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Standardization of Herbal Drugs Derivatives with Special Reference to Brazilian Regulations

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1. Introduction

The development of herbal medicine requires a careful selection and unambiguous identification of plant species that will be the exclusive active principle, either as extract or as fraction, embedded in the product. An isolated substance, even if obtained from natural sources, does not give rise to a phytomedicine.

The technical standardization of an intermediary of a phytomedicine is a decisive step for the quality standard that the product will show. After the validation of the alleged use, popular form of use, posology and agronomic certification of the plant material, for which phytochemical and pharmacognostic pattern will be developed to monitor the physical, chemical and physico-chemical characteristics of the plant, ensuring homogeneity of samples and the similarity to specimens tested in experimental stage. The plant material is then extracted and the obtained extract is used in the development of the formulation. This extract must be in accordance to the Brazilian Pharmacopoeia, respecting as far as possible the characteristics of the popular form of use. Before the next step, the pharmacotechnical handling, the extract will be phytochemically analysed, in order to determine the metabolic substance classes present in the sample. Then it must be pharmacologically investigated to assure that its activity, is similar to that originally alleged and experimentally tested; its physical and physico-chemical characteristics will be determined, and, subsequently, the extract undergoes chemical and chromatographic analysis to have identified substances that could characterize the plant and serve as chemical and, preferably, pharmacological quality markers. Substances which are chemically stable, responsible for the activity to be presented by the phytomedicine and able to be detectable and quantifiable by usual analytical methods, such as chromatography and ultraviolet spectroscopy or mass spectrometry, are potential candidates to be used as markers.

To compose a pharmacopoeial monography of a medicinal plant it is recommended to describe the characteristics concerning the anatomy of organelle or tissues of the plant organ to be notified or from which a medicine will be developed. These structures of the herbal

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drug can be observed in microscopic anatomical analysis and can be depicted as a micrograph which is the document to be added to the monography.

2. Microscopic anatomical description of leaves of *Echinodorus macrophyllus*

The information produced from these analyses helps to standardize the raw material utilized in the production of a phytomedicine, this is illustrated by the anatomical description of leaves of *Echinodorus macrophyllus* which is used to produce several phytotherapeutical products in Brazil. To obtain the anatomical slices the dried plant material was rehydrated for 24h in distilled water and sectioned with stainless steel blade in different regions of the leaf and midrib. The sections were clarified in 20% NaClO and stained with Astra blue and basic fuchsin (MACHADO et al., 1988).

Fractographs were obtained in light microscope Axiolab model ZEISS coupled to a Canon Power Shot model A640 digital camera for registration of anatomical characters. The electron micrographs of the material were prepared using the process of critical point and metallization with gold dust deposition and suitably organized in circular metal holders (stubs), the images were captured in the scanning electron microscope model LEO 1450 VP (KRAUS & ARDUIN, 1997).

The following figures show the photographs of the cuts obtained by optical microscopy at the referred enlargement (10X or 40X). The leaf shows discrete epicuticular wax deposition of granulation aspect. In frontal view, the epidermal cells on both sides, adaxial and abaxial, are irregularly shaped, ranging from square and rectangular, with tiny and slightly sinuous walls. In transverse section the epidermis of both surfaces are uniseriate, with rectangular cells with flat anticlinal and periclinal walls; they are heterodimensional (Figure 1).

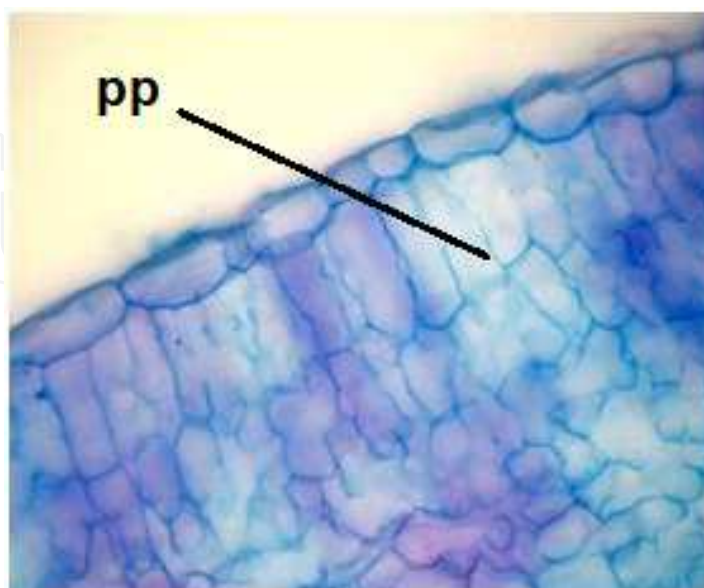


Fig. 1. Transverse section of leaf showing the upper epidermis and palisade parenchyma (pp) in frontal view (40X).

The parenchyma is dorsiventral, consisting generally of two strata of homogeneous cells, slightly Sinuous anticlinal and smooth periclinal walls the first layer being more organized, with cells elongated, juxtaposed and perpendicular to the epidermis; the second layer palisade tissue consists of cells of the same format, but different in size, apparently less than the first. The cells of spongy parenchyma present thin walls, are heterodimensional showing different formats, in addition to well-developed intercellular spaces (Figure 2).

