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Quantitative determination by HPLC of iridoids in the bark and latex of *Himatanthus sucuuba*

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ABSTRACT

The main iridoids from the bark and latex of *Himatanthus sucuuba* were isolated and characterised by spectroscopic methods. HPLC was used for the quantitative analyses of these iridoids and the chromatograms of bark and latex showed a similar iridoid composition. Both parts of the plant are used in folk medicine for the treatment of various ailments.

KEY-WORDS

Apocynaceae, HPLC, *Himatanthus sucuuba,* iridoids, quantitative determination.

Determinação quantitativa por CLAE de iridóides nas cascas e látex de Himatanthus sucuuba

RESUMO

Os iridóides principais das cascas e látex de Himatanthus sucuuba *foram isolados e caracterizados por métodos espectroscópicos. CLAE foi utilizada para a análise quantitativa desses iridóides e os cromatogramas da casca e do látex apresentaram uma composição semelhante de iridóides. Ambas as partes da planta são utilizadas na medicina popular para o tratamento de várias doenças.*

PALAVRAS-CHAVE

Apocynaceae, CLAE, Himatanthus sucuuba, *iridóides, determinação quantitativa.*

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INTRODUCTION

Himatanthus sucuuba (Spruce) Woodson (Apocynaceae) is known in folk medicine for the treatment of various ailments. In Brazil, the latex and bark have been used as an antitumor agent and for their antiulcer and anti-inflammatory activities (Schultes, 1979; van den Berg, 1982; Di Stasi *et al.*, 1989; Silva *et al.*, 1998). However there is limited pharmacology reported to justify, in terms of existing legal requirements, the wide use of the plant by the population. Iridoids are not uncommon in the family Apocynaceae (Perdue *et al.*, 1978; Abe *et al.*, 1984; Abe *et al.*, 1988; Abdel-Kader *et al.,* 1997). There is a continuing interest in these compounds since many of them have shown substantial biological activity e.g.: hypotensive, anti-inflammatory, antifungal, antibacterial, etc (Ghisalberti, 1998). The broad diversity of biological activity exhibited by the iridoids (Abe *et al.*, 1984; Abe *et al.*, 1988; Anderson *et al.*, 1988; Ghisalberti, 1998; Silva, 2000) has stimulated interest in methods for their isolation and determination. The rational use of the latex and bark of *H. sucuuba* in medicine requires careful control of these principles, preferably by a rapid method like HPLC. This paper reports a HPLC method developed for detection and quantification of previously isolated iridoids, the principal constituents of the bark and latex of this plant.

MATERIAL AND METHODS

HPLC ANALYSIS

HPLC was performed with a Shimadzu LC-8A gradient chromatograph equipped with two LC-8A pumps, controlled by a CBM-10A interface module, an automatic injector 10Avp and an SPD-M10A photodiode array detector (PAD) was used for peak purity test and analysis of compounds. Solvents were filtered using a Millipore system and analysis was performed on a Merck Lichrospher C18 column (250 mm x 4.6 mm I.D., 5 mm particle size). All the samples were filtered through 0.45 μm filter (Sartorius) before being analysed. The mobile phase was acetonitrile (A) and water containing 0.05% trifluoroacetic acid (TFA) (B) and the composition gradient was: 5% (A) → 25% (A) for 20 min; 25% (A) for 5 min and then to 40% (A) for 15 min. The injection volume was 20 μl and the samples were monitored at 230 nm. A constant flow-rate of 1 $ml.min^{-1}$ was used during analysis and a PAD range 200 – 500 nm. HPLC grade solvents and bidistilled water were used in the chromatographic studies. All chromatographic experiments were performed at room temperature.

SAMPLES

The bark and latex of *H. sucuuba* were collected in Santarém, Pará. A voucher specimen was deposited at the Herbarium of the Institute of Biological Sciences, University of Amazonas, Manaus, Brazil, under number 5436.

SAMPLE PREPARATION

BARK (SAMPLE A)

The ground bark (1 g) was extracted by infusion by addition of boiling water (10 ml) and allowed to stand for 30 min, followed by filtration and lyophilisation. This preparation (30 mg) was dissolved in MeOH/H_2O (1:1, 1 ml) and analysed by HPLC.

LATEX (SAMPLE B)

Methanol/H_2O (1:1, 10 ml) was added to the lyophilised latex (1 g). The resulting suspension was centrifuged, filtered and evaporated. The residue (30 mg) was dissolved in MeOH/H_2O (1:1, 1 ml) and injected into the HPLC.

IRIDOIDS

EXTRACT FRACTIONATION

n-Buthanol (5 x 20 ml) was added to the latex (3 l) of *H. sucuuba* to promote the clotting of the polyisoprene present. The remaining organic fraction, after evaporation of the solvents, was dissolved in methanol/H_2O 8:2, (200 ml), and partitioned with hexane. One part of the methanol/H_2O fraction (5 g), after evaporation, was submitted to MPLC chromatography using RP-18 columns (25 – 40 mm particle size, 460 x 36 mm i.d., 460 x 15 mm i.d.). Elution was made with H_2O, H_2O/MeOH (90 to 50%) and 50 ml fractions were collected. Fractions 19-26 produced a white solid, identified as plumieride (**2**) (12 mg). Fractions 9-17 and 31-42 were refractionated in semi-preparative columns of RP-18 with the same chromatographic technique above. Elution with H_2O/MeOH (75:25) and (65:35), respectively, produced two solids, identified as 15-demethylplumieride (**1**) (10 mg) and isoplumieride (**3**) (5.0 mg). The isolated compounds (Figure 1) were characterised by spectral methods (^1H and ^{13}C NMR, MS, UV and IV) (Silva, 2000). The iridoids were dissolved in H_2O/MeOH (1:1) to prepare the stock solutions.

