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Artigo

PREPARATIVE SEPARATION OF FLAVONOIDS FROM THE MEDICINAL PLANT *Davilla elliptica* ST. HILL. BY HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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High-speed counter-current chromatography (HSCCC) is a major tool for the fast separation of natural products from plants. It was used for the preparative isolation of the flavonoid monoglucosides present in the aerial parts of the *Davilla elliptica* St. Hill. (Dilleniaceae). This species is used in Brazilian folk medicine for the treatment of gastric disorders. The optimum solvent system used was composed of a mixture of ethyl acetate-*n*-propanol-water (140:8:80, v/v/v) and led to a successful separation of quercetin-3-*O*-α-L-rhamnopyranoside and myricetin-3-*O*-α-L-rhamnopyranoside in approximately 3.0 hours with purity higher than 95%. Identification was performed by ^{1}H NMR, ^{13}C NMR and HPLC-UV-DAD analyses.

Keywords: HSCCC; *Davilla elliptica*; flavonoids.

INTRODUCTION

Davilla elliptica St. Hill. (Dilleniaceae) is a small tree that grows wild in cerrado lands of Brazil. It is called "lixinha" (sandpaper) due to the presence of tricomes on the leaves. It has been used as a folk medicine for the treatment of gastric ulcers. Studies on *Davilla* species are scarce. Guaraldo *et al.* verified the gastroprotective effect of *D. rugosa* against lesions induced by HCl/ethanol[1,2]. David *et al.*[3] isolated α-tocoferol, myricetin, quercetin and myricetin-3-*O*-α-L-rhamnopiranoside from *D. flexuosa*.

Preliminary phytochemical investigation by TLC using authentic standards led to the detection of flavonoids in the EtOH extract of the aerial parts of this species. Since the presence of flavonoids is related to the anti-ulcer action of plant extracts[4], biological assays with pure compounds can contribute to a better understanding of the healing processes.

Several classes of natural products were already isolated using high-speed counter-current chromatography (HSCCC), including flavonoids[5]. Degenhardt *et al.*[6] isolated flavonol glycosides and other phenolics compounds from tea leaves from *Camellia sinensis* L. (Theaceae). Degenhardt e Winterhalter isolated isoflavones from soy flour (Fabaceae), whereas Chen *et al*. isolated chrysin and baicalein from *Oroxylum indicum* (L.) Vent. (Bignoniaceae)[7,8]. Our group reported the isolation of quercetine derivatives and the biflavonoid amentoflavone from the leaves of *Byrsonima crassa* (Malpighiaceae)[9].

Separations using are based on the distribution of a solute between two immiscible phases and submitted to a centrifugal force that retains the stationary phase[10,11]. It provides natural products chemists with a very efficient method for the separation of compounds derived from plant origin in short separation times and with the possibility of a large range of organic solvents[12]. Particularly for polar compounds it presents the advantage of eliminating the problem of irreversible adsorption of the sample on the solid support[10,11]. The solvent system selection is the first and most important step in performing HSCCC separations.

We report here the fast and efficient preparative separation of flavonoid monoglucosides from the aerial parts of *Davilla elliptica*.

EXPERIMENTAL PART

Chemicals

All solvents used for HSCCC were of p.a. grade from Merck. The solvents used for HPLC were of analytical grade from J.T. Baker, USA. Water was nanopure quality. Hydrochloric acid, ethanol (Nuclear, Brazil), cimetidine (Sigma Chemical Co., St Louis, MO, USA) and carbenoxolone (Medley, Brazil) were used in this study. Extract was dissolved in NaCl solution 0.9% (vehicle). All substances were prepared immediately before use.

Preparation of crude sample and sample solution

Aerial parts of *Davilla elliptica* were collected at Porto Nacional, Tocantins State, Brazil and authenticated by Prof. E. R. dos Santos, from the Institute of Biosciences of the Tocantins University. A *voucher* specimen (4583) was deposited at the Herbarium of the Tocantins University.

The air-dried and powdered aerial parts (1.0 kg) were extracted exhaustively with ethanol (8 L) at room temperature for 1 week. Solvents were evaporated at 60 °C under reduced pressure and affording the EtOH extract (187.0 g). The EtOH extract (10.0 g) was partitioned with a mixture of *n*-BuOH/H_2O (1:1, v/v for 3 times), affording 4.5 g of the *n*-BuOH phase and 4.7 g of the aqueous phase. An aliquot of the *n*-BuOH fraction (0.3 g) was dissolved in

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20 mL of a mixture consisting of 10 mL lower phase + 10 mL upper phase of the solvent system ethyl acetate-*n*-propanol-water (140:8:80, v/v/v).