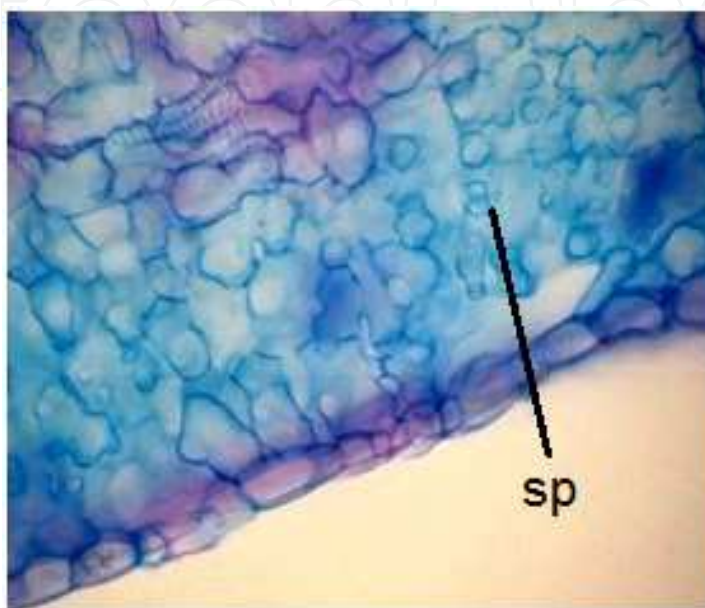


Fig. 2. Transverse section of the leaf showing the lower epidermis with sinuous anticlinal walls and spongy parenchyma (sp) (40X).

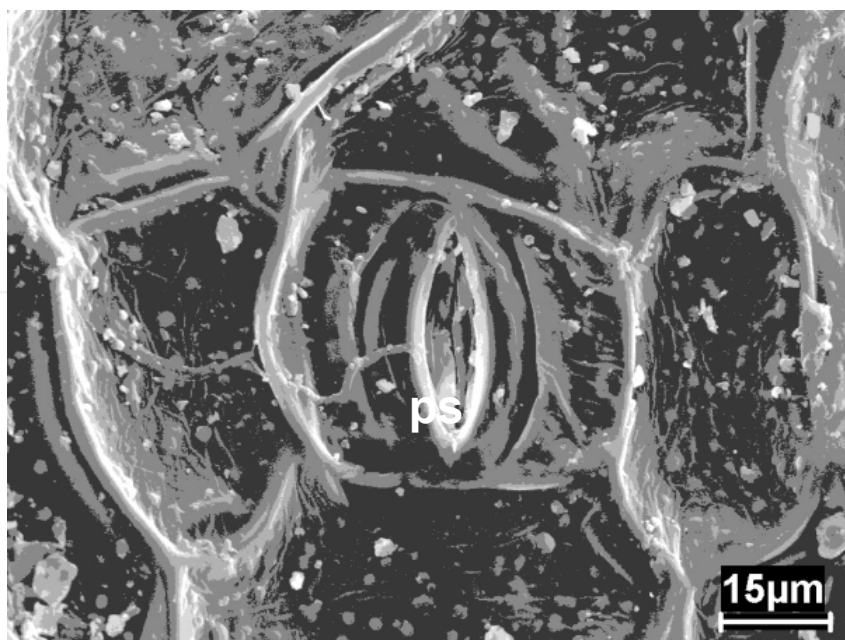


Fig. 3. Scanning electron microscopy showing paracytic stomata (ps).

In the parenchyma paracytic stomata can be observed, where stomatal complex is formed by a pair of lateral subsidiary cells connected to the guard cells, corroborating the description made by Tomlinson (1982). Picture obtained by electronic microscopy (Figure 3).

The vascular bundles have xylem at the upper face and phloem occurring at the opposite side, in polar regions cells of sclerenchyma can be seen. This set appears enclosed in a sheath of parenchyma.

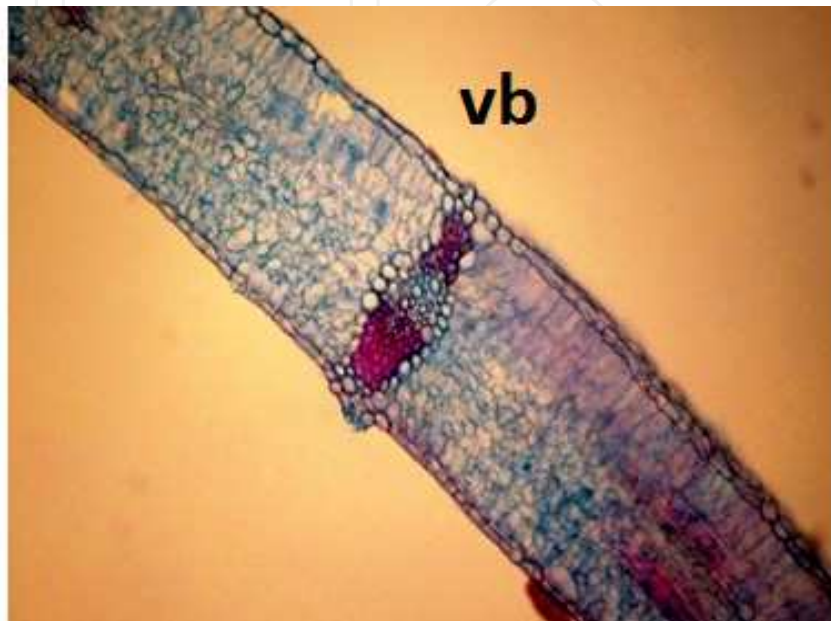


Fig. 4. Transverse section of leaf showing vascular bundle (vb) (10X).

The next figures show the transverse section of a petiole, which exhibits triangular to hexagonal shape, anatomically resembling the petiole of *Sagittaria lancifolia*, also an Alismataceae (TOMLINSON, 1982) (Figure 5).

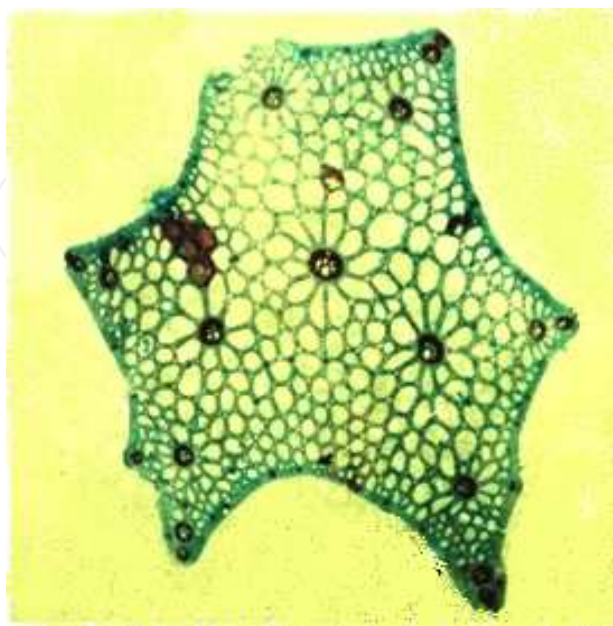


Fig. 5. Transverse section of petiole showing the hexagonal shape (10X).