HPLC ANALYSIS

CALIBRATION CURVES

Standard curves for authentic samples of the iridoid **1**, **2** and **3** were prepared with 1, 5, 10, 20 and 30 $\mu g.ml^{-1}$. Each solution was injected in triplicate and the calibration curves were constructed with the averages. The retention times, capacity factor (k'), regression equations, coefficient of variation (CV) and correlation coefficients (R) determined for standards are given in Table 1.

LIMITS OF DETECTION

The standard solutions were diluted with H_2O/MeOH (1:1) to provide appropriate concentrations. The limit of detection for each compound was determined as 0.21 $\mu g.ml^{-1}$ (compound 1) and 0.20 $\mu g.ml^{-1}$ (compounds 2 and 3) when ratio of testing peak signal-to-noise was 4.

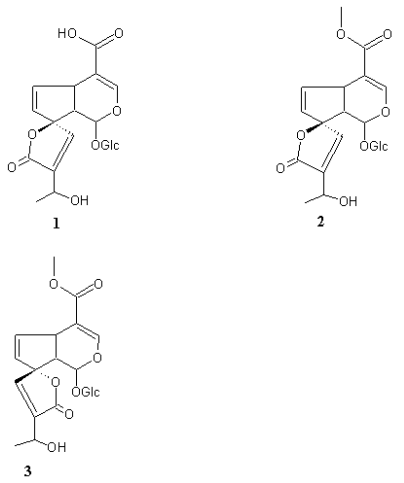

Figure 1 - The structures of iridoids from *H. sucuuba*

Table 1 - Results of the quantitative analysis

Compound	Ret. Time (min)	k	Regression equation	R	Bark*	Latex*	CV (%)
1	18.4	5.1	y = 1e+06x-873596	0.9932	3.8	10.5	0.30
2	26.8	7.9	y = 1e+06x-497670	0.9981	26.7	34.7	0.81
3	31.3	9.4	y = 1e+06x+134438	0.9996	4.4	4.7	0.30

*($\mu g.m^{-1}$)

CV – coefficient of variation

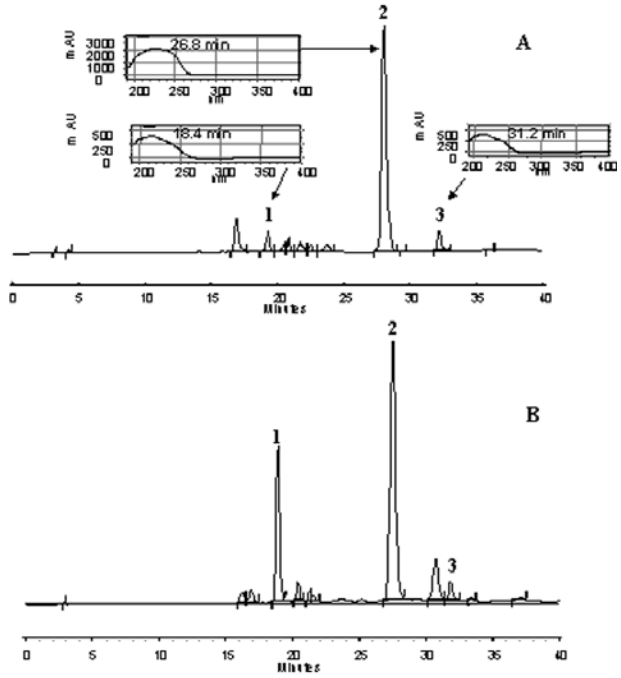

Figure 2 - HPLC chromatogram showing the separation of iridoids (1), (2) and (3) from the bark (chromatogram A with UV spectra) and latex (chromatogram B) of H. sucuuba

RESULTS AND DISCUSSION

The method developed for HPLC fingerprinting provided an analysis of the iridoids from the bark (sample A, Chromatogram A) and latex (sample B, Chromatogram B) samples. The conditions here applied led to a good separation of the peaks which could be identified in the Figure 2. The chromatogram A showed the UV spectra of 15-demethylplumieride (**1**; R_t = 18.4 min), plumieride (**2**; R_t = 26.8 min) and isoplumieride (**3**; R_t = 31.3 min). These iridoids in the extracts were characterised by comparing with their chromatograms and UV spectra with those obtained from the pure substances used to prepare the standard curves. The calibration curves showed linearity in the concentration range used for the standards. Results are summarised in Table 1.

ANALYSIS OF HPLC CHROMATOGRAMS

The HPLC chromatograms of samples A and B showed a similar iridoid composition, plumieride (**2**), the main iridoid present, constituting 26.7 $\mu g.ml^{-1}$ of the bark and 34.7 $\mu g.ml^{-1}$ of the latex. The iridoids glycosides **1**, **2** and **3** have the same basic carbon skeleton with a glucose moiety at C-1 but differ in the functional groups at C-4 and the stereochemistry at C-8. Compound **1** with a -COOH group attached to C-4 was the first to be eluted (R_t = 18.4 min), once its retention time in the C-18 column decreases, by protonation in the acidic mobile phase, with relation to the other iridoids. The order of elution between **2** (R_t = 26.8 min) and **3** (R_t = 31.3 min) and the comparison of their structures indicated that they might differ only by the stereochemistry at C-8. These retention times can be explained by the close proximity of H-1*a* to the oxygen at C-8, which decreases the polarity of compound **3**. The addition of TFA to the mobile phase facilitates the optimisation of peak resolution. The presence of -COOH and -CH_2OH groups decreases the solute retention on the C-18 column. The results indicated the quantity of plumieride (**2**) is present.

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