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Fast preparative separation of flavones from capitula of *Eriocaulon ligulatum* (Vell.) L.B.Smith (Eriocaulaceae) by High-speed countercurrent chromatography (HSCCC)

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) is a leading method for the fast separation of natural products from plants. It was used for the preparative isolation of two flavone monoglucosides present in the capitula of *Eriocaulon ligulatum* (Vell.) L.B.Smith (Eriocaulaceae). This species, known locally as ″botão-dourado″, is exported to Europe, Japan and North America as an ornamental species, constituting an important source of income for the local population of Minas Gerais State, Brazil. The solvent system, optimized in tests prior to the HSCCC run, consisted of the two phases of the mixture ethyl acetate: *n*-propanol: water (140:8:80, v/v/v), which led to the successful separation of 6-methoxyluteolin-7-*O*-β-D-allopyranoside and 6-methoxyapigenin-7-*O*-β-D-allopyranoside in only 3 hours. The two flavonoids were identified by NMR (1-D and 2-D) and ESI-MS, comparing their spectra with published data.

Keywords: HSCCC, *Eriocaulaceae*, flavone, *E. ligulatum.*

INTRODUCTION

Eriocaulon ligulatum (Vell.) L.B.Smith. (of the pipewort family, Eriocaulaceae), called ″botão-dourado″ (golden button) locally, is exported to Europe, Japan and North America as an ornamental flower, representing an important source of income to the population of Minas Gerais State.

Scientific research on *Eriocaulon* is scarce and very little is known about its chemical constituents. Bate-Smith & Harborne (1969) identified the flavonols quercetagetin and patuletin from the leaves of *E. septangulare* (common pipewort), *E. brownianum*, *E. nilagirense*, *E. decangulare*, *E. sexangulare*, *E. wightianum*. These results were the first record of flavonols bearing an extra 6-hydroxyl (or methoxyl) substituent being isolated from monocotyledons. Ho & Chen (2002) identified four flavonoids, including (2S)-3',4'-methylenedioxy-5,7-dimethoxyflavan and hispidulin [7-(6-*E*-*p*-coumaroyl-β-*D*-glucopyranoside)], as well as tocopherol, in the capitula of *E. buergerianum* Koern.

The separation and purification of flavonoids by conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) is tedious and usually requires multiple chromatography steps. As a modern liquid–liquid partition chromatography without a solid sorbent phase, high-speed countercurrent chromatography (HSCCC) uses no solid support, so the effects of adsorption and artifact formation on stationary-phase material can be eliminated. This technique has a greater maximum capacity, coupled with excellent sample recovery and fine separation of closely-related natural products, and a wider choice of solvent systems, than HPLC. Furthermore, crude sample can be introduced directly into the hollow coiled separation column.

We report here the isolation of taxonomically relevant flavones from the capitula (flower head) and scape (leafless flower stalk) of *E. ligulatum* by HSCCC.

MATERIAL AND METHODS

Preparation of crude sample and sample solution: Capitula of *E. ligulatum* (Vell.) L.B.Smith were collected in May 1999, in Diamantina, Minas Gerais State, Brazil and authenticated by Professor Dr. Paulo Takeo Sano at the Instituto de Biociências (IB) of the Universidade de São Paulo (USP), São Paulo. A voucher specimen (catalogued as SANO 2978) was deposited at the Herbarium of the IB-USP.

Capitula and scapes of *E. ligulatum* (500 g) were separated, powdered and successively macerated at room temperature with *n*-hexane (2 L), methylene choride (2 L) and methanol (2 L) for one week each solvent. Solvents were evaporated under reduced pressure to yield gummy extracts.

The methanol extract (10.0 g) was partitioned with a mixture of *n*-butanol/water (1:1, v/v for 3 times), affording 4.5 g of extract in the *n*-butanol phase and 4.7 g in the aqueous phase. An aliquot of the *n*-butanol fraction (400

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mg) was dissolved in 20 mL of a mixture consisting of 10 mL lower (aqueous) phase + 10 mL upper (organic) phase of the solvent system chosen for HSCCC: ethyl acetate:*n*-propanol:water (140:8:80, v/v/v).

