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Application of preparative high-speed counter-current chromatography for the separation of flavonoids from the leaves of *Byrsonima crassa* Niedenzu (IK)

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Abstract

The methanolic extract of the leaves of the medicinal plant *Byrsonima crassa* (Malpighiaceae) contain flavonoids with antioxidant activity. They were separated in a preparative scale using high-speed counter-current chromatography. 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 between monoglucosilated flavonoids (quercetin-3-*O*-α-L-arabinoside, quercetin-3-*O*-β-D-galactoside) and the biflavonoid amentoflavone in only 3.5 h. The purities of quercetin-3-*O*-α-L-arabinoside (95 mg), quercetin-3-*O*-β-D-galactoside (16 mg) and the biflavonoid amentoflavone (114 mg) were all isolated at purity over 95%. Identification was performed by ^{1}H NMR, ^{13}C NMR and UV analyses.

Keywords: Counter-current chromatography; *Byrsonima crassa*; Plant materials; Flavonoids; Amentoflavone

1. Introduction

Byrsonima species (Malpighiaceae) are popularly known as 'murici-vermelho' or 'murici-cascudo' and some members are used as juice, jellies and liquor in Northest and Northeastern of Brazil. This genus produces mainly sulphonoglycolipid, steroids, triterpenes, aromatic esters, amino acids and proanthocyanidins [1–6]. *Byrsonima crassa* Niedenzu (IK) is used in Brazilian folk medicine for the treatment of diseases related mainly to gastric and peptic ulcer. Preliminary phytochemical investigation by TLC using authentic standards led to the detection of flavonoids with antioxidant activity in the polar extracts of the leaves of this species. Since the antioxidant activity is related to the anti-ulcer action of plant extracts [7], biological assays with pure compounds can contribute to a better understanding of the healing processes.

Despite the fact that many Brazilian herbal preparations consist of crude plant extracts and are not further purified prior to use, isolation and structure elucidation of the lead structures is essential in order to provide a better understanding of the alleged bioactivity. However, isolation is still a key step, because most of the vegetable extracts are composed of complex mixtures. The separation of the compounds present in a crude extract is often performed by repeated processes based on adsorption column chromatography. In our case, we investigate mainly polar extracts containing glycosilated compounds, which can be irreversibly adsorbed on the stationary phase or even be degraded because of the catalytic activity of some solid supports. Besides, the isolated compounds are submitted to in vivo pharmacological tests, which require fast separation and amounts of sample varying from few milligrams to grams of pure compounds.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates the problem of irreversible adsorption of the sample on the solid support [8]. The method has been successfully applied to the analysis and separation of

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various natural products [9–11]. Among the advantages of HSCCC compared to the other liquid–liquid chromatography techniques (e.g. droplet counter-current chromatography, DCCC) are the possibility of shorter separation times and wide range of suitable solvent systems. The solvent system selection is the first and most important step in performing HSCCC separations.

Therefore, the scope of our analysis was to search for an efficient method to isolate in preparative scale the flavonoids contained in the methanolic extract of *B. crassa*.

2. Experimental

2.1. Chemicals

All solvents used for HSCCC were of analytical-reagent grade from Merck. The solvents used for HPLC were of analytical grade from J.T. Baker, USA. Water was nanopure quality.

2.2. Preparation of crude sample and sample solution

Leaves (2.0 kg) of *B. crassa* were collected at Porto Nacional, Tocantins State, Brazil and authenticated by Eduardo Ribeiro dos Santos. A *voucher* specimen (No. 3377) was deposited at the Herbarium of the Tocantins University.

The air-dried and powdered leaves were exhaustively extracted (three times) with chloroform, methanol and 80% aqueous methanol (48 h, 4 l each) successively at room temperature. Solvents were evaporated at 60 °C under reduced pressure and affording the $CHCl_3$ extract (53.8 g), MeOH extract (158.3 g) and MeOH–water extract (95.7 g). The MeOH extract was partitioned with a mixture of EtOAc–water (1:1 (v/v), for three times). The EtOAc fraction (0.7 g) was dissolved in 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)).

2.3. High-speed counter-current chromatography

The preparative HSCCC instrument employed in this study was from P.C. Inc., Potomac, USA. It was equipped with a multiplayer 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 0.5 at the internal 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 was adjusted with a controller to an optimum speed of 800 rpm. The flow rate was controlled with a Waters 4000 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^{-1}. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 20 ml of the sample solution containing 0.7 g of the EtOAc fraction of the methanolic extract was injected through the injection module at a flow rate 3.0 ml min^{-1}. We collected 95 fractions of 10 ml each with a Redifrac automated fraction collector (Pharmacia, Sweden), in approximately 5 h. After TLC analyses, fractions with similar retention fractions were combined.