Figure 6 displays a thin cuticle, showing a uniseriate epidermis, with polygonal shaped cells, and smooth walls. Reference to this format of petiole in this species has already been made by Matias (2007).

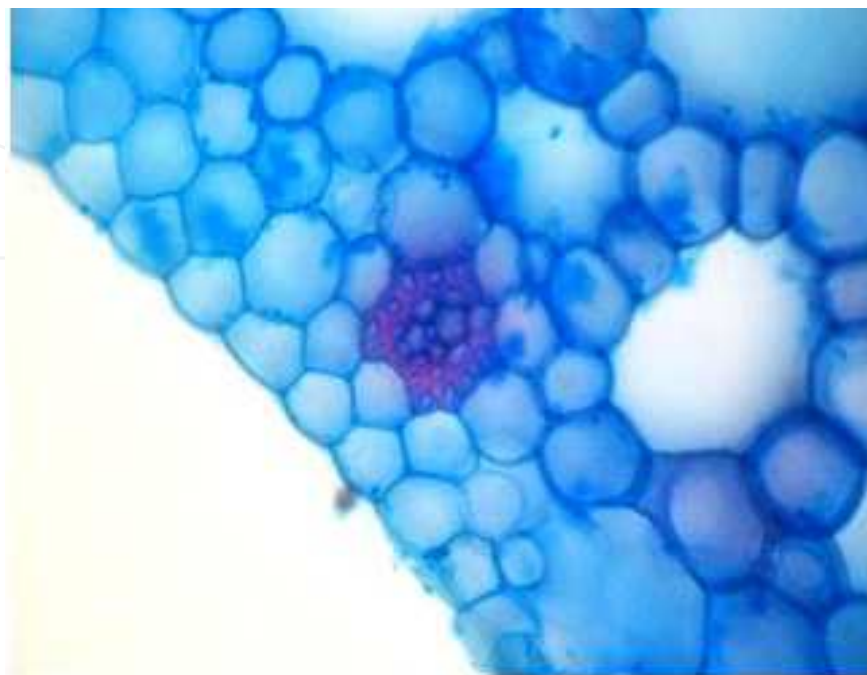


Fig. 6. Transverse section of petiole showing the epidermis (40X).

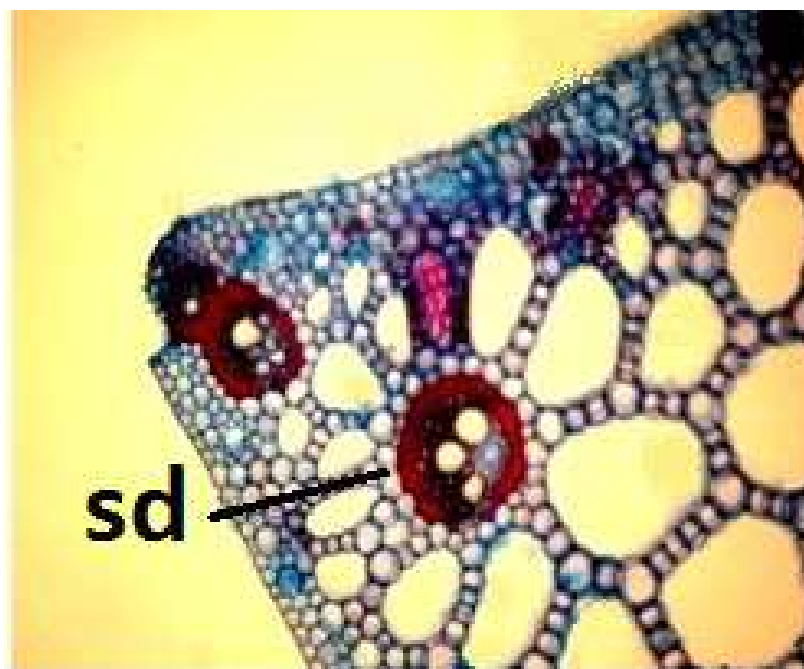


Fig. 7. Transverse section of the petiole highlighting the presence of secretory ducts (sd) (10X).

The figure 7 shows that the petiole has two to three layers of compact parenchyma cells, in which secretory ducts are to see as well vascular bundles that have smaller diameter than

the ones observed in the leaf's parenchyma. This plant organ presents a great amount of parenchyma formed by a layer of isodiametric parenchyma cells, where bundles of different sizes are distributed, as seen in the figure.

In the parenchyma of the petiole diaphragms can be observed (Figures 8 and 9). These structures are septate brachiform cells that interrupt the existing intercellular spaces, preventing the collapse of the organ if there is an injury in the submerged part of the plant. Leite (2007) describes in less detail this structure.