High-speed countercurrent chromatography (HSCCC): The preparative HSCCC instrument employed in this study was from P.C. Inc., Potomac, (Buffalo, NY, USA). It was equipped with a multilayer helical column with two coils of 1.68 mm i.d. polytetrafluoroethylene (PTFE) tubing of approximately 80 mL and 240 mL connected in series with a total capacity of 320 mL. The β value varied from 0.5 at the internal to 0.85 at the external terminal and the revolution radius was 10 cm (β= *r*/*R*, where *r* is the distance from the coil to the column holder axis, and *R*, the revolution radius or the distance between the holder axis and the central shaft). The speed was adjusted with a controller to an optimal 850 rpm. The flow rate was controlled with a Waters 4000 constant-flow pump (Milford, MA-USA). The sample was injected through a P.C. Inc. Injection Module (Buffalo, NY, USA) with a 20 mL sample injection loop. The coiled column was first entirely filled with the stationary (lower) phase. Then the apparatus was rotated forward at 850 rpm, while the mobile (upper) phase was pumped into the column in a head to tail (H→T) direction at a flow-rate of 1.0 mL min^{-1}. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 20 mL of the sample solution containing 0.3 g of the *n*-butanol fraction of the methanol extract was injected through the injection module at a flow-rate of 1.0 mL min^{-1}. We collected 70 fractions of 4 mL each with a Redifrac automated fraction collector (Pharmacia, Uppsala, Sweden), in approximately 4.5 h. After TLC analysis, fractions with similar retention fractions (R_F) were combined.

Preparation of the two-phase solvent system: The solvent system, composed of ethyl acetate: *n*-propanol: water (140:8:80, v/v/v), was thoroughly equilibrated overnight in a separator funnel at room temperature and the two phases were separated shortly before use.

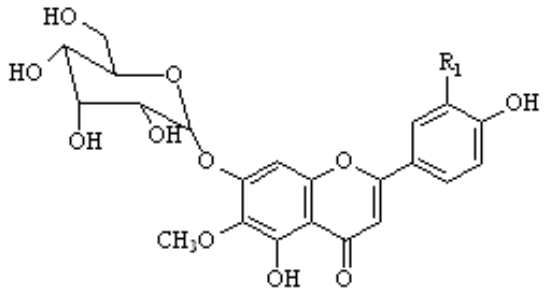
Analyses of the compounds by TLC: Aliquots of the *n*-butanol fraction and the collected fractions 70 fractions were analyzed on silica gel TLC plates on glass (20 x 20 cm, Aldrich), the mobile phase being chloroform: methanol: water (43:37:20, v/v/v). The spots on the TLC plates were observed under a UV lamp (254 nm), and then developed with NP/PEG reagent (Wagner et al., 1984).

Structural identification of the compounds: Nuclear Magnetic Resonance (NMR) spectra of compounds **1** and **2** (see Figure 1) in DMSO-d_6 were obtained with a Varian, INOVA 500 spectrometer, operating at 500 MHz for ^1H and 150 MHz for ^{13}C. 2D experiments: ^1H-^1H-COSY (chemical shift correlation spectroscopy), inverse-detected ^1H-^{13}C HSQC (heteronuclear single quantum coherence) and HMBC (heteronuclear multiple bond connectivity). Chemical shifts were given in δ (ppm) using TMS as internal standard.

The two compounds were also submitted to electrospray ionization mass spectrometry (ESI-MS) in a Fisons VG Platform with a single quadrupole, operated in the positive mode (70 V). Samples were dissolved in methanol and injected directly into the mass spectrometer through a Rheodyne injector. Acetonitrile: water (1:1 v/v) flowing at 10 mL min^{-1} was used as mobile phase and nitrogen gas was used as the drying gas and for nebulization. The range of mass analyzed was 50-1050 u.m.a.

RESULTS

HSCCC fractions 86-90 (35 mg) and 64-72 (25 mg) afforded the pure flavonoid compounds **1** and **2**, respectively, whose structures are shown in Figure 1. Their $\mathbf{R_F}$ values, obtained from the TLC plates, were 0.20 and 0.30, respectively. The two main compounds were successfully identified by comparing the results of spectroscopic analyses with literature data (Santos et al., 2005): 6-methoxyluteolin-7-O-β-D-allopyranoside **1** and 6-methoxyapigenin-7-O-β-D-allopyranoside **2**. These compounds were obtained more than 95% pure in a single step, eliminating the need for time-consuming clean-up and thus minimizing the loss of material due to decomposition.