High-speed counter-current chromatography (HSCCC)

The preparative HSCCC instrument employed in this study was from P.C. Inc., Potomac, (Buffalo, NY-USA). It was equipped with a multiplayer 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 holder, 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 optimum speed of 850 rpm. The flow rate was controlled with a Waters 4000 constant-flow pump (Milford, MA-USA). The sample was injected with 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 phase (lower phase). Then the apparatus was rotated forward at 850 rpm, while the mobile phase (upper phase) was pumped into the column in a tail to head (T→H) 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*-BuOH fraction of the EtOH extract was injected through the injection module at a flow rate 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 analyses, fractions with similar retention fractions (R_F) were joined.

Preparation of the two-phases solvent system

We first selected a suitable two-phase solvent system for HSCCC using small amounts of the *n*-BuOH fraction. They were dissolved into two immiscible liquid phases consisting of organic solvents and water[10,11] and analyzed by TLC in order to estimate the best distribution of the flavonoids between the two phases, with partition coefficient value K' of approx. 1. Silicagel plates were eluted with mixtures of $CHCl_3$/MeOH 8:2 (v/v) and visualized under UV light (254 nm). The solvent system composed of ethyl acetate-*n*-propanol-water (140:8:80, v/v/v) was thoroughly equilibrated overnight in a separatory funnel at room temperature and the two phases separated shortly before use.

Analyses of the compounds by TLC and HPLC

An aliquot of the *n*-BuOH and the collected fractions were analyzed using silica gel TLC plates on glass (20 x 20 cm, Aldrich) developed with a solvent mixture composed of $CHCl_3$/MeOH (85:15, v/v). The spots on the TLC plates were observed under UV lamp (254 nm). Fractions of similar retention factors (R_F) were combined, weighed and further analyzed using a Varian, *ProStar* HPLC system (Walnut Creek, CA-USA) equipped with a RP-18 column (250 x 4.60 mm i.d., 5 μm, Phenomenex Luna). Conditions: column: reversed-phase Phenomenex Luna, C_{18} column (250 x 4.60 mm I.D., 5 μm); mobile phase: 0.05% trifluoroacetic acid (TFA) in acetonitrile (A) and 0.05% TFA in water, linear gradient elution: A-B (20:80, v/v) to A-B (40:60, v/v) in 20 min, then to 100% B in 55 min, flow rate: 0.8 mL min^{-1}, and the effluent was monitored using a *ProStar* 330 photodiode-array ultraviolet detection (PDA) system (Walnut Creek, CA-USA) at 330 nm.

Structural identification of the compounds

The NMR spectra in DMSO-d_6 were obtained using a Varian, INOVA 500 spectrometer (Palo Alto, CA-USA), operating at 500 MHz for ^1H and 150 MHz for ^{13}C and 2D-NMR (^1H-^1H COSY, HMQC, TOCSY and HMBC). Chemical shifts were given in δ (ppm) using TMS as internal standard.

RESULTS AND DISCUSSION

Our group investigates Brazilian plants used in folk medicine to treat gastric problems. *D. elliptica* is one of the species used popularly to treat ulcers. Preliminary pharmacological assays showed that the EtOH extract of *D. elliptica* (dose 100 g/kg) inhibited 46% of the ulcerogenic lesions caused by the HCl/ethanol solution, whereas the positive control carbenoxolone (250 mg/kg) inhibited 42% of the gastric ulcers. Therefore, results obtained are promising. Thus, in order to perform *in vivo* pharmacological tests with isolated compounds or enriched fractions we need fast preparative methods that can provide us with enough amount of pure material.

Flavonoids are also involved in anti-ulcer processes[13]. Due to the presence of tannins in the EtOH extract of *Davilla* elliptica, the extract was first partitioned between *n*-BuOH and water. Polymeric tannins remained in the water phase, while flavonoids were transferred to the *n*-BuOH phase. TLC analyses with authentic standards of the *n*-BuOH phase suggested the presence of flavonoid monoglucosides with close retention factors. Since this type of compound has similar size, gel permeation chromatography is not a method of choice to separate these compounds. Therefore, we decide to investigate the use of HSCCC for the separation the flavonoid monoglucosides from *D. elliptica*.

Concerning the solvent system used in this separation, the best result was obtained with the mixture of ethyl acetate-*n*-propanol-water (140:8:80, v/v/v), with flavonoids almost equally distributed between the two phases (partition coefficient value K' of approx. 1). Since the high proportion of EtOAc in the solvent mixture and the R_F values of the compounds indicate the medium polarity flavonoids of *D. elliptica,* the upper phase was chosen as mobile phase and the lower phase was used as stationary phase for the CCC separation. This choice also represents an additional advantage, since the upper phase consists of large amounts of the volatile EtOAc, easily eliminated before performing the biological assays.

