Journal of Chromatography A, 1218 (2011) 6200–6205

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Evaluation of different solvent systems for the isolation of *Sparattosperma leucanthum* flavonoids by counter-current chromatography

Fernanda das Neves Costa, Gilda Guimarães Leitão*

Universidade Federal do Rio de Janeiro, Núcleo de Pesquisas de Produtos Naturais, CCS, Bloco H, Av. Carlos Chagas Filho, 373, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 5 November 2010
Received in revised form 19 June 2011
Accepted 20 June 2011
Available online 24 June 2011

Keywords:
Counter-current chromatography
Flavonoids
Sparattosperma leucanthum
Bignoniaceae

ABSTRACT

Three flavonoids – 2′,4′,6′-trihydroxy-4′-*O*-β-D-glucopiranosyl dihydrochalcone, **1**, pinocembrin-7-*O*-(-neohesperidoside, **2** and pinocembrin-7-*O*-(-(6″-*O*-acetyl) neohesperidoside, **3** – were successfully isolated from the EtOAc extract of leaves of *Sparattosperma leucanthum* (Vell.) K. Schum (Bignoniaceae) using a two-step counter-current chromatography (CCC). Two different CCC machines were used, with different column axes (P.C. Inc., vertical orientation axis and AECS Quattro HTPrep, horizontal orientation axis). Detailed studies of flavonoids behaviour in several solvent systems made possible the use of the best system for their isolation. HEMWat and its modifications – exchange of alcohol and addition of a fifth solvent – were tested for isolation of the three compounds in a single run, but good K and α values were not achieved. So, HEMWat 4:10:4:10, with upper phase as mobile, was used to isolate compound **3**. A mixture of compounds **1** and **2** was recovered and submitted to a new CCC fractionation using a more polar solvent system: EBuWat 8:2:10, upper phase as mobile. Butironitrile–acetonitrile–water (BuCN–ACN–H_2O) 5:10:10, upper phase as mobile, was also used for the isolation of the mixture containing compounds **1** and **2**, in order to increase the solubility of the compounds in the CCC solvent system. It is the first time that the system BuCN–ACN–H_2O is described in literature.

1. Introduction

The family Bignoniaceae is composed of 113 genera and 800 species of trees, shrubs and climbing shrubs. Species of this family occur in the tropical regions of the world, especially in the American continent [1]. In Brazil, plants of this family occur from the Amazon region to the State of Rio Grande do Sul and can be found at three different biomes such as the Cerrado, the Atlantic Forest and the Amazon forest. Species of Bignoniaceae present medicinal and pharmacological potential, being frequently cited in ethnobotanical studies. Some biological activities were proved experimentally, such as antitumor, anti-inflammatory and antimicrobial [2–5].

Sparattosperma leucanthum (Vell.) K. Schum is a native tree of Brazil, popularly known as "caroba branca" or "ipê branco" [6]. Previous phytochemical studies on the genus *Sparattosperma* described the isolation of the flavanone pinocembrin-7-*O*-(-D-neohesperidoside, **2** (Fig. 1), from fruits of *S. vernicosum* [7]. The crude ethanolic extract of this plant was previously assayed by our group showing a good inhibitory effect on the ATPase activity of the Pdr5p enzyme from yeast plasma membrane, responsible for multiple drug resistance phenotype in yeast cells [8].

The conventional methods of preparative separation and purification of natural products, like silica gel, polyamide and Sephadex LH-20 column chromatography consume a large amount of solvent or are time consuming. Counter-current chromatography (CCC) has become an effective alternative to the conventional chromatographic techniques because it is a support-free liquid–liquid partition chromatography technique, which can eliminate irreversible adsorption of samples, having an excellent sample recovery [9,10].

The present work describes an efficient method for the preparative isolation and purification of the major flavonoids from the EtOAc extract of leaves of *S. leucanthum*: 2′,4′,6′-trihydroxy-4′-*O*-β-D-glucopiranosyl dihydrochalcone, **1**, pinocembrin-7-*O*-(-neohesperidoside, **2** and pinocembrin-7-*O*-(-(6″-*O*-acetyl) neohesperidoside, **3** (Fig. 1). A detailed study on these flavonoids behaviour on several solvent systems was performed after which the best solvent systems for their isolation were defined. Two different CCC machines were used, with different column orientation axes (P.C. Inc., vertical orientation axis and AECS Quattro HTPrep, horizontal orientation axis). The structures of the three compounds were elucidated by ^{1}H and ^{13}C NMR.

* Corresponding author. Tel.: +55 21 2562 6513; fax: +55 21 2562 6512.
E-mail addresses: ggleitao@yahoo.com.br, ggleitao@nppn.ufrj.br (G.G. Leitão).