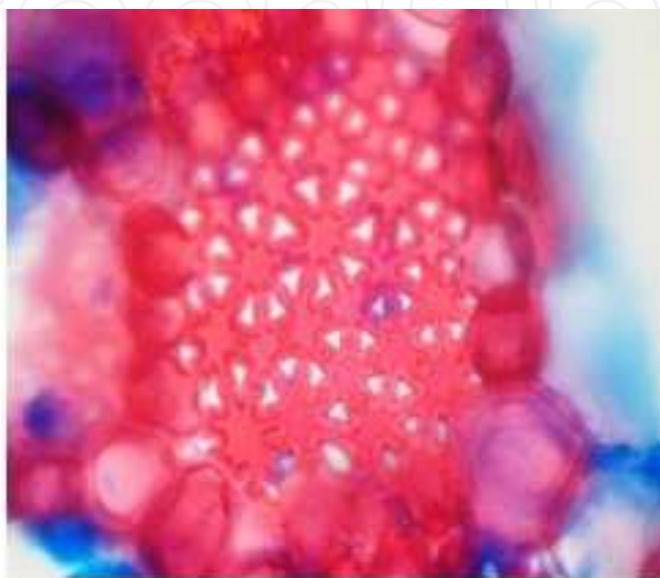


Fig. 8. Transverse section of petiole showing the presence of diaphragms. 40X.

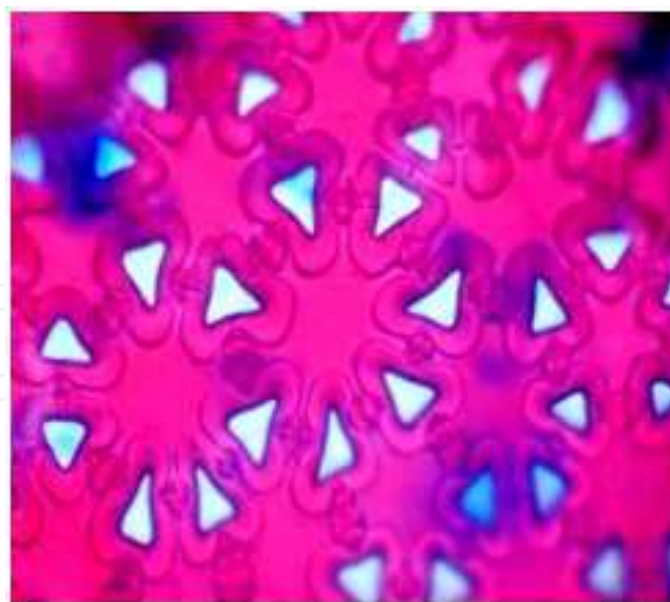


Fig. 9. Details of diaphragms in a higher enlargement (100X).

The presence, in the analysed sample, of petioles with different sizes and shapes could also be observed. Figure 10 illustrates this observation since the shape of the organ differs from that showed in figure 5. The characteristic of the petiole here presented, according to Matias

(2007), seems closely similar to that from *E. subalatus* and *E. palaefolius*, indicating a possible introduction of parts of one of these species in the commercial sample, object of this study.

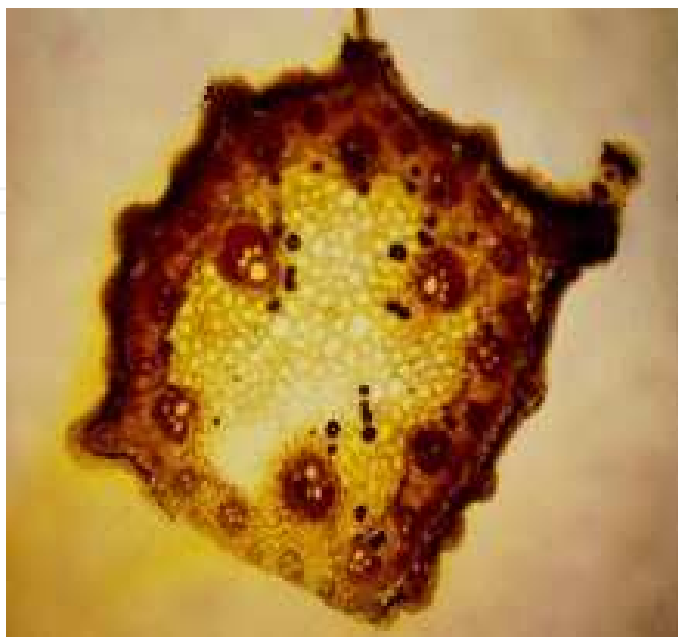


Fig. 10. Transverse section of a petiole showing a shape different from that already described (10X).

As a conclusion about the results here presented it could be assumed that the raw material to be used in the production of a phytomedicine must be analysed by microscopic anatomical techniques to ensure the identity of the species here in question. More specifically, although most of the fragments analysed can be attributed to *Echinodorus macrophyllus*, there is the possibility that fragments of petiole from *E. subalatus* and *E. palaefolius* were introduced in the material supplied for the work reported in this section. Anyway, it is important to note that for the production of herbal medicines based on *E. macrophyllus*, the part used is the leaf, where the chemical markers and the possible active principles are present, which this feature can threaten the credibility of the medicines prepared having a medicinal plant as active pharmaceutical matter.

Once the raw material is anatomically characterized, in case of the development of a phytomedicine, a set of procedures is followed, which includes processing of plant material providing the herbal drug, that is used to obtain an extract, both drug and extract, are physically and physico-chemically analysed. Afterwards, or simultaneously, this extract undergoes the next steps in developing the proposed medicine, where chemical markers, or rather, pharmacological active markers, must be detected, characterized and quantified in the herbal drug derivative – a tincture, for instance.

3. Characterization of potential markers for an *Eleutherine* species using TLC, LC-DAD e NMR

The following case illustrates the characterisation of pro-oxidizing components found in an *Eleutherine* species (Iridaceae) that presents an excellent anti-protozoan activity in its extract. The substances belong to a class of metabolites which is easily detectable and quantifiable

by hyphenated techniques like LC-DAD. A tincture prepared from this medicinal plant was phytochemically investigated and provided two components which have been characterized by LC-DAD and by NMR. As the substances have this biochemical characteristic, induce oxidative stress, which allows combating microorganisms and protozoa, and can support the use of the plant as antiprotozoal agent, they can be used as markers for derivatives and products derived from this species.

In attention to Brazilian regulation the marketing of herbal drugs for use in the form of tea, the phytochemical approach of an extract obtained from the medicinal plant in question and its chloroform fraction was performed and shows the presence of naphthoquinones.

