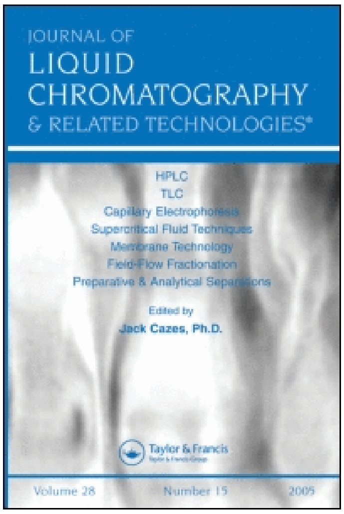

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Step-Gradient CCC Separation of Phenylpropanoid and Iridoid Glycosides from Roots of *Stachytarpheta cayennensis* (Rich.) Vahl

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Abstract: Glycosylated phenylpropanoids and iridoids were isolated from the ethyl acetate extract from the roots of *Stachytarpheta cayennensis* by step gradient countercurrent chromatography (CCC). The chosen gradient utilized a stepwise increase of the butanol ratio in a normal phase separation, utilizing a biphasic EtOAc:BuOH:H_2O solvent system, 1:X:1 v/v/v, in four steps: $A - X = 0.05$, $B - X = 0.2$, $C - X = 0.5$ and $D - X = 1.0$. The sequential increase of BuOH in the organic phase of the CCC solvent system, and wash-off of stationary phase allowed the isolation of 4 compounds identified as martinoside, isoverbascoside, verbascoside, ipolamiide, and two more iridoid glycosides. These compounds covered a wide span of polarities.

Keywords: *Stachytarpheta cayennensis*, Verbenaceae, Gradient elution, Countercurrent chromatography

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INTRODUCTION

Stachytarpheta cayennensis (Rich.) Vahl, Verbenaceae, is commonly known in Brazil as "Gervão–roxo,"[1] "Erva–gervão," "Verbena," "Verbena-falsa," "Verbena-negra," etc.[2] The roots of this plant are used in Brazilian folk medicine for rheumatism and back pain.[3] Topical application of the macerated leaves and roots is also recommended to treat sore skin wounds.[4] The most frequent ethnomedical usages of roots and leaves of *S. cayennensis* are as diuretics, analgesics, or tranquilizers.[5] They are also useful in the treatment of hemorrhoids, bronchial problems, coughs, rheumatism, and back pain.[5]

Plants of the family Verbenaceae are known to be rich in both iridoids and phenylpropanoids and the genus *Stachytarpheta* is considered to be most rich in iridoids, mainly of the ipolamiide type.[3,4]

This research describes the isolation of glycosylated phenylpropanoids and iridoids from the ethyl acetate extract from the roots of *Stachytarpheta cayennensis* by step gradient countercurrent chromatography (CCC). Some examples of isolation of these kinds of compounds, by DCCC[6–8] and HSCCC[9,10] can be found in the literature. The chosen gradient utilizes a stepwise increase of the butanol ratio in a normal phase separation, utilizing a biphasic EtOAc:BuOH:H_2O solvent system, to elute initially lower polarity components, and then progressively more polar ones.

EXPERIMENTAL

Collection and Extraction of Plant Material

Roots of *Stachytarpheta cayennensis* (Rich.) Vahl were collected at Florianópolis Island, Santa Catarina State, Brazil, dried in the shade, ground, and exhaustively extracted with hexane and ethanol in a Soxhlet apparatus. The dried ethanol extract was suspended in MeOH/H_2O 3:2 and extracted with hexane, chloroform, ethyl acetate, and butanol, in this order.

Choice of the Solvent System by Test Tube Experiments

Small amounts of the ethyl acetate extract of the roots of *S. cayennensis* were dissolved in separate test tubes containing the solvent systems (volume ratio) EtOAc:H_2O 1:1, BuOH:H_2O 1:1, and EtOAc:BuOH:H_2O 1:0.2:1, 1:0.5:1, and 1:1:1. 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 TLC plates, developed with the organic phase of the solvent system EtOAc:Acetone:H_2O 25:8:5. The results were visualized after spraying with vanillin in sulfuric acid (1%). The step gradient was defined as EtOAc:BuOH:H_2O (1:X:1 v/v/v), where X = 0.05 (solvent system A); 0.2 (solvent system B); 0.5 (solvent system C), and 1.0 (solvent system D).

CCC Separation by Gradient Elution

A P.C. Inc countercurrent chromatograph equipped with a multi-layer coil equilibrated by a counterweight was used. The volume of the coil was 80 mL. The pump was Model SD-200 Dynamax. A Dynamax FC-1 fraction collector was also used. The CCC column was filled with the lower aqueous phase of solvent system A. The rotor was started and set at 850 rpm. The upper organic phase of system A was pumped in the tail to head direction at 2 mL/min. In these conditions, the aqueous stationary phase, V_S, initially retained in the CCC column was 63 mL ($S_f = 79\%$; $V_M = 17$ mL).