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doi:10.1016/j.chroma.2011.06.069

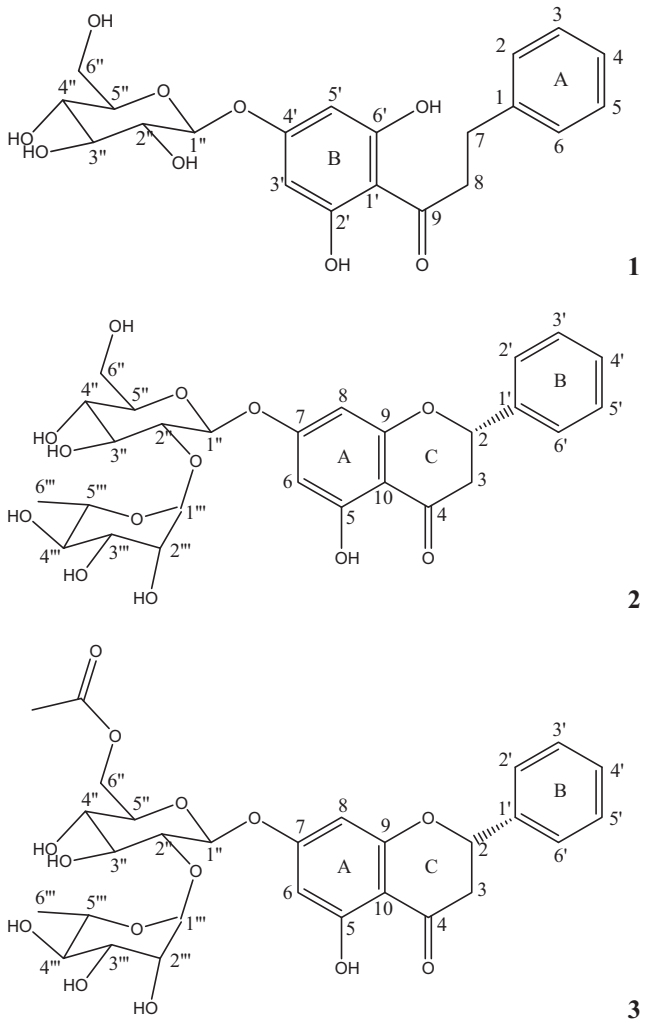

Fig. 1. Chemical structures of 2′,4′,6′-trihydroxy-4′-*O*-β-D-glucosyl dihydrochalcone, **1**, pinocembrin-7-*O*-(-neohesperidoside, **2** and pinocembrin-7-*O*-(-(6″-*O*-acetyl) neohesperidoside, **3**.

2. Experimental

2.1. Reagents

Organic solvents used for preparation of crude extracts and HSCCC separation were of analytical and/or HPLC grade and purchased from Tedia Brazil (Rio de Janeiro, Brazil). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system.

2.2. HSCCC equipments

Preparative HSCCC was performed on the following two equipments:

(1) Multilayer Coil Separator – Extractor counter-current chromatograph (P.C. Inc., Potomac, Maryland, USA) equipped with a polytetrafluoroethylene triple multi-layer coil (15 ml, 1.0 mm i.d. + 80 ml, 1.6 mm i.d. + 210 ml, 1.6 mm i.d.) equilibrated by a counterweight. The rotation speed is adjustable from 0 to 1000 rpm and the column axis is vertical.
(2) HT-Prep Quattro counter-current chromatograph (AECS, Bridgend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene multi-layer coils each (26 ml, 1.0 mm i.d. + 234 ml, 3.2 mm i.d. and 95 ml, 2.0 mm i.d. + 98 ml, 2.0 mm i.d.). The rotation speed is adjustable from 0 to 860 rpm and the column axis is horizontal.

The HSCCC systems were connected to a constant flow pump Series II (Scientific Systems Inc., Lab Alliance) and a Merck fraction collector L-7650 (Merck, Darmstadt, Germany). A 5 ml sample loop was used to inject the sample, except when the 26 ml coil was used, when in this case a sample loop of 1 ml was used.

2.3. Preparation of crude extract

S. leucanthum was collected at Mata Boa Vista, near Levy Gasparian city – Rio de Janeiro State, Brazil. A voucher specimen is deposited at the Herbarium of Federal University of Rio de Janeiro under the number RFA 31775. Dried and ground leaves (840 g) were submitted to extraction with ethanol 96 °GL (degrees Gay-Lussac) by maceration. The solvent was evaporated under reduced pressure on a rotary evaporator. About 50 g of the crude extract was partitioned between water and hexane, chloroform, ethyl acetate and butanol, in this order, affording four different extracts: hexanic (5.2 g), chloroformic (7.7 g), ethyl acetate (4.5 g) and butanolic (5.0 g). During the liquid–liquid chloroform extraction procedure, a large amount of a yellow fine powder precipitated (approximately 8 g), which was identified by ^{1}H and ^{13}C NMR as pinocembrin-7-*O*-(-neohesperidoside, **2**.