Metabolic class	Crude extract	Chloroform
Steroids & Triterpenoids	+	+
Azulen	+	+
Reducer Sugars	+	-
Anthraquinones	+	+
Naphthoquinones	+	+

Table 1. Metabolic classes detected by phytochemical analysis.

The lyophilized chloroform fraction prepared from the tincture, was used in a sequence of chromatographic experiments performed using normal phase silica gel column chromatography and preparative Thin Layer Chromatography - TLC, with chloroform and acetone as mobile phase and with an 10% ethanolic solution of KOH for TLC monitoring of the separation and isolation of the naphthoquinones. The characterisation of the substances was achieved by comparison of the ¹H e ¹³C NMR spectra of them with the spectra of substances already isolated from other species of Eleutherine

Still in compliance with the Brazilian standards for herbal drugs (BRASIL, 2010), TLC analyses were conducted aiming to establish the chromatographic profile of the crude extract (EE), Hexan Fraction (HF) and the Chloroform Fraction (CF), and thus contribute to the quality control of drugs and their derivatives. Figure 10, observed under visible light, shows two zones of yellow color at Rfs 0.31 and 0.25, and pink areas at 0.44 pink Rf 0.62, both in EE and CF.

When the chromatogram is observed under ultraviolet light at 254 nm, three absorption zones in EE and CF can be observed with the following retention factor (Rf) 0.25 and 0.31 0.44, respectively. The chromatograms, after treatment with a 10% KOH methanolic solution, which is the spray reagent to detect quinones in the sample, show brownish coloured zones at Rf 0.25, 0.31 and 0.44 when observed under day light, this feature indicates the presence of naphthoquinones (WAGNER; BLAT .2001) in EE and CF.

Naftoquinones are chemical compounds that present biological activities such as bactericide, fungicide, giardicide and amebicide and in recent years have aroused interest in the medicinal chemistry because they can induce oxidative stress, causing apoptosis (cell death) via topoisomerase inhibition (ANAZETTI; MELO, 2007). The isolated naftoquinones had their antioxidant capacity evaluated through the reaction with 2.2-diphenyl-1-picrilhidrazil (DPPH. +) a stable free radical. The reaction was monitored by colour change and the

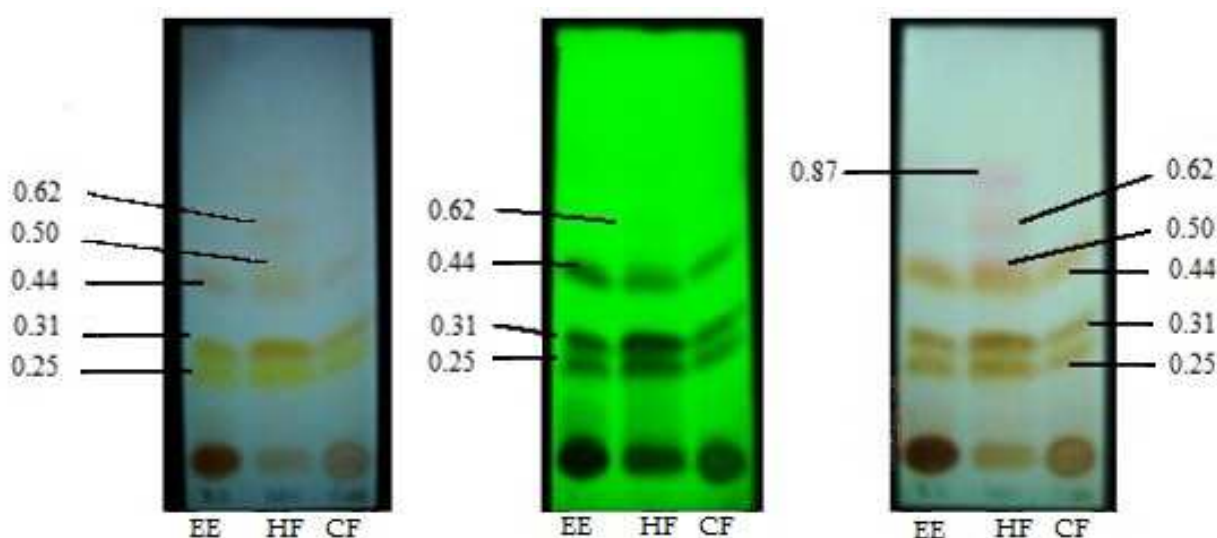


Fig. 10. Chromatograms obtained on normal silica gel using as eluent Chloroform/ Aceton 99:1. From left: observed under visible light; b) ultraviolet light by 254 nm, and c) after treatment with 10% methanolic KOH.

activity was measured by absorbance decrease of the sample measured at 517nm in relation to the corresponding blank (BLOIS, 1958).

In order to verify the suitability of the substances to function as quality markers, the chromatographic profile of chloroform fraction and ethanolic extract was determined by High Performance Liquid Chromatography. To accomplish this task a Merck Hitachi® LaChrom7000 chromatographic system equipped with a diode array detector (DAD) was employed. The analysis were performed on a Agilent LiChrospher100 (250mmx 4.6 mm) column using ultra purified water (A) and acetonitrile (B) as mobile phase, which was pumped at 1.0mL/min., in gradient, starting with 15% of B during 10 minutes, followed by periods of 10 minutes by 30%, 50% and 80%. The detection range was set between 200nm and 400 nm, using the method developed by Paramapojn et al (2008), adapted to our sample conditions.

The chromatogram of EE registered at 250nm presented two peaks (Ep1 and Ep2) of high intensity with R_t =18.93min. and 20.83min., areas of 45854675 and 60180902 and purity of 99.97% and 99.72% (fig. 12).

The chromatogram of the chloroform fraction presents as more intense peaks those with R_t of 19.12min. (Ep2) and 21.18min. (Ep1), areas of 1900571 and 7813739 and purity of 98.29% and 99.75, respectively, (Fig. 13).