One gram of the ethyl acetate extract of roots of *S. cayennensis* was dissolved in 2.5 mL of each phase of solvent system A. The 5 mL were injected in the 80 mL coil using a classical injection valve. Fractions of 5 mL were collected. A total volume of 80 mL of the organic phase of solvent system A was used filling tubes 1 to 16 (40 min). Another 80 mL of the upper organic phase of solvent system B was used to fill tubes 17 to 32 (40 min). Next, only 50 mL of the organic phase of solvent system C was collected in tubes 33 to 42 (25 min) and, finally, 100 mL of the most polar upper organic phase of solvent system D filled tubes 34 to 54 for 50 min. Then, the rotor rotation was stopped and the column content (organic and aqueous phases) was "washed-off." Sixteen fractions (5 mL) were collected (80 mL). This assures that any remaining solutes are collected either in the aqueous stationary phases (very polar compounds) or in the organic phase (less polar compounds).

RESULTS AND DISCUSSION

The crude ethanolic extract from the roots of *Stachytarpheta cayennensis* (Rich.) Vahl has been previously assayed by our group for antioxidant and anti-microbial activities. The ethyl acetate extract resulting from the original ethanol extract afforded the best results (unpublished data) in the two assays and was therefore, chosen for this study.

Gradient elution in CCC can be very useful when samples have a wide range of polarities. However, gradients are much more difficult to use in CCC than in classical LC. It should never be forgotten that any change in the composition of one liquid phase can change the composition of the other phase (phase diagrams).[11] This fact explains why the choice of an appropriate biphasic solvent system for a gradient elution in CCC can be a difficult task.[11] According to Foucault,[12] the system EtOAc:1-BuOH:H_2O can be used for gradient separations in the normal phase mode because all aqueous phase compositions in this system contain more than 90% water. The organic phases of this system are mixtures of ethyl acetate and 1-butanol with a small amount of water. Foucault further comments that compounds preferring water to ethyl acetate and butanol rather than water can be purified using this system.[12]

The basic solvent system EtOAc:BuOH:H_2O was tested for the optimization of the ratios of butanol in the solvent system. As suggested by Foucault,[12] the less polar system (EtOAc:H_2O) and the more polar one (BuOH:H_2O) were tested first for partitioning with the sample. A further advantage of this system[12] is that the composition of the polar water-rich phase does not vary so much when adding butanol, since the latter goes preferentially into the ethyl acetate-rich phase. It was found that a great part (*ca.* 90%) of the compounds remained in the aqueous lower phase of the EtOAc:H_2O system while in the BuOH:H_2O, *ca.* 50% of the compounds remained in each phase (Figure 1). These results show the ability of BuOH

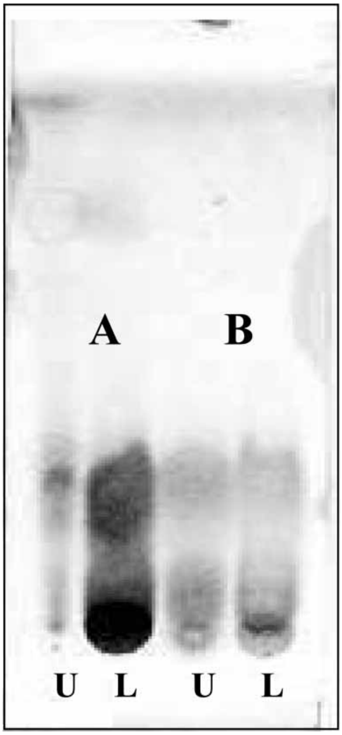

Figure 1. TLC results of the test tube partitioning test of the ethyl acetate extract of roots of *Stachytarpheta cayennensis.* Solvent system A:EtOAc:H_2O 1:1 v/v; solvent system B: BuOH:H_2O 1:1 v/v. U = upper phase, L = lower phase. Spray reagent: Vanillin 1% in sulfuric acid, developing solvent for TLC: EtOAc:acetone:H_2O 25:8:5 v/v/v, upper organic phase.

to extract the compounds into the organic upper phase, meaning that the polarity can be finely tuned by adjusting the ratios of this solvent in the system.

Next, the various ratios of butanol in the system were tested in order to establish an appropriate step gradient. Figure 2 shows the results of the partitioning test tube experiments for EtOAc:BuOH:H_2O-1:0.2:1, 1:0.5:1, and 1:1:1 v/v/v. These results suggest that the sample is very sensitive to small amounts of BuOH. With X = 0.5 BuOH, a large amount of the less polar compounds are in the upper organic phase. Based on Figure 2 results, the butanol content in the organic phase (ethyl acetate rich) was increased from 0.05 to 1.0 in four steps. The gradient was established as EtOAc:BuOH:H_2O 1:X:1 where X = 0.05 (solvent system A), X = 0.2 (solvent system B), X = 0.5 (solvent system C), and X = 1 (solvent system D).

