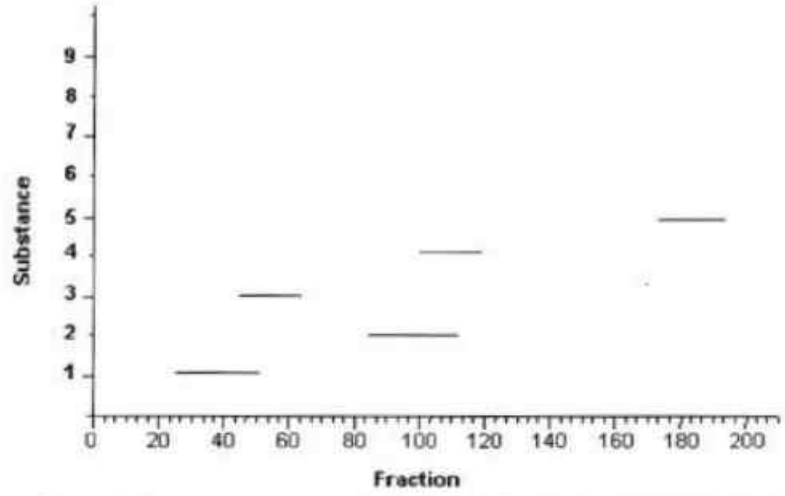


FIGURE 2: Fractions collected after separation by DCCC showing the separation of substances **1-5** using solvent system (**I**) $CHCl_3$:MeOH:H_2O, 43:37:20 v.v.v. descending mode.

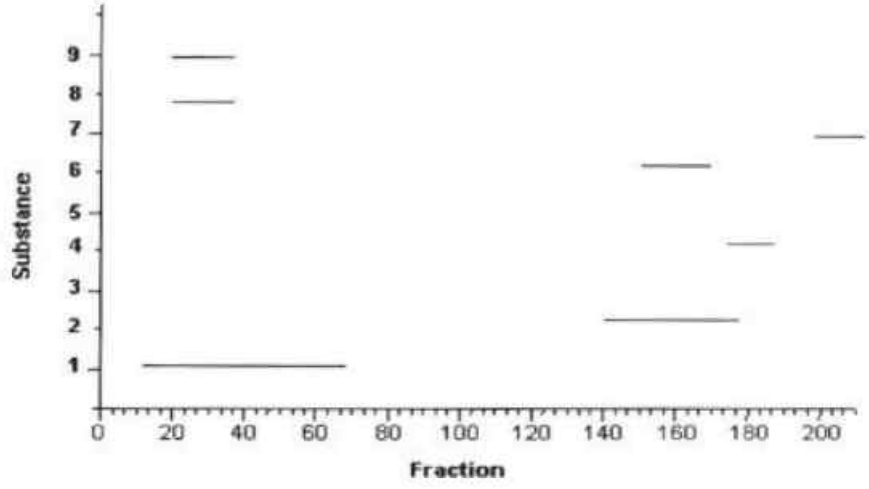


FIGURE 3: Fractions collected after separation by DCCC showing the separation of substances **1, 2, 6, 7, 8** and **9** using solvent system (**II**) $CHCl_3$:MeOH: H_2O 13:7:4 v:v:v, descending mode.

It is interesting to note that substance **3**, aglycone of **4**, eluted after substance **1**, a naphthopyrone monoglycoside, and substance **4**, a flavonoid monoglucoside, eluted after substance **2**, a naphthopyrone diglucoside. It seems that the phenolic hydroxyls of the flavonoids led to a higher solubility of the compounds in the stationary (aqueous) phase of system **I** than do the sugar moieties bound to the naphthopyrone nucleus, a fact which helped to provide a good separation among these substances.

System **II** (Figure 3) led to a faster elution of **1**, but to slower elution of **2** due to the lower polarity of this compound in the mobile phase. **1** was obtained in a mixture with the aglycones **8** and **9**, while **2** was mixed with **4** and **6** (Figure 3). Therefore, other chromatographic methods were used for final separations. The mixture **2 + 6** was separated by gel permeation CC (Sephadex LH-20) (HANS, 1995) and the mixture **1 + 8 + 9** was separated by CC on polyvinylpyrrolidone (PVP) (OLSSON, 1974).

Although the four species **A**, **B**, **C** and **D** produce different flavonoids, the naphthopyrones **1** and **2** are present in all those studied. These substances are relatively rare in nature and more often isolated from microrganisms (HILL, 1986).

Our results indicated that DCCC with system **I** is a good method for the isolation without column losses of polar constituents from *Paepalanthus* spp.

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REFERENCE

GIULIETTI,A. M .Modificações taxonômicas no gênero *Eriocaulon* L. **Boletim de Botanica**, v.6 , p. 39-47, 1978

HANS,H.**Preparative gel chromatography on sephadex** LH-20. Heidelberg: Huthig, 1995.

HARBORNE, J.B. , MABRY, T.J. **The flavonoids in advances in research.** New York : Chapman and Hall, 1982. 744p.

HILL, A. Naturally occuring isocoumarins. **Fortschritte der Chemie Organischer Naturstoffe,** v. 49, p.1-78. 1986.

HOSTETTMANN, K .Droplet countercurrent chromatography and its applications to the preparative scale separation of natural products. **Journal of Medicinal Plants,** v. 39, n. 1, p. 1-18, 1980.

MERFORT, I., WENDISCH, D. Flavonoidglycoside aus *Arnica montana* and *Arnica chamissonis*. **Planta Medica**, v.53, p. 434-7, 1987.

OLSSON, L., SAMUELSON, O. chromatography of aromatic acids and aldehydes and phenols on crosslinked poly(vinylpyrrolidinone). **Journal of Chromatography,** v. 93, n.1, p. 189-99, 1974.

SANO, P.T. Fenologia de *Paepalanthus hilairei* Koern., *P.polyanthus* (Bong.) Kunth e *P.robustus* Silveira: *Paeplanthus* Sect.*Actinocephalus* Koern. Eriocaulaceae. **Acta Botanica Brasilica,** v.10 , n. 1, 1996.

VARANDA, E.A., RADDI, M.S.G., DIAS, F.L., ARAÚJO, M.C.P., GIBRAN, S.C.A., TAKAHASHI, C.S. , VILEGAS, W. Mutagenic citotoxic activity of an isocoumarin (paepalantine) isolated from *Paepalanthus vellozioides*. **Teratatogenesis Carcinogenesis and Mutagenesis**, v. 17, p. 85-95, 1997.

VILEGAS, W.; ROQUE, N.F.; SALATINO, A.; GIESBRECHT, A M. AND DAVINO, S. Isocoumarin from *Paepalanthus bromelioides* **Phytochemistry,** v. *29*, n. 7, p. 2299-301, 1990.

VILEGAS, W., SANTOS, L.C., ALÉCIO, A C.; PIZZA, C., PIACENTE, S., DE PAUW E ,SANO, P.T. Naphthopyranone glycosides from *Paepalanthus bromelioides*. **Phytochemistry**, v. 49, n.1, p. 207-10, 1998.

WAGNER, H , BLADT, S. , ZGAINSKI, E. M. **Plant drug analysis.** Berlin: Springer, 1984. 303p.

WOLLENWEBER, E. , LEBRETON, P, CHADENSON, M. *Z.* Flavonoide im Knospen-Exkret von *Prunus*- und *Rhamnus*-Arten. **Zeitschrift fur Naturforschung Teil B,** v. 27 b, p. 567-70,1972.