Table 1
The distribution coefficient (K) and separation factor (α) of the major compounds from the EtOAc extract of *S. leucanthum* in different solvent systems.

Experiment number	Solvent systems	Solvent ratio	$K_{\text{Compound 1}}$ [a]	α	$K_{\text{Compound 2}}$ [b]	α	$K_{\text{Compound 3}}$ [c]
1	HEMWat	5:9:5:9	47.0	1.3	36.3	2.9	12.7
2	HEMWat	4:10:4:10	25.7	1.5	17.1	12.2	1.4
3	HEMWat	2:12:2:12	5.6	1.3	4.4	6.3	0.7
4	HE–EtOH–Wat	4:10:4:10	58.0	2.8	20.6	5.3	3.9
5	HE–*n*PrOH–Wat	4:10:4:10	12.6	0.9	14.1	1.0	14.4
6	HE–*i*PrOH–Wat	4:10:4:10	12.4	1.2	15.0	1.1	14.1
7	HE–*n*BuOH–Wat	4:10:2:10	4.6	2.1	2.1	1.6	1.3
8	HE–*i*BuOH–Wat	4:10:2:10	4.5	1.6	2.9	3.2	0.9
9	HE–*n*BuOH–MWat	4:10:0.5:4:10	8.1	1.0	7.9	5.3	1.5
10	HE–*n*BuOH–MWat	4:10:0.7:4:10	7.0	1.4	4.9	3.5	1.4
11	HE–*n*BuOH–MWat	4:10:1:4:10	5.7	1.8	3.1	2.8	1.1
12	HE–*n*BuOH–MWat	4:10:1.2:4:10	4.1	1.4	3.0	3.0	1.0

[a] Retention time: 6.59 min.
[b] Retention time: 10.45 min.
[c] Retention time: 16.21 min.
H = hexane, E = ethyl acetate, M = methanol, EtOH = ethanol, nPrOH = *n*-propanol, iPrOH = *iso*-propanol, nBuOH = n-butanol, iBuOH = *iso*-butanol.

Table 2
The distribution coefficient (K) and separation factor (α) of compounds **1** and **2** from the EtOAc extract of *S. leucanthum* in different solvent systems.

Solvent systems	$K_{Compound\ 1}$	α	$K_{Compound\ 2}$
EBuWat 9:1:10	1.9	1.9	1.0
EBuWat 8:2:10	1.2	2.0	0.6
EBuWat 7:3:10	0.4	1.0	0.4
BuCN–ACN–H_2O 10:5:10	2.3	1.2	2.0
BuCN–ACN–H_2O 5:5:10	2.3	1.2	1.9
BuCN–ACN–H_2O 5:10:10	2.3	1.8	1.3

2.4. Selection of the two-phase solvent system

A number of two-phase solvent systems were tested by changing the volume ratio of the solvents in the system to obtain the optimum composition that gave suitable distribution coefficient (K) and selectivity factor (α) values. Small amounts of the sample (concentration: 0.5 mg/ml) were dissolved in a small test tube (100 × 13 mm) containing the equilibrated two-phase solvent system. The test tubes were shaken and the compounds allowed to partition between the two phases. Equal aliquots of each phase were spotted beside each other separately on silica gel TLC plates, developed with the organic phase of the solvent system ethyl acetate–acetone–water 25:10:5. The results were visualized under UV light (254 nm). Then, 2 ml of each phase was taken and evaporated in a rotary evaporator under reduced pressure. The residues were dissolved in 5 ml of methanol and analyzed by HPLC in order to calculate the distribution coefficient and selectivity factor. The K value was expressed as the peak area of the compound in the stationary phase divided by the peak area of the compound in the mobile phase. The $\alpha = K_2/K_1$, $K_2 > K_1$. All solvent systems tested and the resulting K for compounds **1**, **2** and **3** along with α values are summarized in Tables 1 and 2.

2.5. Preparation of the two-phase solvent system and sample solution

All selected solvent systems were thoroughly equilibrated in a separatory funnel at room temperature. The two phases were separated shortly before use and degassed by sonication for 15 min. The aqueous lower phase was used as stationary phase while the organic upper phase was used as mobile phase, in tail to head direction. The sample solution was prepared by dissolving the sample in the solvent mixture of aqueous lower phase and organic upper phase (1:1, v/v) of the solvent system used for HSCCC separation.