Substances Ep1 and Ep2 were analysed by HPLC under the same conditions as EE and CF, and showed peaks at R_t of 18.13min. and 21.71 min., areas of 3641711 and 24727851 and purity around 99.14% and 99.92% respectively. Using the reversed search function where recorded data are compared to others stored in the equipment library, it could be observed that peaks 01 in EE (fig. 12) and CF (fig. 13) present a similarity correlation of 97.40% and 99.89%, respectively, to substance Ep2. The peaks 02 in EE (fig. 12) and CF (fig. 13) showed correlation of 99.84% and 99.98% to substance Ep1.

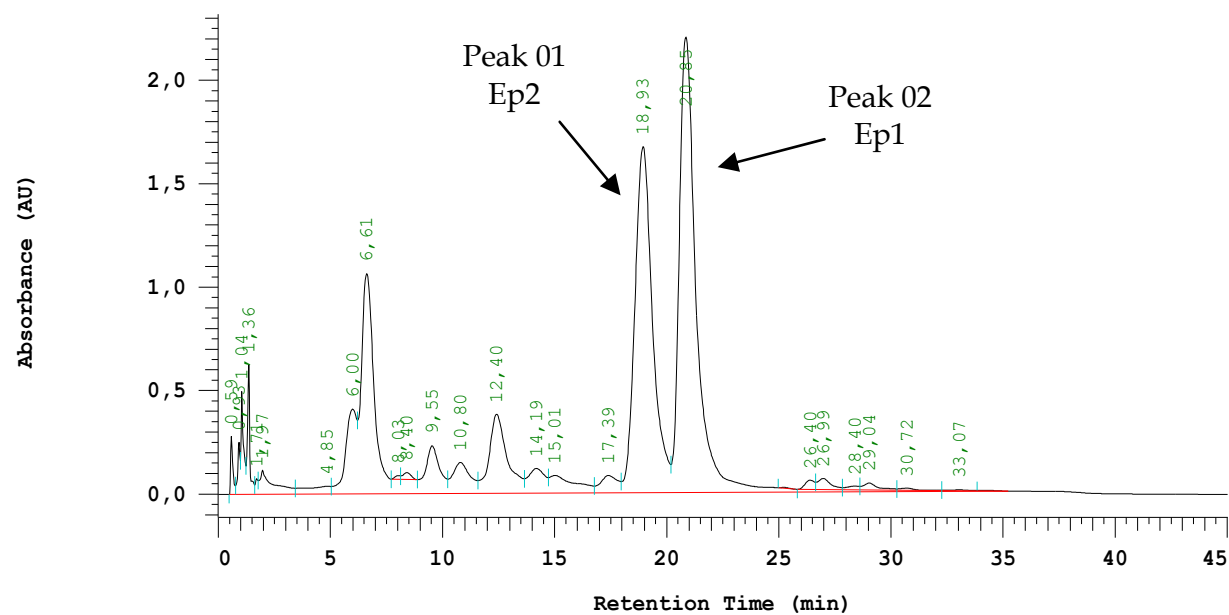


Fig. 12. CLAE - Chromatogram registered at 250nm from EE.

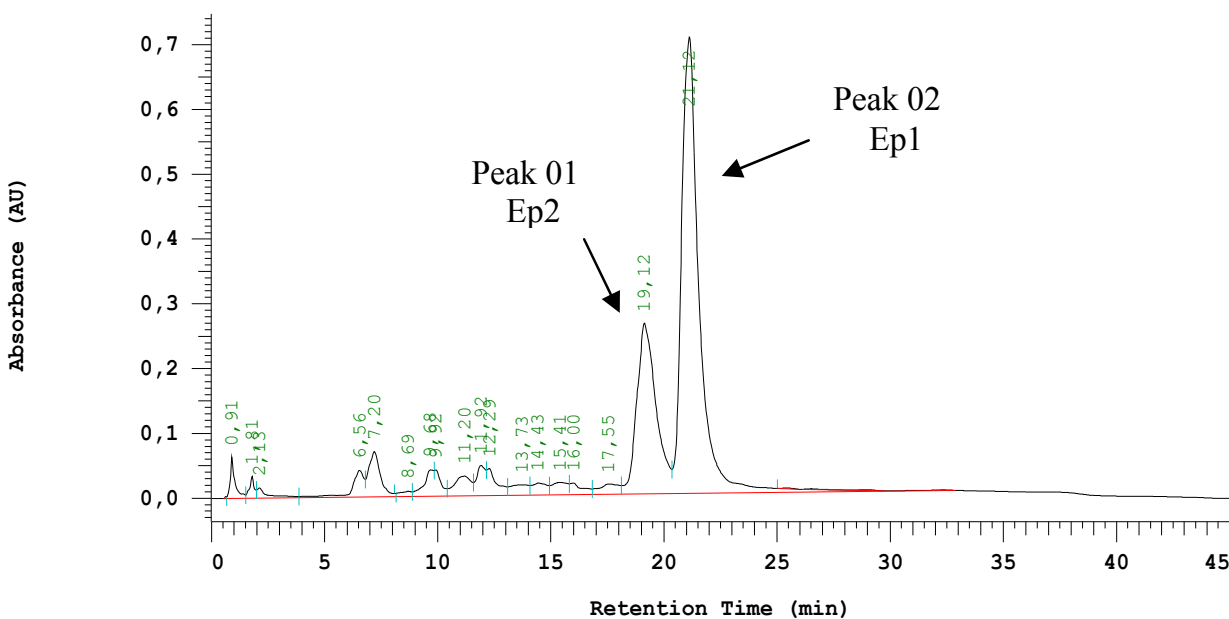


Fig. 13. HPLC - Chromatogram obtained from the Chloroform Fraction, registered at 250nm.

The detection of these substances by LC-DAD is based on diode arrangement and generates a diagram showing retention time, wavelength and absorbance as the three dimensions considered in the picture, which evidence the resolution of the chromatogram in the region where the two naphthoquinones are eluted (Figure 14).

