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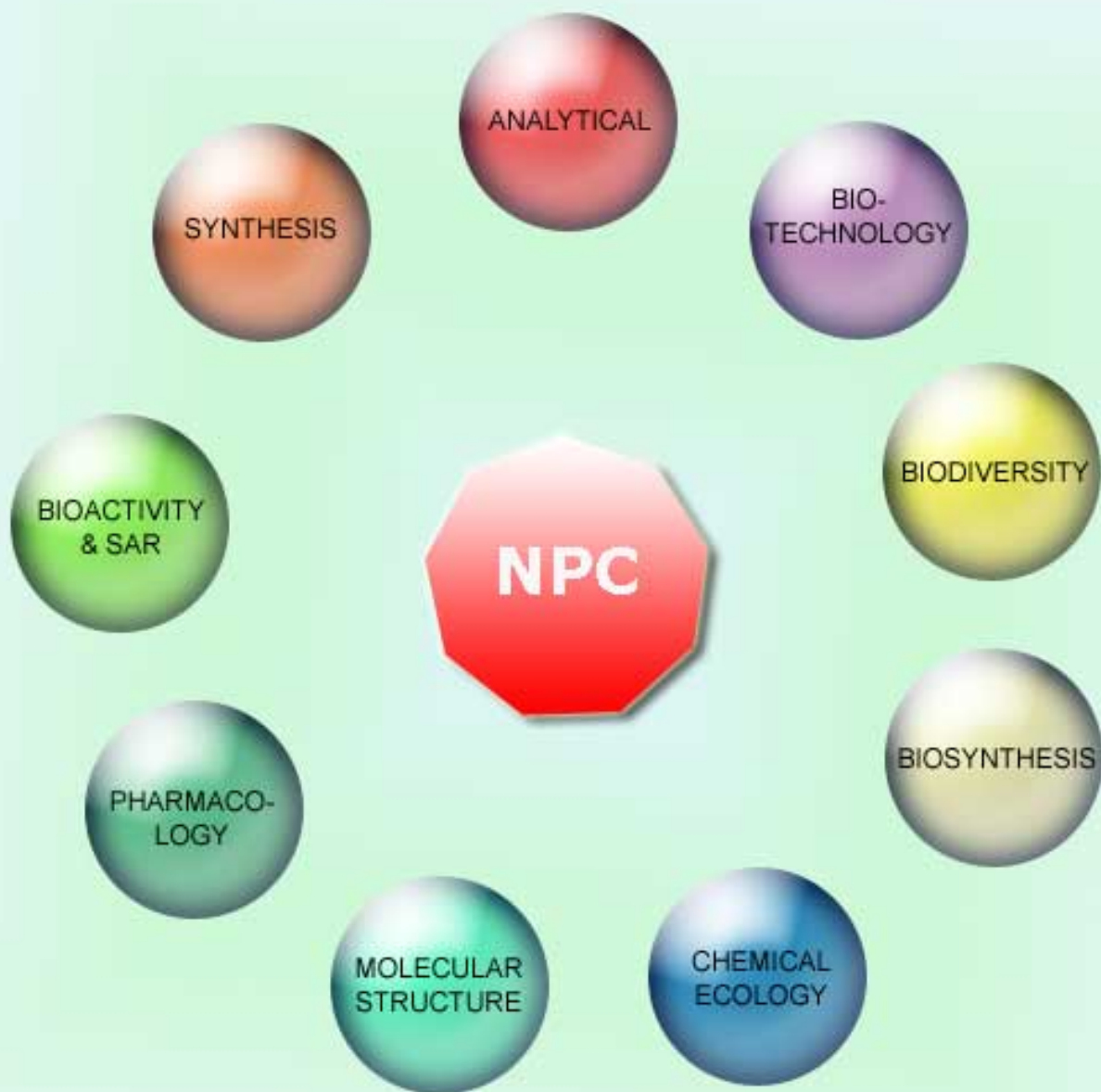
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Preparative Isolation of Antimycobacterial Shoreic Acid from *Cabralea canjerana* by High Speed Countercurrent Chromatography

Gilda G. Leitão^{a*}, Lisandra F. Abreu^a, Fernanda N. Costa^a, Thiago B. Brum^a, Daniela Fernandes Ramos^b, Pedro Eduardo A. Silva^b, Maria Cristina S. Lourenço^c and Suzana G. Leitão^d

^aNúcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, Bl. H, CCS. Ilha do Fundão, Rio de Janeiro, RJ, Brazil, 21.941-590

^bUniversidade Federal do Rio Grande, FURG, Departamento de Patologia, Laboratório de Micobactérias, Rua General Osório S/N Área Acadêmica da Saúde, CEP: 96200-190 Rio Grande/RS, Brazil