2.6. Separation procedure

In each separation, the coil was first entirely filled with the stationary phase (lower aqueous phase in all cases), and then the apparatus was rotated at 850 rpm, while the mobile phase was pumped into the column at the appropriate flow rate. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 5 ml of the sample solution was injected into the column through the injection valve (Rheodyne model 5020, USA). For experimental conditions see Table 3.

2.7. HPLC analyses and identification of flavonoids

The EtOAc extract and each purified fraction from HSCCC were analyzed by HPLC with a Lachron Merck HPLC (Merck, Darmstadt, Germany) equipped with an interface D-7000, pump L-7100, diode array detector (DAD) L-7450A and solvent degasser L-7612. The injections were done manually with an injection valve equipped with a 20 μl sample loop. A Lichrosorb RP-18 column (5 μm particle size, 250 × 4.6 mm i.d.) was used. The mobile phase used was MeOH–H_2O (acidified with acetic acid until pH 3.0) 40:60 until 100:0 in 35 min, the flow-rate was 1 ml/min and detection was done at 240 nm.

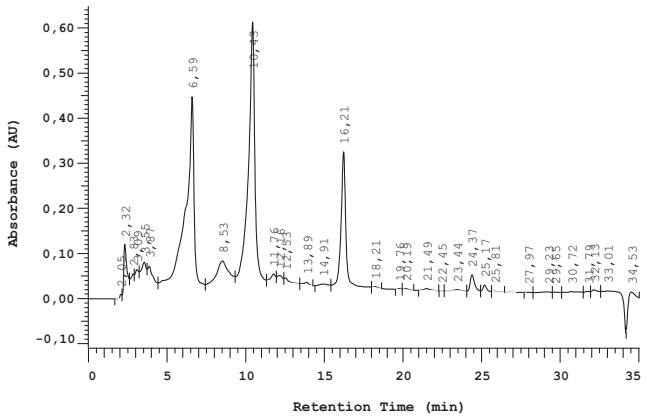

Fig. 2. HPLC analysis of the EtOAc extract from *S. leucanthum* showing compounds compounds **1**, **2** and **3**. For experimental conditions see Section 2.7.

^1H and ^{13}C NMR data measurements for the flavonoids isolated from HSCCC were recorded on a Bruker Avance DRX400 (Karlsruhe, Germany) at 25 °C, operating at 400.13 MHz for ^1H and 100.61 for ^{13}C. NMR spectra were recorded in pyridine–C_5D_5N or methanol–MeOD using TMS as internal standard.

3. Results and discussion

The EtOAc extract of leaves of *S. leucanthum* was initially analyzed by HPLC. The chromatographic profile of the extract (Fig. 2) showed the presence of three major peaks which UV spectra (λ_{max} = 261.8, 256.0; 324.6, 284.0, and 322.6, 284.0 nm; compound **1**, compound **2** and compound **3** respectively) were consistent with flavonoid derivatives [11]. The small tailing in the peak of compound **1** may have been caused by the excess of sample injected in the column.

Choosing the correct solvent system is crucial for a successful CCC separation and some basic requirements such as settlings times, distribution coefficient (K) and separation factor (α) of target compounds, among others [12,13] should be taken into account. We recently proposed some strategies of solvent system selection for the purification of flavonoids [14]. When dealing with glycosylated flavonoids it can be expected that the hexane–ethyl acetate–methanol–water (HEMWat) solvent system will only be useful at its higher polarity ratios. We have then selected HEMWat two-phase solvent system as it provides a broad range of polarities by modifying the volume ratio of the four solvents. Table 1 shows K and α values in HEMWat system ranging from 5:9:5:9; 4:10:4:10 to 2:12:2:12 for compounds **1**, **2** and **3** in the ethyl acetate extract of *S. leucanthum*. These three solvent systems were not able to provide suitable ranges of K and α values for the isolation of all compounds in a single run. K values for compounds **1**, **2** and **3** in HEMWat 5:9:5:9 were too large which would imply too long runs. More polar HEMWat systems, 4:10:4:10 and 2:12:2:12, furnished better K for compound **3** but compounds **1** and **2** still had large K values and a low α value in HEMWat system 2:12:2:12. The ratio 4:10:4:10 was found to be appropriate as a starting point to do modifications in order to achieve satisfactory K and α values.