3.1 Characterization of substance Ep1 as isoeleutherol

The Hydrogen Nuclear Magnetic Resonance spectrum obtained from Ep1 shows at $\delta=1.74\text{ppm}$ a duplet, due to a coupling with H-1, corresponding to three equivalents Hydrogen atoms from the Methyl group at C-1 in the furan ring. H-1 ($\delta\ 5.72\text{ppm}$) couples with the

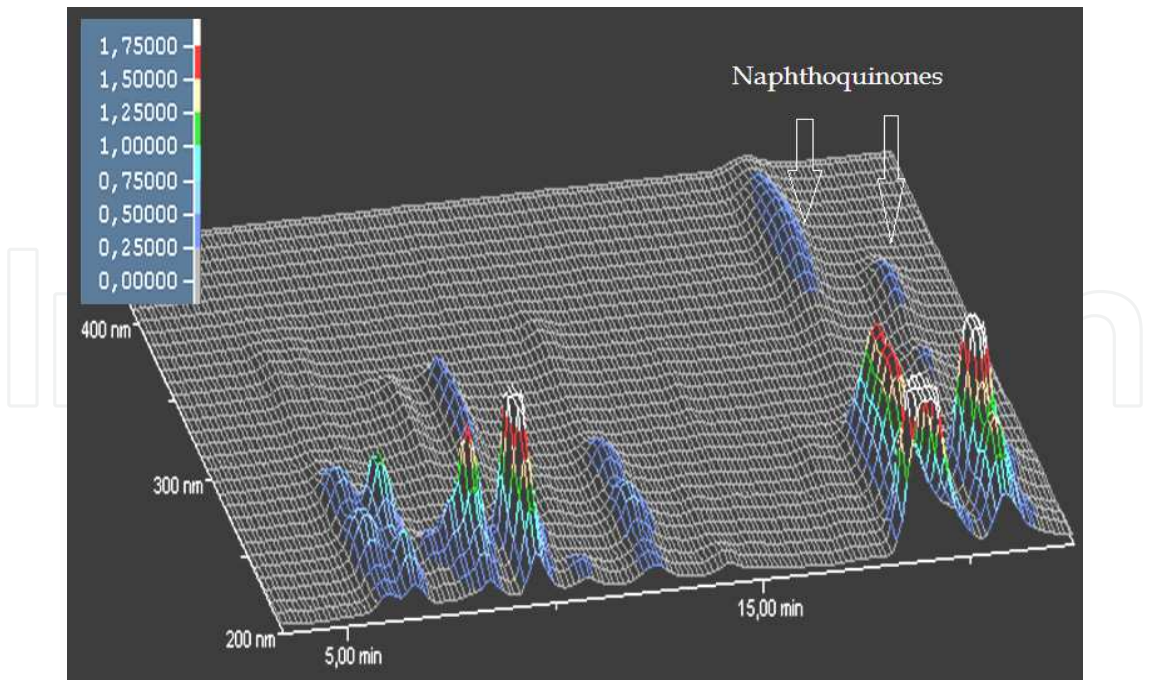


Fig. 14. Chromatographic diagram showing the chemical constituents of EE. In detail the two isolated naphthoquinones and the absorbance scale.

three Methyl Hydrogen atoms and produces a quadruplet. Furthermore four non equivalent aromatic Hydrogen atoms, *e. g.* H-4, H-6, H-7 and H-8 (Fig. 15) can also be observed. H-4 appears as a singlet by $\delta=7.86\text{ppm}$, H-5 ($\delta=6.93\text{ppm}$) couples with H-6 ($\delta=7.40\text{ppm}$) giving rise to a duplet, H-6 besides the coupling with H-5 also couples with H-7 ($\delta=7.54\text{ppm}$) originating a signal like a superposed double duplet or false triplet, H-7 couples with H-6 and produces a duplet. The Hydrogen atoms from the Methoxyl group at C-8 ($\delta=4.11\text{ppm}$) appears as a singlet. Finally a phenolic Hydrogen atom generates a singlet at $\delta=9.64$.

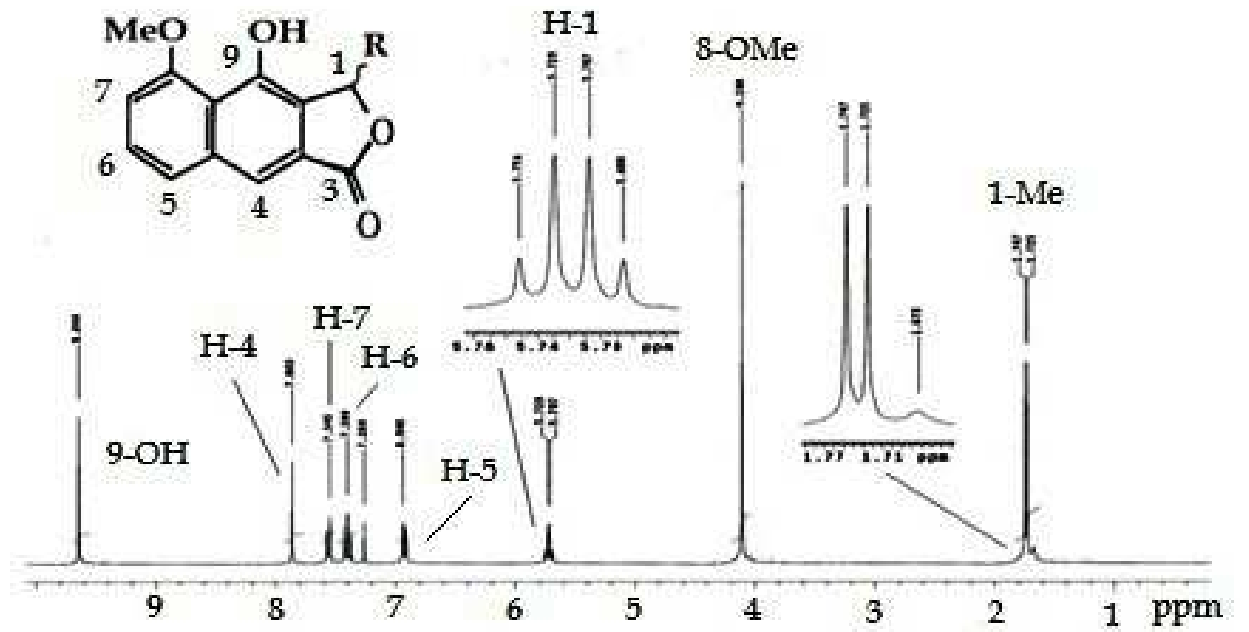


Fig. 15. ¹HNMR spectrum of Isoeleutherol.