^cInstituto de Pesquisa Clínica Evandro Chagas, Fiocruz Laboratório de Bacteriologia e Bioensaios em Micobactérias, Plataforma de Bioensaios II, FIOCRUZ, 21045-900, Rio de Janeiro, Brazil

^dFaculdade de Farmácia, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, Bl. A, CCS. Ilha do Fundão, Rio de Janeiro, RJ, Brazil, 21.941-590

ggleitao@nppn.ufrj.br

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High speed countercurrent chromatography (HSCCC) was used to isolate the dammarane type triterpene shoreic acid from the dichloromethane extract of leaves of *Cabralea canjerana*, which showed activity against *Mycobacterium tuberculosis*. A preparative scale-up of the process was also developed.

Keywords: High-speed countercurrent chromatography, HSCCC, *Cabralea canjerana*, tuberculosis, antimycobacterial activity, Meliaceae.

The separation of bioactive secondary metabolites from crude plant extracts has always been a challenge to natural products researchers and countercurrent chromatography (CCC) offers many advantages compared with traditional phytochemical techniques of purification, especially those where chromatography with solid supports is used. The main advantage of CCC is that it is a form of liquid-liquid chromatography, which does not use a solid support and, therefore, there can be no loss of compounds or bioactivity due to interactions between the solid phase and the target compounds [1]. In the course of our investigation of bioactive plants from the Brazilian Atlantic forest, the dichloromethane extract of leaves of *Cabralea canjerana* (Meliaceae) showed antimycobacterial activity (minimal inhibitory activity, MIC of

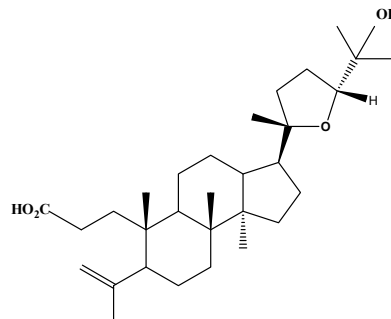


Figure 1: Shoreic Acid.

100 µg/mL) and was fractionated by high-speed countercurrent chromatography (HSCCC). The success of any HSCCC fractionation is dependent on the correct choice of solvent system, as this form of chromatography is based on the partition of solutes

Table 1: Amount of dichloromethane extract injected into the HSCCC equipment (sample size) and corresponding amount of shoreic acid obtained in the isolation process. Solvent system *n*-hexane:EtOAc:MeOH:H₂O 1:1.5:2.5:1.

Sample size (g)	Fraction no. ^a	Fraction size (mL)	Shoreic acid (mg)
0.8	17-25	4	213
1	14-24	4	321
1.5	17-26	5	642

^afractions where shoreic acid was eluted.

between two immiscible liquid phases. Compounds are separated according to their distribution constants (K_D), expressed as the ratio of their concentration in the stationary phase to their concentration in the mobile phase [2]. The test-tube partitioning test [3] is a good way of predicting the distribution ratios of target compounds to be separated and was used here for the choice of the appropriate solvent system. As the bioactive extract of *C. canjerana* contained medium polarity compounds, the solvent system *n*-hexane-ethyl acetate-methanol-water was chosen. Various ratios of the solvents in the biphasic solvent system were tested: *n*-hexane-ethyl acetate-methanol-water 1:2:1:2, 1:1.5:2.5:1, 1:2:2:1; 1:2:2.5:0.5, 1:2:2.5:1, (v:v:v:v). The best solvent system is that when the K_D of the target compounds remains around 1. Also, the volumes of upper and lower phases should be equivalent. When this is not the case (as in some of the solvent systems tested), the chosen system should be that where the mobile phase has a larger volume. Bearing this in mind, the final solvent system chosen for this fractionation was *n*-hexane-ethyl acetate-methanol-water 1:1.5:2.5:1 (v:v:v:v), with the upper organic layer acting as the stationary phase and the lower aqueous layer as the mobile phase. In this mode of CCC fractionation (reversed phase mode), the more polar compounds in the extract elute first. The separation was initially run with 820 mg of the dichloromethane extract of leaves of *C. canjerana*, affording *ca.* 213 mg of a dammarane triterpene, shoreic acid (K_D approx. 1) (Fig. 1), the major compound in this extract. The structure of this compound was confirmed by ¹H and ¹³C NMR spectroscopic data, which were in accordance with those in the literature [4]. The activity of this compound against *Mycobacterium tuberculosis* was tested by the MABA as well as the REMA assays, showing a MIC of 100 µg/mL. Less polar triterpenes were retained in the stationary organic phase, which was also fractionated, affording other triterpenes (structures under investigation).