1 - R_1 = OH
2 - R_1 = H

Figure 1 - Compounds isolated from *E. ligulatum* by HSCCC.

DISCUSSION

Several classes of natural products have already been isolated by HSCCC, including flavonoids (Marston et al., 1991). Degenhardt et al. (2000) isolated flavonol glycosides and other phenolic compounds from tea leaves of *Camellia sinensis* L. (Theaceae). Degenhardt et al. (2001) isolated isoflavones from soy flour (Fabaceae), whereas Chen et al. (2005) isolated chrysin and baicalein from *Oroxylum indicum* (L.) Vent. (Bignoniaceae). Our group reported the isolation of naphthopyranone glycosides from *Paepalanthus microphyllus* (Eriocaulaceae) (Santos et al., 2001).

The separation mechanism of HSCCC is based mainly on the differential solubility of the compounds between the two immiscible phases, one flowing along the other, allowing the fast and efficient isolation of several compounds without decomposition. Particularly for polar compounds, this technique has the advantage of eliminating the problem of irreversible adsorption of the sample on the solid support (Conway et al., 1990; Conway, 1995).

Selection of the solvent system is the first and most important step in performing HSCCC separations. We first selected a suitable two-phase solvent system for HSCCC using small amounts of the *n*-butanol fraction. The samples were dissolved into two immiscible liquid phases consisting of organic solvents and water (Conway et al., 1990; Conway, 1995), and analyzed by TLC in order to estimate the distribution of the flavone fraction between the two phases. Silica-gel plates were eluted with mixtures of chloroform: methanol: water, 43:37:20 (organic phase, v/v/v) and visualized under UV light (254 nm). The best result was obtained with the mixture of ethyl acetate: *n*-propanol: water (140:8:80, v/v/v); the flavone was almost equally distributed between the two phases (partition coefficient K' ≈ 1). Since the high proportion of ethyl acetate in the solvent mixture and the R_F values of the compounds indicated medium polarity flavones from *E. ligulatum*, the upper (CH_3COOEt-rich) phase was chosen as mobile phase and the lower (H_2O-rich) phase remained stationary in the coiled column during CCC separation. This choice has the additional advantage that the upper phase consists largely of the volatile ethyl acetate, readily eliminated by evaporation before performing chemical analyses and eventual biological assays on the pure, unaltered residues. Under the conditions described, the retention of the stationary phase in the HSCCC was 87%.

The flavonoids from the flowering parts of *E. ligulatum* were separated on a preparative scale in about 3.0 h, and the process was completed in 4.5 hours. Despite the similar polarity of these compounds when analyzed by TLC ($R_{F\underline{1}}$ = 0.20; $R_{F\underline{2}}$ = 0.30), 6-methoxyapigenin-7-*O*-β-D-allopyranoside **2** was eluted after approximately to 2.5 hours, and was very well separated from 6-methoxyluteolin-7-*O*-β-D-allopyranoside **1** (eluted after 3 hours). In the experimental conditions used, the presence of the one additional hydroxyl group on ring B of compound **1** was enough for HSCCC to separate these two flavonoids.

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RESUMO

Separação rápida de flavonas dos capítulos de ***Eriocaulon ligulatum*** *(Vell.) L.B.Smith (Eriocaulaceae) por cromatografia de contra-corrente (HSCCC)*

Cromatografia em contra corrente de alta velocidade é uma técnica rápida para separação de produtos naturais de plantas. Esta técnica foi utilizada em escala preparativa para separar duas flavonas monoglicosiladas presentes nos capítulos de *Eriocaulon ligulatum* (Vell.) L.B.Smith (Eriocaulaceae). Esta espécie é conhecida como "botão dourado" e é exportada para a Europa, Japão e América do Norte como uma espécie ornamental, constituindo uma importante fonte de renda para a população do Estado de Minas Gerais. O sistema de solvente utilizado foi uma mistura de acetato de etila:*n*-propanol:água (140:8:80, v/v/v) que levou ao isolamento de 6-metoxiluteolina-7-*O*-β-D-alopiranosideo 1 e 6-metoxiapigenina-7-*O*-β-D-alopiranosideo 2 em apenas 3.0 horas. A identificação dos compostos foi feita por RMN (mono e bi-dimensionais), EM-ES e comparado com dados da literatura.

Palavras-chave: HSCCC, *Eriocaulaceae*, flavona, *E. ligulatum.*

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