2.4. Preparation of two-phases solvent system

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.

2.5. Analyses of the compounds by TLC and HPLC

An aliquot of the ethyl acetate fraction and the collected fractions were analyzed using silica gel TLC plates on glass (20 cm × 20 cm, Aldrich) developed with a solvent mixture composed of $CHCl_3$–CH_3OH (85:15 (v/v)). The spots on the TLC plates were observed under a UV lamp (254 nm). Fractions of similar retention factors (R_F) were combined, weighed and further analyzed using a Varian, *ProStar* HPLC system equipped with a RP-18 column (250 mm × 4.60 mm i.d., 5 μm, Phenomenex Luna). The mobile phase used a linear gradient of 10–100% acetonitrile in water over 30 min eluted at a flow rate of 0.8 ml min^{-1}, and the effluent was monitored using a *ProStar* 330 photodiode-array ultraviolet detection (DAD) system at 254 nm.

2.6. Structural identification of the compounds

The NMR spectra in [$^2H_6$]dimethyl sulfoxide DMSO-d_6 were obtained using a Varian INOVA 500 spectrometer, operating at 500 MHz for ^1H and 150 MHz for ^{13}C and two-dimensional NMR (^1H–^1H COSY, HMQC, TOCSY and HMBC). Chemical shifts were given in δ (ppm) using tetramethylsilane (TMS) as internal standard.

The spectral data of the three flavonoids are given as below:

Amentoflavone (**1**): ^1H NMR (DMSO-d_6, 500 MHz): *unity 1* δ 6.33 (1H, s, H-6), δ 6.69 (2H, *d*, $J = 8.5$ z, H-3′,5′), δ 6.76 (1H, s, H-3), δ 7.58 (2H, *d*, $J = 8,5$ Hz, H-2′,6′). *Unity 2* δ 6.18 (1H, *d*, $J = 2.5$ Hz, H-6″), δ 6.43 (1H, *d*, $J = 2.5$ Hz, H-8″), δ 6.81 (1H, s, H-3″), δ 7.09 (1H, *d*, $J = 8.5$ Hz, H-5‴), δ 7.97 (1H, dd, $J = 8.5$ and 2.5 Hz, H-6‴), δ 7.98 (1H, d, $J = 2.5$ Hz, H-2‴). ^{13}C NMR (DMSO-d_6, 125 MHz): *unity 1* δ 98.9 (C-6), δ 103.7 (C-10), δ 104.0 (C-8), δ

115.8 (C-3′,5′), δ 102.6 (C-3), δ 121.4 (C-1′), δ 128.2 (C-2′,6′), δ 160.5 (C-7), δ 159.7 (C-9), δ 161.0 (C-4′), δ 161.5 (C-5), δ 163.8 (C-2), δ 182.2 (C-4). *Unity 2* δ 94.0 (C-8″), δ 98.7 (C-6″), δ 102.9 (C-3‴), δ 103.6 (C-10″), δ 116.3 (C-5‴), δ 120.0 (C-3‴), δ 120.9 (C-11″), δ 127.8 (C-6‴), δ 131.4 (C-2‴), δ 161.4 (C-5″), δ 163.7 (C-2″), δ 164.1 (C-7″), δ 181.7 (C-4″).

Quercetin-3-*O*-α-L-arabinoside (**2**): ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.21 (1H, dd, J = 11.5 and 2.5 Hz, H-5a″), δ 3.51 (1H, dd, J = 7.0 and 3.5 Hz, H-3″), δ 3.59 (1H, dd, J = 11.5 and 5.5 Hz, H-5b″), δ 3.64 (1H, m, H-4″), δ 3.75 (1H, dd, J = 8.3 and 5.0 Hz, H-2″), δ 5.27 (1H, d, J = 5.0 Hz, H-1″), δ 6.19 (1H, d, J = 2.0 Hz, H-6),δ 6.39 (1H, d, J = 2.0 Hz, H-8), δ 6.84 (1H, d, J = 8.5 Hz, H-5′), δ 7.50 (1H, d, J = 2.5 Hz, H-2′), δ 7.66 (1H, dd, J = 8.5 and 2.5 Hz, H-6′). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 64.3 (C-5″), δ 66.1 (C-4″), δ 70.7 (C-2″), δ 71.6 (C-3″), δ 93.5 (C-8), δ 98.7 (C-6),δ 101.4 (C-1″), δ 103.9 (C-10), δ 115.4 (C-5′), δ 115.7 (C-2'), δ 121.9 (C-1′), δ 122.1 (C-6′), δ 133.7 (C-3), δ 144.9 (C-3′), δ 148.6 (C-4′), δ 156.2 (C-9*), δ 156.3 (C-2*), δ 161.2 (C-5), δ 164.3 (C-7), δ 177.5 (C-4).

