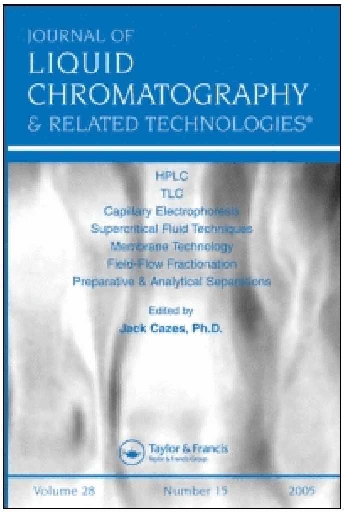

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Abstract: Countercurrent chromatography was used for the separation of neolignans from stems of *Ocotea elegans*. This separation was performed using a clean up system as prefractionation and the concentrated neolignans were submitted to CCC using Hexane:EtOAc:MeOH:H_2O (1:2:2:1 v/v/v/v) as the solvent system. It was possible to isolate burchellin, guaianin, and canelin, showing the high efficiency of CCC for the neolignan mixture separation.

Keywords: *Ocotea eligans*, CCC, Neolignans, Isolation

INTRODUCTION

Lignans and neolignans are plant compounds biogenetically derived by oxidative dimerization of cinnamyl alcohols/cinnamic acids and

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allylphenols/propenylphenols, respectively.[1] These natural products are of great importance due to their biological properties and they have anti-oxidant, anti-hypertensive, hepatoprotective, antimicrobial, and citotoxic effects.[2]

The genus *Ocotea* belongs to the family Lauraceae, and comprises 300 species of arboreal habit widely distributed in the Americas. In Brazil, these plants are used in folk medicine as anti-rheumatics, uterine tonics, and anti-syphilitics. Wood from the *Ocotea* species are used in civil construction and in the furniture industry.[3]

The chemical profile of plants belonging to the family Lauraceae is characterized by the presence of flavonoids, alkaloids, terpenes, and, especially lignoids.[4]

Ocotea elegans is known as "canela ferro" or "canela preta" in Brazil, where it is well widespread.[3] Preliminary phytochemical studies indicated the production of three major neolignans. Separation of these compounds from plant extracts by classical chromatographic techniques often involves irreversible adsorption phenomena on solid stationary phase that usually cause loss of time and sample. Countercurrent chromatography (CCC) is a separation technique in which there is no solid matrix to hold the liquid stationary phase, thus eliminating the problem of sample adsorption observed in traditional adsorption chromatography.[5]

This paper describes an elegant and quick method using CCC for the isolation of neolignans from stems of *O. elegans*.

EXPERIMENTAL

Apparatus

The high speed countercurrent chromatograph used in this work was the CCC-1000 model from Pharma-Tech Research Corp, equipped with three columns connected in series with total capacity of 325 mL. The 2.6 i.d. Teflon colis show β value ranges 0.5 to 0.75. It was used as a solvent delivery system based on a Pharma-Tech Research Corp constant flow pump; an injection valve with a 5 mL loop; and a Spectra-Chrom CF1 Fraction Collector from Spectrum Chromatography.

GC/MS analysis was carried out using a Hewlett-Packard 6890 gas chromatograph equipped with a fused silica capillary column HP-5 (30 m × 0.20 mm, 0.2 μm film thickness). The carrier gas was helium in a flow rate of $1.0\,\mathrm{mL \cdot min^{-1}}$. GC experiments were performed at temperatures ranging from 70°C to 290°C. The GC detector was a HP 5972 mass spectrometer, operating at 70 eV and 250°C. ^{1}H and ^{13}C NMR spectra were performed on a Brucker model DRX 400 at 400.13 and 100.61 MHz, respectively, with TMS as internal standard.

