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A validated higher-performance liquid chromatography method for quantification of cinchonain Ib in bark and phytopharmaceuticals of *Trichilia catigua* used as Catuaba

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Abstract

The hydroalcoholic extract, prepared from authentic chopped barks of *Trichilia catigua,* was evaluated by high-performance liquid chromatography using a diode array detector (200–400 mn). The crude extract was purified by rotation locular counter-current chromatography and the chloroform fraction obtained was clean-up by solid-phase extraction. With the aim of getting preliminary structure information on-line, the methanol fraction thus obtained was analyzed by gradient elution using the diode array detector coupled to a mass spectrometer. The presence of flavalignan in this extract was inferred by the chromatographic band, in the total ion current trace, that had an $[M–H]^-$ = 451. With this information, cinchonain Ib was isolated as a pure compound from the crude hydroalcoholic extract using a solid-phase extraction procedure for the sample clean-up followed by a semi-preparative separation using the reverse mode of elution. The isolated compound, after complete characterization, was used as an external standard for the development and validation of a method for the analysis of this compound in herbal medicines using the ultraviolet as the detector. The validated method has been successfully applied for quantification of cinchonain Ib in commercialized herbal medicines sold as Catuaba in Brazil and also in standard chopped barks of *T. catigua*.

Keywords: *Trichilia catigua*; Flavalignan; Validation; HPLC; Catuaba

1. Introduction

With a huge vegetal biodiversity, having more than 55,000 species listed, the Brazilian phytotherapy industry holds a comfortable 10th biggest position in the world market, and represents 20–25% of the local pharmaceutical market [1]. Despite this, the majority of the phytopharmaceuticals commercialized in Brazil is developed abroad and from non-native plants. However, a number of native plants are used on large scale as folk medicines and sometimes there are problems in identifying the plant used.

A good example of this is the plant that is harvested in Brazil and sold around the world as Catuaba. In Brazil, different genera and families are known as Catuaba such as *Anemopaegma* (Bignoniaceae), *Erythroxylum* (Erythroxylaceae), *Illex* (Aquifoliaceae), *Micropholis* (Sapotaceae), *Secondatia* (Apocynaceae), *Tetragastris* (Bursereceae), *Trichilia* (Meliaceae). They all are used as a sexual stimulant, as an aphrodisiac, as a purgative and for the treatment of rheumatism and hydropsy [2–5].

The specie registered as true Catuaba for medicinal purposes in the Brazilian Pharmacopoeia [6] is *Anemopaegma arvense* (Veil.) Stellfeld (Bignoniaceae) and the use of its roots is recommended. A small number of works on this species have been published and the alkaloid yohimbine has been attributed to this plant [7].

However, *Trichilia catigua* A. Juss (Meliaceae) is also widely used in Brazil as Catuaba [8]. *T. catigua* is also known as catiguá, catuaba, catiguá vermelho, pau ervilha and catuaba do norte [2]; its bark that is used for medicinal purposes, and the literature registers some pharmacological studies with this plant [9–12].

The chemical constituents of these species are entirely different, although the plants are indicated for the same medicinal purposes. This use of plants of different families and genera with the same popular name has resulted in erratic identification. This

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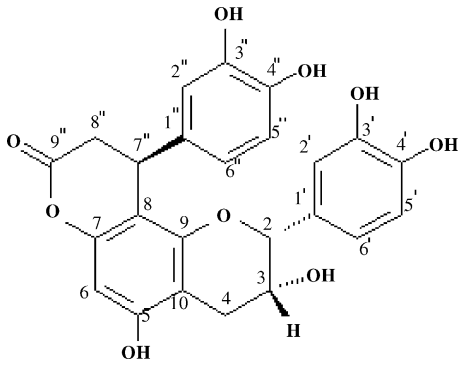

Fig. 1. Structure of cinchonain Ib.

is thoroughly discussed in the recent publication by Kletter et al. [4].

It is well accepted that for evaluating the quality of a herbal medicine is necessary to standardize the content of the active principles. However, when the active principles are not known, a compound, which is the most representative of the chemical composition of the extract may be selected as the marker [13–16].

For evaluating the quality and authenticity of herbal medicines that use *T. catigua* A. Juss as the herbal product, a method for quantification of the flavalignan (Fig. 1) isolated from the barks of *T. catigua* A. Juss was developed, validated and used.