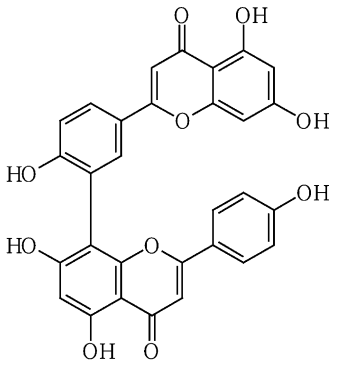
Quercetin-3-*O*-β-galactoside (**3**): ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.30 (1H, m, H-6a″), δ 3.32 (1H, m, H-5″), δ 3.38 (1H, m, H-3″), δ 3.46 (1H, m, H-6b″), δ 3.58 (1H, m, H-2″), δ 3,66 (1H, m, H-4″), δ5.38 (1H, d, J = 7.5 Hz, H-1″), δ 6.20 (1H, d, J = 2.0 Hz, H-6), δ 6.41 (1H, d, J = 2.0 Hz, H-8), δ 6.82 (1H, J = 8.5 Hz, H-5′), δ 7.54 (1H, d, J = 2, 5 Hz, H-3′), δ 7.76 (1H, dd, J = 8.5 and 2.5Hz, H-6′). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 60.1 (C-6″), δ 67.9 (C-4″), δ 71.2 (C-2″), δ 73.2 (C-3″), δ 75.9 (C-5″), δ 93.5 (C-8), δ 98.7 (C-6), δ 103.9 (C-1″ and C-10), δ 115.2 (C-5′), δ 115.9 (C-2′), δ 122.0 (C-6′), δ 121.1 (C-1′), δ 133.5 (C-3), δ 144.8 (C-3′), δ 148.5 (C-4′), δ 156.2 (C-2), δ 156.3 (C-9), δ 161.2 (C-5), δ 164.1 (C-7), δ 177.5 (C-4).

3. Results and discussions

A series of experiments was performed to determine a suitable two-phase solvent system for HSCCC [12,13]. Small amounts of the EtOAc fraction (10 mg) were dissolved into 2 ml of two immiscible liquid phases consisting of organic solvents and water (e.g. $CHCl_3$–CH_3OH–water and EtOAc–*n*-PrOH–water).

From this mixture, 10 μl of the upper phase and 10 μl of the lower phase were spotted onto TLC plates and eluted with several solvent systems [12,13]. After elution, plates were dried and observed under UV light (254 nm) in order to compare the intensity of the spots containing flavonoids in each phase. TLC analyses allowed to estimate the distribution of the flavonoids between the two phases. The mixture of ethyl acetate–*n*-propanol–water (140:8:80 (v/v/v)) gave the best result, with the compounds of interest almost equally distributed between the two phases (partition coefficient value K' of approximately 1). A good chromatographic resolution was obtained with the upper phase of this solvent system, with R_F values of 0.9, 0.7 and 0.6 for compounds **1**–**3**, respectively. The relatively high proportion of EtOAc in the solvent mixture and the R_F values of the compounds indicate the medium polarity flavonoids of *B. crassa*. Thus the upper phase was chosen as mobile phase and the lower phase was used as stationary phase for the CCC separation of the ethyl acetate fraction of the methanolic extract of *B. crassa*. 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 73%. After the HSCCC separation, the collected fractions were monitored by TLC and combined. Spectroscopic analyses allowed to identify three main compounds, recognized by comparison with literature data [14,15]: the biflavonoid amentoflavone (**1**), quercetin-3-*O*-α-L-arabinoside (**2**) and quercetin-3-*O*-β-D-galactoside (**3**) (Fig. 1). The identity and purity of the isolated substances were also checked by HPLC–DAD analyses using authentic samples from a collection of our laboratory. The biflavonoid amentoflavone (**1**) (114 mg) was isolated with purity over



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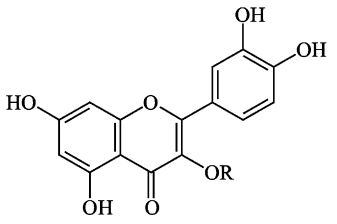

R=α-ara **2**

β-gal **3**

Fig. 1. Structure of the isolated flavonoids: **1**, amentoflavone; **2**, quercetin-3-*O*-α-arabinoside and **3**, quercetin-3-*O*-β-galactoside.