Changing the alcohol component of the HEMWat solvent system can cause variations on the properties of each phase to a different extent, depending on which alcohol is used. According to Foucault [15] the variation in the ratios of methanol and ethanol cause modification on the properties of the aqueous phase while propanol causes variations on both phases and butanol is a major organic

Table 3
CCC chromatographic conditions.[a]

Experiment number	Equipment	Flow rate (ml/min)	Solvent system	Stationary phase retention (%)	Sample (mg in 5 ml)	Fractions
13	P.C. Inc.	2	HEMWat 4:10:4:10	80	150	40 fractions with rotation on + 20 fractions with rotation off ($2K+K$)
14	HT-Prep Quattro CCC	2	HEMWat 4:10:4:10	85	150	50 fractions with rotation on + 25 fractions with rotation off ($2K+K$)
15	P.C. Inc.	1	EBuWat 8:2:10	83	50	80 fractions with rotation on + 20 fractions with rotation off ($K+K$)
16	HT-Prep Quattro CCC	1	EBuWat 8:2:10	81	50	100 fractions with rotation on + 20 fractions with rotation off ($K+K$)
17	P.C. Inc.	2	BuCN–ACN–H_2O 5:10:10	85	50	40 fractions with rotation on + 20 fractions with rotation off ($2K+K$)
18	HT-Prep Quattro CCC	2	BuCN–ACN–H_2O 5:10:10	82	50	50 fractions with rotation on + 25 fractions with rotation off ($2K+K$)

[a] All separations were performed using the aqueous phase as stationary phase and organic phase as mobile phase.

phase modifier. Table 1 shows the K and α values of the major compounds in the modified systems. The exchange of methanol by ethanol (Experiment number 4, Table 1) increases K values of compounds **1** and **2**, rendering them more retained in the lower stationary phase whereas the exchange of methanol by *n*-propanol or *iso*-propanol (experiments number 5 and 6, Table 1) lowers these values which are still large. When methanol was replaced by *n*-butanol or *iso*-butanol (experiments number 7 and 8, Table 1), more satisfactory K and α values were achieved, but they were not used as the solvent system in experiment 8 would still give long runs and because of the low solubility of the EtOAc extract in these systems.

The addition of butanol, as an organic phase modifier, to the quaternary HEMWat system will ease the partitioning of rather polar solutes from the aqueous phase to the organic phase. Table 1 (experiments 9–12) shows the K and α values of the major compounds in these five solvent systems. The increase of the butanol ratio added to this solvent system, caused the decrease of K values for compounds **1** and **2** and all three compounds were more evenly distributed in the phases but still long runs would be needed for the complete separation of compounds **1** and **2**.

HEMWat system in the ratio 4:10:4:10 was chosen to separate compound **3** from the mixture of compounds **1** and **2** in the EtOAc extract of leaves of *S. leucanthum*. Semi-preparative HSCCC of 150 mg of the EtOAc extract, using HEMWat system 4:10:4:10, was performed in two different equipments: P.C. Inc. with a 80 ml and 1.6 mm i.d. column (vertical column axis) and HT-Prep Quattro CCC with a 95 ml and 2.0 mm i.d. column (horizontal column axis). For experimental conditions see Table 3, experiments 13 and 14. Both separations resulted in four main fractions combined according to thin layer chromatography (TLC) similarity, and then, analyzed by HPLC. Compound **3** was isolated in Fraction 2 (purity: 89.0 and 88.4% for the compound obtained from P.C. Inc. and HT-Prep Quattro CCC respectively) and a mixture of compounds 1 and **2** were isolated in Fraction 4. The HPLC chromatograms of Fractions 2 and 4 are shown in Fig. 3.

The mixtures of the more polar compounds were submitted to a second CCC step. As proposed before [14], in cases where the hexane–ethyl acetate–methanol–water solvent system is not effective for the separation of more polar target compounds, the use of the following two families of ternary solvent systems is suggested: ethyl acetate–butanol–water (EtOAc–BuOH–H_2O) or chloroform–methanol–water ($CHCl_3$–MeOH–H_2O) as both solvent systems were found to be used in more than 60% of the separations of glycosylated flavonoid derivatives. In order to isolate compounds **1** and **2** from Fraction 4, we have selected the two-phase solvent system EtOAc–BuOH–H_2O (EBuWat) because it provides a good polarity window, targeting compounds of moderate hydrophobicity [16]. The general description of this solvent family can be as follows: organic solvent–organic modifier–water, BuOH acting as the modifier as it goes preferentially into ethyl acetate rather than into water [15]. Table 2 shows the K and α values in EBuWat system ranging from 9:1:10, 8:2:10 and 7:3:10. After trying these systems, the ratios of 9:1:10 and 8:2:10 were found to be suitable for the separation of compounds **1** and **2**.