This work reports the isolation and the use of the flavalignan cinchonain Ib as the marker for an HPLC quality control method for commercialized herbal medicines and also for the crude material sold as Catuaba in Brazil.

2. Experimental

2.1. Plant material

T. catigua A. Juss bark was obtained in Maringá, Brazil, in October 2001. The plant was identified and a voucher (Exsiccate 9908) was deposited on the Herbarium of the Universidade Estadual de Maringá.

2.2. Chemicals

Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) were HPLC-grade (J.T.Baker, Philipsburg, PA, USA). Acetic acid (AcOH) and triethylamine (TEA) were analytical grade (Synth, Diadema, SP, Brazil). Water was purified with a Millipore Mlli-Q system (Mllipore, São Paulo, SP, Brazil) and it was used for all experiments. The silicas used were C_{18} (Hypersil®, 5 μm particle size, 120 Å pore size) and phenylhexil (Luna®, 10 μm particle size and 100 A pore size).

2.3. Instrumentation and chromatographic columns

The higher performance liquid chromatographic–diode array–mass spectrometer (HPLC–DAD–MS) system consist of two Shimadzu LC-10AD pumps (Kyoto, Japan), a SUS mixer, an auto injector model SIL 10A, a photodiode array model SPD-10AVP, and it was used with a CBM 10A interface. HPLC data acquisition was done on CLASS LC10 software. The LC system was coupled to a triple quadrupole QuattroLC mass spectrometer (Micromass), operating in the ESI (negative ion, megaflow) mode. The MS experiment setup and data acquisition were conducted using the Masslinxk (V. 3.5) software.

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker DRX-400 spectrometer, using TMS as an internal standard or by reference to solvent signal.

The semi-preparative HPLC system with one LC-6A pump, a manual RHEODYNE 7125 injector and an SPD 10D UV detector with a CBM 10A interface was used to purify cinchonain Ib. Data acquisition was done on CLASS LC10 software.

For the quantification analysis a gradient Shimadzu HPLC system was employed and it was composed of two LC-10AD pumps (Kyoto, Japan), a SUS mixer, an auto injector model SIL 10A and a SPD 10D UV–vis detector. For the peak purity a photodiode array model SPD-10AVP was used. A CBM 10A interface was used for both detectors. Data acquisition was done on CLASS LC10 software.

The rotation locular counter-current chromatography was performed using a RLCC-100 apparatus (Tokyo Rikakikai, Tokyo, Japan), which consisted of 16 columns (45 cm × 11 mm I.D.) divided by centrally perforated PFTE disks into 37 loculi each. Injections were made manually by filling the RLCC loop volume (3 ml).

The columns were packed by the ascending slurry method, using methanol for the preparation of the slurry (50 ml) and for the packing. These were carried out at a pressure of 7500 psi [17]. The dimensions for the analytical columns were of 15 cm × 0.4 cm I.D. and the column used for semi-preparative HPLC was a 25 cm × 0.7 cm I.D.

2.4. The HPLC analysis

2.4.1. HPLC–DAD–MS analysis

A 20% hydroalcoholic tincture of air-dried bark (200 g) of *T. catigua* was prepared in water:ethanol (34:66, v/v) at room temperature for 7 days. The ethanol was removed under vacuum at 40 °C to give an aqueous extract. The aqueous extract was lyophilized and yielded 43 g. The lyophilized extract (500 mg) was then submitted to rotation locular counter-current chromatography (RLCC) using methanol:water:chloroform (17:33:50, v/v) as the solvent system. The methanol:water phase was used as stationary phase while chloroform as the mobile phase.

The solvents were prepared using a separator funnel (2 l) with time allowed to equilibrate and separate the two phases. RLCC was employed in the descendent mode at 100 rpm and 0.5 psi of pressure at a flow rate of 0.8 ml/mim.

The chloroform fraction (the lower phase) was collected over a period of 12 h (as one fraction only) and after drying under vacuum it was cleaned using the same conditions described in Section 2.5.3 and then analyzed using the C_{18} column by gradient elution with methanol (B) in water (A)—(38 to 100% in 35 mim, Δ%B = 1.77), an isocratic run at 100% of B was main-