Table 1
Flavonoids isolated from the ethyl acetate fraction of the methanolic extract from *B. crassa* leaves by HSCCC

Flavonoid	Fractions	Isolated amount (mg)	Purity (%)	Yield (%)
1	12–14	114	95	16
2	17	95	97	14
3	20	16	97	2

Yield based on the mass of the crude EtOAc fraction.

95%, whereas quercetin-3-*O*-α-L-arabinoside (**2**) (95 mg) and quercetin-3-*O*-β-D-galactoside (**3**) (16 mg) were obtained with purity over 97% (Table 1).

Fig. 2 shows the HPLC chromatographic analysis of the EtOAc fraction of the methanolic extract of *B. crassa* leaves. Three intense peaks could be detected with retention times 28.20, 19.44, 18.33 min, corresponding to the isolated compounds **1**–**3**. The UV spectra of the peaks 1–3 show the characteristics spectra of flavonoids, with bands at 270 and 350 nm. Two other minor peaks with retention times were detected at 20.42 (UV bands at 268.2 and 354.4 nm) and 14.33 min (UV bands at 215.3 and 272.1 nm) were not identified, but probably correspond to a flavonoid and a catechin, respectively [16].

This approach led to the isolation of the three flavonoids in approximately 3.5 h, while the whole process was completed in 5 h. The biflavonoid amentoflavone (**1**) was the first compound to be eluted (after 2 h), because of its lower polarity compared to the flavonoid monoglucosides. Despite the similar polarity of the monoglucosides, quercetin-3-*O*-α-L-arabinoside (**2**) eluted before quercetin-3-*O*-β-D-galactoside (**3**). Thus, the separation of these glucosides seems to depend of the total number of the hydroxyl groups rather than the number of sugar unities attached to the aglycone. Both flavonoids have one sugar moiety at position 3 of the aglycone, but the galactosyl unit has four free OH groups while the arabinosyl moiety has only three. Our results showed that the conditions used provided a very efficient method for the separation of the flavonoids from *B. crassa*.

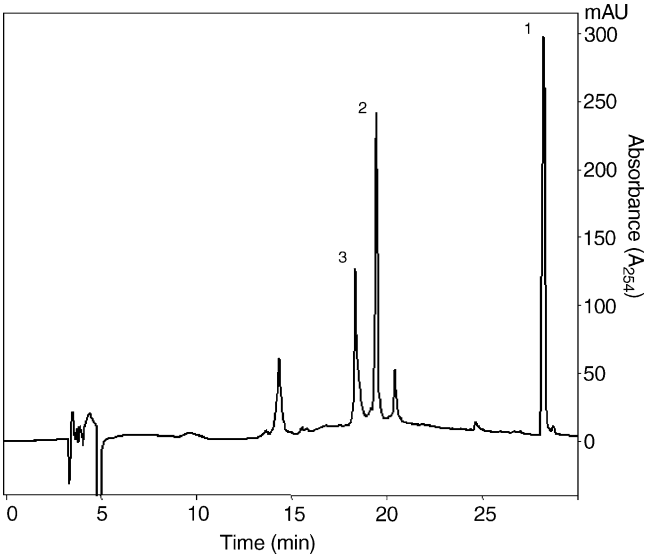

Fig. 2. Chromatogram of the ethyl acetate fraction of the methanolic extract from *B. crassa* leaves by HPLC analysis. Conditions—column: reversed-phase Phenomenex Luna, C_{18} column (250 mm × 4.60 mm i.d., 5 μm); mobile phase: linear gradient of 10–100% acetonitrile in water over 30 min; flow rate: 0.8 ml min^{-1} and detection at 254 nm.

It is well known that the anti-ulcerogenic effect and the antiviral activity of the amentoflavone (**1**) [17–19]. Flavonoid are also involved in anti-ulcer processes [20]. Preliminar assays with *B. crassa* crude extracts exhibited a significative anti-ulcerogenic effect (results not shown). Since **1**–**3** were obtained in good amounts and purity, without the need of further steps of purification, we can conclude that the HSCCC conditions used in our work fits well with our objective of obtaining these substances to perform future pharmacological tests. The process will be repeated several times in order to furnish larger amounts of material. To the best of our knowledge, no report has been published on the use of HSCCC for the isolation between a mixture monoglucosilated flavonoids and a biflavonoid in analytical or preparative scale.

4. Conclusion

The results of our studies clearly demonstrate the potential of HSCCC for the preparative isolation of amentoflavone (**1**), quercetin-3-*O*-α-L-arabinoside (**2**) and quercetin-3-*O*-β-D-galactoside (**3**) from the leaves of *B. crassa*. In particular, preparative HSCCC with its speedy separation and minimum solvent consumption offers a very efficient method for the separation and purification of natural products.

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