EBuWat 8:2:10 was chosen for the HSCCC separation of the mixture of flavonoids in Fraction 4 as it provided a better α value for compounds **1** and **2** and shorter run times. Semi-preparative HSCCC of 50 mg of the flavonoid mixture, using EBuWat solvent system 8:2:10, was performed in two different equipments: P.C. Inc. with a 80 ml and 1.6 mm i.d. column and HT-Prep Quattro CCC with a 95 ml

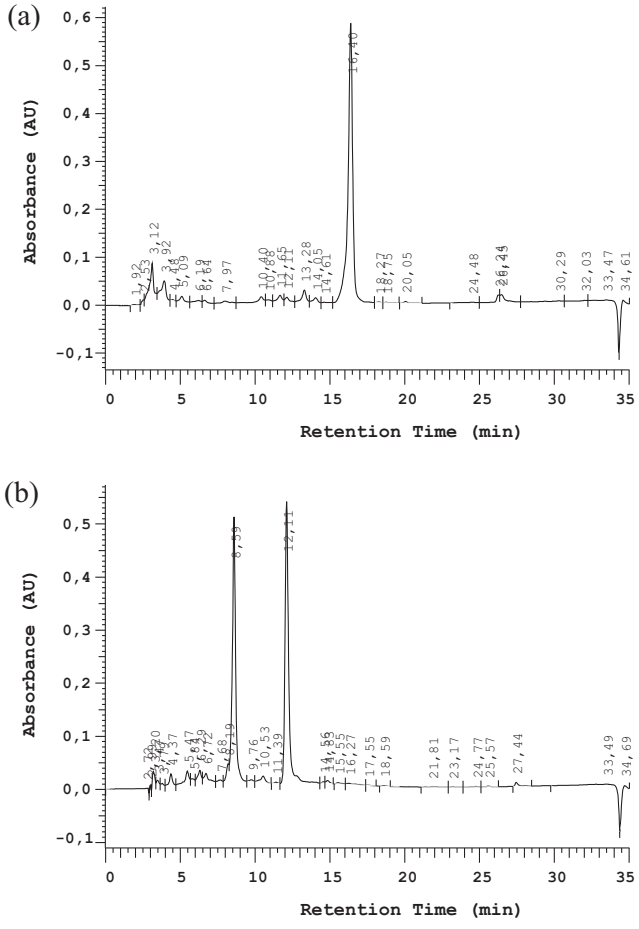

Fig. 3. HPLC analyses of the HSCCC fractionation of the EtOAc extract of leaves of *S. leucanthum* with HEMWat 4:10:4:10, (a) Fraction 2 and (b) Fraction 4. For experimental conditions see Section 2.7.

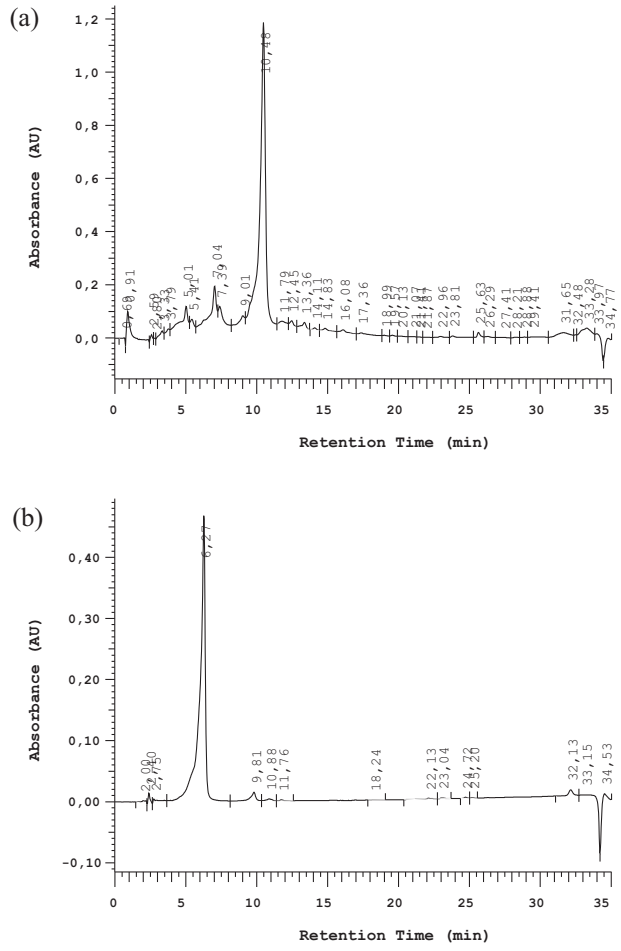

Fig. 4. HPLC analyses of the HSCCC fractionation of Fraction 4 with EtOAc–BuOH–H_2O 8:2:10, (a) Fraction 1 and (b) Fraction 3. For experimental conditions see Section 2.7.