Preparation of Lignoids Pool from *Ocotea elegans*

Stems of *Ocotea elegans* were collected in July, 2003 at Rio de Janeiro, Brazil. The species was collected by Prof. Marcus Naduz and a voucher is deposited at the RB herbarium of the Instituto de Pesquisas do Jardim Botânico do Rio de Janeiro, Rio de Janeiro, Brazil, under the number 337.002. Crushed stems of *O. elegans* (435 g) were exhaustively extracted with hexane at room temperature. The dried extract (3.22 g 0.7%) was partitioned with hexane and dichloromethane. The dichloromethane fraction was purified through the partition technique using Hex:MeOH:H_2O (6:4:1 v/v/v) as solvent system, resulting in a rich neolignan fraction (1.9 g). This material was submitted to a CCC separation.

Choice of Solvent System

The empirical screening of the solvent system was made using the direct measurement of distribution coefficient by the shake-flask method.[6] In this test, the solute is simply partitioned between the two liquid phases of the proposed solvent system in a test tube. The relative concentration of the substances in each phase (lower and upper phase) was determined by TLC. An aliquot of each phase was spotted on a silica TLC plate and eluted with hexane:ethyl acetate 40:60 v/v. The spots were visualized after spraying with $CeSO_4$ and heating in air.

CCC Separation Procedure

The separation of compounds from the lignoid rich fraction was carried out in countercurrent chromatography using as solvent system: hexane (Hex):EtOAc:MeOH:H_2O (1:2:2:1). The mixture of the four solvents was mechanically shaken for 30 min and then briefly degassed.

The CCC machine was first entirely filled with 325 mL of the organic upper phase of the solvent system. The apparatus rotor was started at 900 rpm and the aqueous lower phase of the solvent system was pumped into the column at a flow-rate of 2.0 mL/min in head to tail direction. Part of the organic phase (62 mL) was pushed out of the CCC column and the remaining stationary phase (263 mL) correspondedto 81% of stationary phase retention. When the CCC column was well equilibrated, 5 mL of the lignoid fraction dissolved in the organic stationary phase were injected through the injection valve. The eluting mobile phase was collected in 5 mL fractions. After running 3.5 h (exactly 225 min), 450 mL of mobile phase was used and 90 fractions were collected. The rotor was stopped and distilled water was pumped into the coil at 2 mL/min to wash the column content off. The two phases (organic stationary phase and aqueous mobile phase) were collected in 65 fractions of 5 mL each. All fractions were monitored by TLC.

The isolated neolignans burchellin, guaianin, and canelin were identified by comparison of spectral data (^{1}H NMR, ^{13}C NMR and MS) with literature values.[7,9]

RESULTS AND DISCUSSION

Partitions of the hexane extract from *Ocotea elegans* stems successively with hexane and dichloromethane were used as clean up in order to concentrate the neolignans in the dichloromethane fraction. According to GC-MS analysis (Figure 1), this mixture contained mainly three neolignans that were later identified as burchellin, guaianin, and canelin.

The dichloromethane fraction was then purified by partition with the solvent system Hex:MeOH:H_2O (6:4:1) the neolignans remaining in the lower phase (aqueous phase); other substances present in the mixture were shifted to the organic phase. This pre-purification has economized a fractionation step and so the neolignans were separate from the fatty acids.

The solvent system Hex:EtOAc:MeOH:H_2O (1:1:1:1)[10] tested by the shake-flask method, resulted in undesired distribution of neolignans in both phases. Increase of the ethyl acetate concentration in the mixture did not work since the neolignans remaining concentrated mainly in the upper phase. Increase of the methanol concentration resulted in a very good distribution of the neolignans in both phases of the solvent system, once the methanol content reduced the lower phase polarity.

It is important to point out the difficulty to separate neolignans by traditional chromatographic techniques, especially when they are so similar to each other. Countercurrent chromatography represents a valuable tool in comparison with open-column silica gel or HPLC methods, resulting in very low solvent consumption and good separation.