CCC is particularly useful in the preparative range (mg to grams) and the time required for preparative

separations is no more than a few hours. The previous separation took about 3 h and consumed about 1.5 L of solvent. The isolation of shoreic acid was scaled-up from 820 mg to 1.5 g, using the same column volume, with good reproducibility (Table 1). This method proved to be fast, economic and highly effective in the scaled-up isolation of shoreic acid directly from a crude plant extract.

Experimental

General procedures: The NMR spectra were recorded using a Bruker Avance DRX400 spectrometer (Karlsruhe, Germany) at 25°C, operating at 400.13 MHz for ¹H and 100.61MHz for ¹³C. NMR spectra were recorded in CDCl₃ using TMS as internal standard. TLC analyses were carried out on pre-coated silica gel plates G_{F254} from Merck, and visualized by UV (254 nm) and reaction with vanillin in sulfuric acid (2%), followed by heating. For HSCCC separations a P.C. Inc countercurrent chromatograph equipped with a triple multi-layer coil equilibrated by a counterweight was used. Solvents were pumped into the coil with a Rainin Dynamax Model SD-200 pump. A Rainin Dynamax FC-1 fraction collector was also used.

Plant material and extraction: Leaves of *Cabralea canjerana* (Vell.) Mart. were collected in May 2003 at Mata Boa Vista Farm, Levy Gasparian, Rio de Janeiro State, Brazil. The plant was identified by Sebastião José Silva Neto, from the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, and a voucher specimen is deposited at the herbarium of the Federal University of Rio de Janeiro. The dried and ground leaves (890 g) were exhaustively extracted with ethanol 96°GL. The resulting dried ethanolic extract was suspended in water-methanol 70:30 (v:v) and extracted with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol, in this order.

Choice of solvent system by test tube experiments: Small amounts of the dichloromethane extract from leaves of *C. canjerana* were dissolved in separate test tubes containing *n*-hexane-ethyl acetate-methanol-water 1:2:1:2, 1:2:2.5:0.5, 1:2:2:1; 1:2:2.5:1, and 1:1.5:2.5:1 (v:v:v:v). 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 and developed with CHCl₃:MeOH 6:0.5 (v:v). The results were visualized by spraying with vanillin in sulfuric acid (2%), followed by heating. The final

solvent system was set as *n*-hexane-ethyl acetate-methanol-water 1:1.5:2.5:1.

HSCCC separation: The volume of the coil used in the experiments was 80 mL. The CCC column was filled with the organic stationary phase of the solvent system *n*-hexane-ethyl acetate-methanol-water 1:1.5:2.5:1. After the coil had been filled with the stationary phase, rotation started and the aqueous mobile phase was pumped into the head to tail direction at 2 mL/min until hydrodynamic equilibrium was achieved. In these conditions, the organic stationary phase, V_S , initially retained in the CCC column was 67 mL ($S_f = 75\%$; $V_M = 20$ mL). Eight hundred and twenty milligrams of the dichloromethane extract of leaves of *C. canjerana* was dissolved in 2.5 mL of each phase of the solvent system. The 5 mL was injected in the 80 mL coil using a Rheodyne injection valve at a flow rate of 2 mL/min., 850 rpm; 57 fractions of 4 mL were collected. Rotation stopped at tube 40 (which corresponds to $K_D = 2$). Shoreic acid (213 mg) was obtained from fractions 17 – 25, corresponding to a K_D of approximately 1. Further fractionations were carried out with 1 g and 1.5 g of the dichloromethane extract (Table 1). The same 80 mL coil was used, but the injection volumes were now 10 mL for both experiments. All other conditions were the same, except for the volume of fractions collected in the 1.5 g separation, which was 5 mL.

Antimycobacterial tests: Samples were simultaneously screened by both microbiology laboratories (FURG and FIOCRUZ), using the MABA and REMA bioassays, respectively, as described previously [5]. Final concentration of plant extracts/substances was either 200 µg/mL or 100 µg/mL. Media plus bacteria with and without rifampicin were used as controls. The strain H₃₇Rv (ATCC - 27294) was used for all methodologies. MABA (Microplate Alamar Blue Assay) susceptibility testing was performed at FIOCRUZ according to the method described in [6]. The REMA - Resazurin Microtiter Assay Plate [7] method was used for the determination of the antimycobacterial activity at FURG. In brief, the assay is accomplished in microplates (96 wells) using resazurin as indicator of cellular viability. Medium 7H9 enriched with 10% OADC was used. The minimal inhibitory concentration (MIC) was determined (starting at 200 µg/mL in 1:2 serial dilutions).

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