and 2.0 mm i.d. column. For experimental conditions see Table 3, experiments 15 and 16. The P.C. Inc. separation resulted in three main fractions combined according to TLC similarity, and then, analyzed by HPLC. Compound **2** was isolated in Fraction 1 (containing a small amount of compound **1**) and compound **1** was isolated in Fraction 3 (purities of 74.4 and 95.8%, respectively). The HPLC chromatograms of these fractions are shown in Fig. 4. The separation of the mixture of **1** and **2** was not achieved with the same parameters in the HT-Prep Quattro CCC. This could be due to the different bore size of the 95 ml coil of the HT Prep machine (2.0 mm i.d.) as compared to the PC Inc, (1.6 mm i.d.) The design of Quattro or indeed the design of any CCC will favour certain solvent types over others in some cases, but not so in others. In fact, the stationary phase retention obtained with the HEMWat solvent system was greater for the HT Prep in relation to the PC Inc. (Table 3) but when it comes to the EBuWat solvent system, the stationary phase retention for the HT Prep is slightly smaller.

In an attempt to improve the solubility of compounds **1** and **2** in the solvent system of the CCC separation and, in similarity with the solvent used for NMR analyses of these compounds (deuterated pyridine), a second solvent system composed of butironitrile–acetonitrile–water was tested. This solvent system has been used earlier by Dr. Leslie Brown for the separation of natural products (personal communication, unpublished results). Table 2 shows the K and α values for compounds **1** and **2** in the system BuCN–ACN–H_2O 10:5:10, 5:5:10 and 5:10:10. The ratio of 5:10:10 was found to be suitable for the separation of compounds **1** and **2**. Despite of the following: K values for compounds **1** and **2** are higher in this solvent system (meaning longer run times), the selectivity factor (α) are approximately the same as for EBuWat 8:2:10 and the stationary phase retention of the two systems is almost the same, the selection of butironitrile–acetonitrile–H_2O 5:10:10 is justified as the solubility of compounds **1** and **2** in this solvent system was almost five times higher, meaning higher loading capacity. The experiment was performed with the same amount of sample as used for EBuWat in order to compare the two results.

HSCCC of 50 mg of Fraction 4 from the first step of the purification of the EtOAc extract of leaves of *S. leucanthum*, using the optimized solvent system. resulted in three main fractions which were then, analyzed by HPLC. For experimental conditions see Table 3, experiments 17 and 18. Compound **2** was isolated in Fraction 1 and compound **1** was isolated in Fraction 3 (purities of 73.8 and 97.0%, respectively, for compounds obtained with the P.C. Inc. and 72.3 and 94.1%, respectively, for compounds obtained with the HT Prep). Comparing the results obtained here with those obtained for EBuWat 8:2:10 on the purification of compounds **1** and **2**, we can see that the purification was successfully achieved with both CCC machines with the butironitrile containing solvent system whereas the same is not true for the EBuWat solvent system with the two equipments.

The identification of the obtained compounds was carried out by UV, ^{1}H and ^{13}C NMR spectroscopy as follows.

Compound ***1*** *(2′,4′,6′-trihydroxy, 4′-O-β-*D*-glucopyranosyl-dihydrochalcone)*: UV λ_{nm}^{MeOH} : 261.8; 256.0. ^{1}H NMR (400 MHz, C_5H_5N): δ 3.9–4.6 (5H, glucose); 3.93 (2H, *q*, C-7); 4.25 (2H, *q*, C-8); 5.75 (1H, *d*, C-1^G); 7.17 (2H, *d*, C-3′ and C-5′); 7.18–7.47 (5H, *m*, ring A). ^{13}C NMR (100 MHz, C_5H_5N): δ 40.37 (CH_2, C-7); 47.87 (CH_2, C-8); 63.49 (CH_2, C-6^G); 72.24 (CH, C-4^G); 76.10 (CH, C-5^G); 80.08 (CH, C-2^G); 80.21 (CH, C-3^G); 103.35 (CH, C-1^R); 103.49 (each CH, C-3′ and C5′); 114.63 (each CH, C-3 e C-5); 127.26 (CH, C-4); 129.90 (each CH, C-2 and C-6); 130.18 (C, C-1); 143.75 (C, C-1′); 159.06 (C, C-4′); 162.42 (each C, C-2′ e C-6′); 207.47 (C, C-4). These results are in agreement with those previously published for this compound [17].