The neolignan fraction was submitted to further HSCCC procedure using the solvent system Hex:EtOAc:MeOH:H_2O (1:2:2:1) that was chosen by shake-flask method. The upper phase (organic phase) of this solvent system was used as the stationary phase, in order to retain other less polar constituents found in the neolignan mixture. After the mobile phase front has emerged and the system established, a hydrodynamic equilibrium (S_f 81%) the lignoid fraction was injected and the compounds were eluted through the chromatographic coil and collected in 5 mL fractions. Burchellin (51.6 mg; Kd 0.72) was eluted in 127 min, guaianin (78.7 mg; Kd 0.76) was eluted in 152 min, and canelin (53.0 mg; Kd 0.88) was eluted in 157 min. The procedure showed very good chromatographic resolution. Thus, substances were analysed by GC-MS (Figure 1).

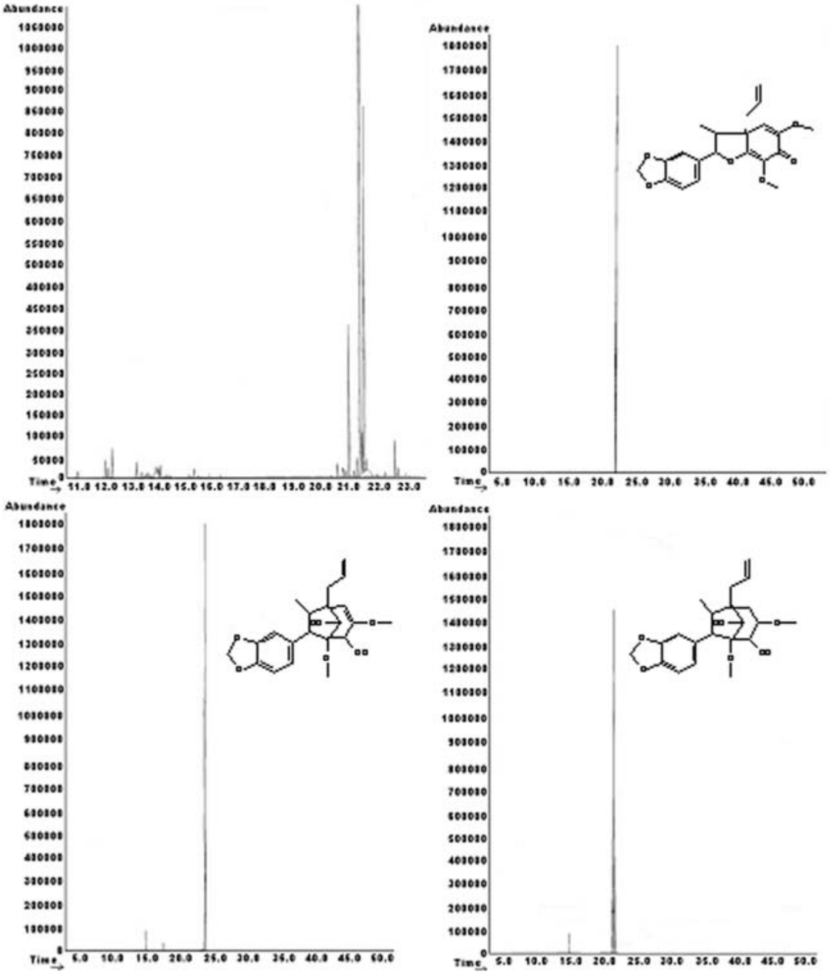

Figure 1. Gas chromatogram of the neolignans mixture after a clean-up of the dichloromethane fraction from *Ocotea elegans* and the neolignans bruchellin, guaianin, and canelin isolated from these mixtures by CCC. Chromatographic conditions: silica capillary column HP-5, helium as carrier gas flow rate 1.0 mL/min, temperature 70°C to 290°C (10°C/min).

CONCLUSIONS

The effectiveness of countercurrent chromatography to separate neolignans with little structural differences showed the value of the partition coefficient in the separation processes.

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