Under the conditions used, the retention of the stationary phase in the HSCCC was 87%. After the HSCCC separation, the collected fractions were monitored by TLC eluted on silica gel plates eluted with mixture of $CHCl_3$/MeOH 8:2 (v/v) and joined. Fractions 26-30 (25 mg) and 38-47 (35 mg) contained a single flavonoid species. Spectroscopic analyses allowed to identify two main compounds, recognized by comparison with literature data[14,15]: myricetin-3-*O*-α-L-rhamnopyranoside **1** and quercetin-3-*O*-α-L-rhamnopyranoside **2** (Figure 1). The identity and purity of the isolated substances were also checked by HPLC-DAD-UV analyses using authentic samples from a collection of our laboratory. Both compounds **1** and **2** were obtained with purity over 95%.

Figure 2 shows the HPLC chromatographic analysis of the *n*-BuOH fraction of the ethanolic extract of *D. elliptica* aerial parts. Three intense peaks could be detected with retention times 9.05 and 15.04 min, corresponding to the isolated compounds **1** and **2**. The UV spectra of the peaks **1**-**2** show the characteristics spectra of flavonoids, with bands at 260 and 350 nm (Figure 2). The peak at 21 min was identified as myricetin by co-injection with an authentic sample. Other minor peaks with retention times at 22.50

min and those ranging from 35-39 min are also due to flavonoids (UV bands at 260 and 350 nm) but could not be isolated in significative amounts to be spectrometrically identified.

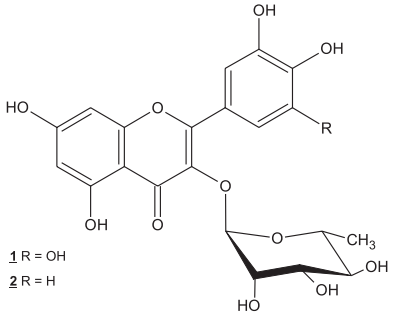

***Figure 1**. Structure of the flavonoids isolated from* Davilla ellliptica*. **1**, myricetin -3-*O*-α-L-rhamnopyranoside. **2**, quercetin -3-*O*-α-L-rhamnopyranoside*

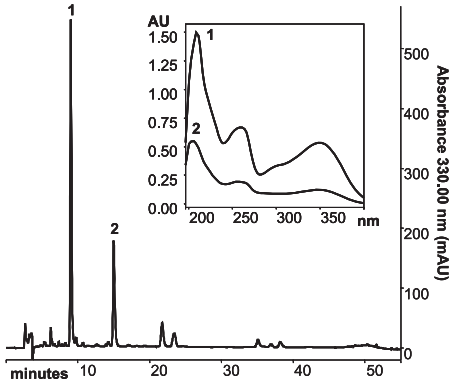

***Figure 2**. Chromatogram of the* n-*BuOH fraction of the ethanolic extract from* D. elliptica *aerial parts by HPLC analysis. Conditions: column: reversed-phase Phenomenex Luna, C_{18} column (250 x 4.60 mm I.D., 5 μm); mobile phase: 0.05% TFA in acetonitrile (A) and 0.05% TFA in water, linear gradient elution: A-B (20:80, v/v) to A-B (40:60, v/v) in 20 min, then to 100% B in 55 min, flow rate: 0.8 mL min^{-1}, detection at 330 nm*

The flavonoids from *D. elliptica* were separated in preparative scale in approx. 3 h, and the process was completed in 4.5 h. Despite the similar polarity of these compounds when analyzed by TLC ($R_{F\underline{1}}$ = 0.20; $R_{F\underline{2}}$ = 0.30), myricetin-3-*O*-α-L-rhamnopyranoside **1** was eluted after approximately in 2 h, very well separated from quercetin-3-*O*-α-L-rhamnopyranoside **2** (eluted after 3 h). Therefore, at the conditions used, the presence of the additional hydroxyl group at ring B of compound **1** was enough to separate these flavonoid monoglucosides from *D. elliptica*.

CONCLUSION

Our results evidenced the advantage of HSCCC for the preparative isolation of myricetin-3-*O*-α-L-rhamnopyranoside **1** and quercetin-3-*O*-α-L-rhamnopyranoside **2** from the aerial parts of *D. elliptica*. In this case, we obtained the fast separation of flavonoids differing in only one OH-group at ring B in only 3 h. Compounds were obtained with purity higher than 95%, thus allowing them to be sent to perform pharmacological tests.

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