Compound ***2*** *(pinocembrin-7-O-neohesperidoside)*: UV λ_{nm}^{MeOH} : 324.6; 284.0. ^{1}H NMR (400 MHz, C_5H_5N): δ 1.75 (3H, *d*, C-6^R); 3.01 (2H, *dq*, C-3); 4.0–4.8 (10H, sugar); 5.37 (1H, *dd*, C-2); 5.67 (1H, *d*, C-1^G); 6.35 (1H, *s*, C-1^R); 6.65 (1H, *s*, C-6); 6.71 (1H, *s*, C-8); 7.34–7.50 (5H, *m*, ring B); 12.4 (1H, *s*, OH C-5). ^{13}C NMR (100 MHz, C_5H_5N): δ 20.16 (CH_3, C-6^R); 44.64 (CH_2, C-3); 63.21 (CH_2, C-6^G); 71.18 (CH, C-5^R); 72.25 (CH, C-4^G); 73.66 (CH, C-3^R); 73.99 (CH, C-2^R); 75.29 (CH, C-4^R); 78.98 (CH, C-5^G); 80.12 (CH, C-2^G); 80.41 (CH, C-3^G); 70.72 (CH, C-2); 97.43 (CH, C-8); 99.09 (CH, C-6); 100.58 (CH, C-1^G); 103.74 (CH, C-1^R); 105.58 (C, C-10); 128.09 (each CH, C-2′ and C-6′); 130.31 (CH, C-4′); 130.38 (each CH, C-3′ and C-5′); 140.52 (C, C-1′); 164.61 (C, C-9); 165.71 (C, C-7); 167.47 (C, C-5); 197.88 (C, C-4). These results are in agreement with those previously published for this compound [18].

Compound ***3*** *(pinocembrin-7-O-(-(6″-O-acetyl) neohesperidoside)*: UV λ_{nm}^{MeOH} : 322.6; 284.0. ^{1}H NMR (400 MHz, MeOD): δ 1.32 (3H, *d*, C-6^R); 1.97 (3H, *s*, MeCO); 2.90 (2H, *dq*, C-3); 3.2–4.0 (9H, sugar); 5.06 (1H, *d*, C-1^G); 5.28 (1H, *s*, C-1^R); 5.49 (1H, *dd*, C-2); 6.17, (1H, *s*, C-6); 6.23 (1H, *s*, C8); 7.38–7.52 (5H, *m*, ring B). ^{1}H NMR (400 MHz, C_5D_5N): δ 1.79 (3H, *d*, C-6^R); 1.99 (3H, *s*, MeCO); 3.07 (2H, *dq*, C3); 4.0–5.0 (8H, sugar); 5.63 (1H, *d*, C-1^G); 6.40 (1H, *s*, C-1^R); 5.46 (1H, *dd*, C2); 6.62 (1H, *s*, C6); 6.72 (1H, *s*, C8); 7.42–7.59 (5H, *m*, ring B). ^{13}C NMR (100 MHz, MeOD): δ 16.87 (CH_3, C-6^R); 19.35 (CH_3, MeCO); 43.00 (CH_2, C-3); 63.31 (CH_2, C-6^G); 68.62 (CH, C-5^R); 70.40 (CH, C-4^G); 70.78 (CH, C-3^R); 70.79 (CH, C-2^R); 72.53 (CH, C-4^R); 74.08 (CH, C-5^G); 77.46 (CH, C-2^G); 77.57 (CH, C-3^G); 79.45 (CH, C-2); 95.64 (CH, C-8); 96.80 (CH, C-6); 98.12 (CH, C-1^G); 101.10 (CH, C-1^R); 103.59 (C, C-10);

126.08 (each CH, C-2′ and C-6′); 128.36 (CH, C-4′); 128.41 (each CH, C-3′ and C-5′); 138.71 (C, C-1′); 162.99 (C, C-9); 163.51 (C, C-7); 165.05 (C, C-5); 171.31 (C, MeCO); 196.73 (C, C-4). These results are in agreement with those previously published for this compound [19].

4. Conclusion

Our results demonstrate that CCC is a powerful technique for the separation, isolation and purification of compounds from natural sources. Using a two-step CCC three flavonoids were successfully isolated from the EtOAc extract of leaves of *S. leucanthum*. Pinocembrin-7-*O*-(-neohesperidoside, **2**, has already been isolated from *Sparattosperma* genus, but it is the first time that 2′,4′,6′-trihydroxy-4′-*O*-β-D-glucopiranosyl dihydrochalcone, **1**, and pinocembrin-7-*O*-(-(6″-*O*-acetyl) neohesperidoside, **3**, are isolated in the Bignoniaceae family.

Detailed studies of flavonoids behaviour in several solvent systems, following our proposed strategy, made possible the use of the best solvent system for their isolation, including a new system BuCN–ACN–H_2O, never before described in literature. The two CCC machines used in these experiments showed similar results with the solvents systems used for the separation of the flavonoids, except for the separations with EBuWat. Further investigations on the effect of different column axes and design of the machines with different solvent systems will be conducted.

Acknowledgements

We are grateful for financial support from CNPq and FAPERJ and CAPES for a fellowship.

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