The step-gradient employed in this separation process afforded the isolation of 6 compounds (Figure 3) from the EtOAc extract: martinoside

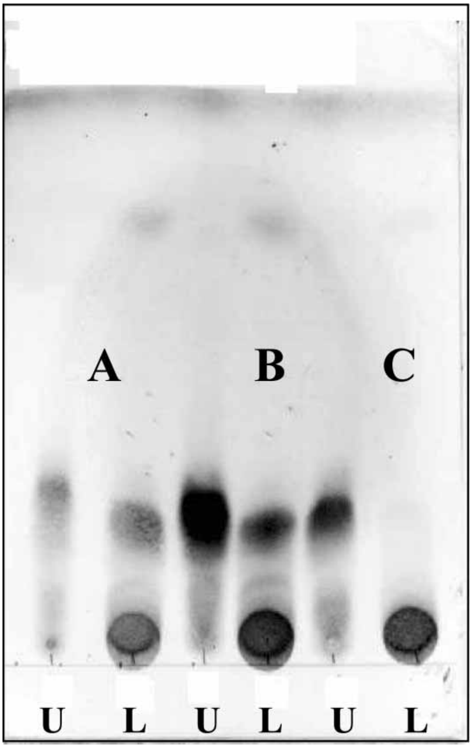

Figure 2. TLC results of the test tube experiments for the optimization of the amount of BuOH in the solvent system EtOAc:BuOH:H_2O 1:X:1 v/v/v. Solvent system A: X = 0.2, B: X = 0.5, C: X = 1. Eluting solvent system for TLC: EtOAc:acetone:H_2O 25:8:5 v/v/v, upper organic phase, spray reagent: vanillin 1% in sulfuric acid. U = upper phase; L = lower phase.

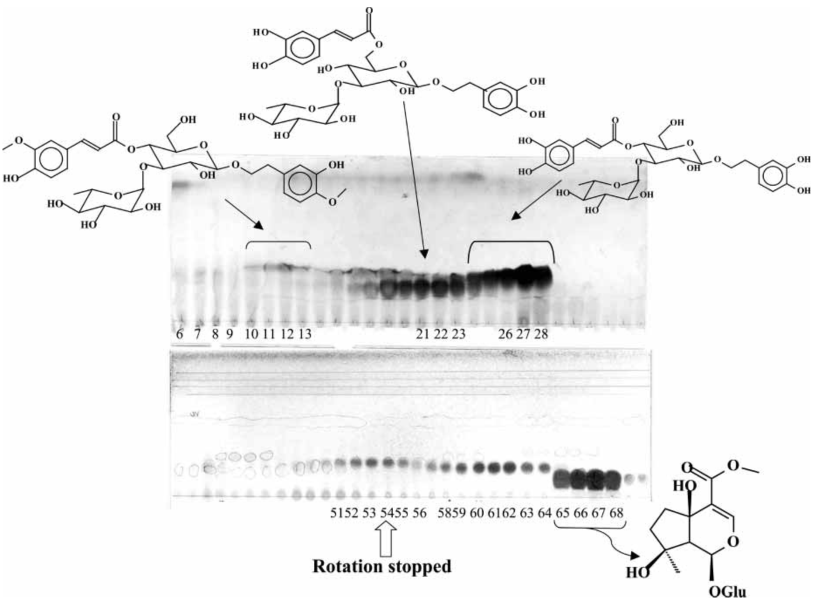

Figure 3. TLC results of the step gradient elution of the ethyl acetate extract of roots of *S. cayennensis.* Solvent system used: EtOAc:BuOH:H_2O (1:X:1 v/v/v), where X = 0.05 (solvent system A); 0.2 (solvent system B); 0.5 (solvent system C), and 1 (solvent system D). Eluting solvent system for TLC EtOAc:acetone:H_2O 25:8:5 v/v/v, upper organic phase, spray reagent: vanillin 1% in sulfuric acid.

(Fr 10–13, 55 mg), isoverbascoside (Fr 21, 37 mg), verbascoside (Fr 23–27, 300 mg), ipolamiide (Fr 65–68, 73 mg), and two other iridoid glycosides (Fr 51–56, 33 mg, and Fr 57–64, 31 mg), in which structures are still under investigation. A mixture of verbascoside/isoverbascoside was obtained in Fr 22. The total step gradient polarity range was thus, from relatively non-polar EtOAc soluble components, to more polar components present in the last drops of washed–off stationary phase. All compounds were identified by ^1H and ^{13}C NMR spectroscopy and the data compared with those from the literature.[13–15]

CONCLUSIONS

The sequential increase of BuOH in the organic phase of an EtOAc:BuOH:H_2O normal phase CCC solvent system, and wash-off of stationary phase allowed the isolation of the bio-active compounds from the ethyl acetate extract of the roots of *Stachytarpheta cayennensis.* This is a good example, showing that CCC can be used with gradient elution when appropriate liquid systems are employed. It should always be kept in mind that any change of mobile phase composition may induce changes in the stationary phase composition. With the EtOAc:BuOH:H_2O liquid system, these changes were used in the separation of glycosides to extend the working polarity window.

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