The ¹HNMR data registered for Isoeleutherol isolated from *Eleutherine americana* MERR. et HEYNE by Hara et al (1997), agrees with that obtained from the substance here described as Ep1, in consequence we can affirm that Ep1 is Isoeleutherol and this is the first report of the occurrence of this substance in the *E. plicata* Herb

Hydrogen Atom	Ep1 δ(ppm)	Isoeleutherol δ(ppm)	Multiplicity	J (Hz)
1-Me	1.74	1.73	d	6.5
8-OMe	4.11	4.11	s	
H-1	5.72	5.70	dd	6.5
H-5	6.93	6.93	d	7.7
H-6	7.40	7.39	dd	7.7
H-7	7.54	7.54	dd	7.7
H-4	7.86	7.84	s	
9-OH	9.64	9.63	s	

From: Hara et al, 1997.

Table 2. ¹HNMR data Ep1, in comparison to Isoeleutherol.

Because Isoeleutherol can show an adequate stability and seems to be one of the major chemical constituent of the tincture prepared from the bulbs of *Eleutherine plicata* EE, this substance can be used as a marker for the standardization of the herbal drug and its derivatives, probably linked to the antimoebian activity alleged to the plant.

3.2 Structural characterization of Ep2 as isoeleutherine

The substance Ep2 was analysed by ¹HRMN, and the characterization of its structure was made by comparison of the obtained spectral data with that found in the literature. In table 3 it can be seen that the Hydrogen atoms H-6, H-7 and H-8 in the aromatic ring, appear at δ=7.73, δ=7.64 and δ=7.27 respectively, with identical coupling constant J_{6H-8H}= 6.7. The signal attributed to 6-H appears in the form of a duplet coupling with H-7, which in turn appears in the form of triplet due to the coupling with 6-H and h-8. The Hydrogen atoms of the Methyl group attached to C-1 and 1-H appear as duplet with δ= 1.33 and δ= 1.54, respectively and the Hydrogen atoms of Methyl group at C-3 appear as a duplet by δ= 1.34.

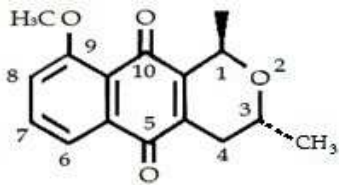


Fig. 16. Chemical structure of isoeleutherine

The bulbs of *Eleutherine plicata* Herb., vernacular “marupazinho”, are widely used in inland areas of the State of Pará, as a tea to treat diarrhoea possibly caused by *Giardia* and/or *Entamoeba*. The isolation of isoeleutherine from the Chloroform fraction of the tincture, suggests that the antiamoebian and anti giardian activity alleged by the population may be due to this chemical constituent, also found in the aqueous extract. Besides isoeleutherol,

Hydrogen Atom	Ep2 δ (ppm)	Isoeleutherine δ (ppm)	Multiplicity	J (Hz)
3-ME	1.33	1.34	d	6.1
1-ME	1.53	1.53	d	6.7
4- β H	2.23	2.23	dd	11.0-19.0
4- α H	2.69	2.68	dd	3.5-19.0
3-H	3.95	3.96	m	- - -
9-OME	4.0	4.0	s	- - -
1-H	5.0	5.01	q	6.7
8-H	7.27	7.27	d	6.7
7-H	7.64	7.64	t	6.7
6-H	7.73	7.74	d	6.7

Based on Hara et al, 1997.

Table 3. ¹HNMR data from Ep2 in comparison to isoeleutherine.

already known to inhibit HIV replication in H9 lymphocytes, these chemical constituents can be used as chemical markers for the quality control of the herbal drug and its derivatives.

4. Characterization of Flavonoid glycosides as potential markers for a Vitaceae using LC-MS

Another plant species widely recognized in Brazil, the *Cissus* species (Vitaceae) used in treating stroke sequels and to control diabetes, had its tincture analysed by LC-MS and is under evaluation for developing a phytomedicine. The tincture was obtained from dried leaves according to the methods described in the Brazilian Pharmacopoeia 5th edition (200g dried plant material macerated in 1 L ethanol 98°GL) (FARMACOPEIA BRASILEIRA, 2010). The analyses were performed in a HPLC system equipped with a diode-array detector. The compounds were separated on a RP-18 column using a mobile phase in linear gradient prepared with Acetonitrile, Methanol and Water (Table 4) containing HCOOH (pH 3.2); the flow rate was 0.8 mL min⁻¹.

Time (min)	H2O+ %	MeOH%	CH ₃ CN%	Flow (mL/min.)
0.1	100	0	0	0.8
5	85	0	15	0.8
25	75	0	25	0.8
40	75	0	25	0.8
42	0	100	0	0.8
47	0	100	0	0.8

Table 4. Mobile phase composition and flow

The tincture of *C. verticillata* was filtered on a PTFE membrane filter before analysis. The UV-Vis spectra were recorded at the range of 190-450nm, and chromatograms were acquired at 230, 254, 280, 330 and 350 nm. Typical chromatograms at 350 nm of *Cissus* (I) and Passionflower (II-reference) samples are reported in Figure 17.

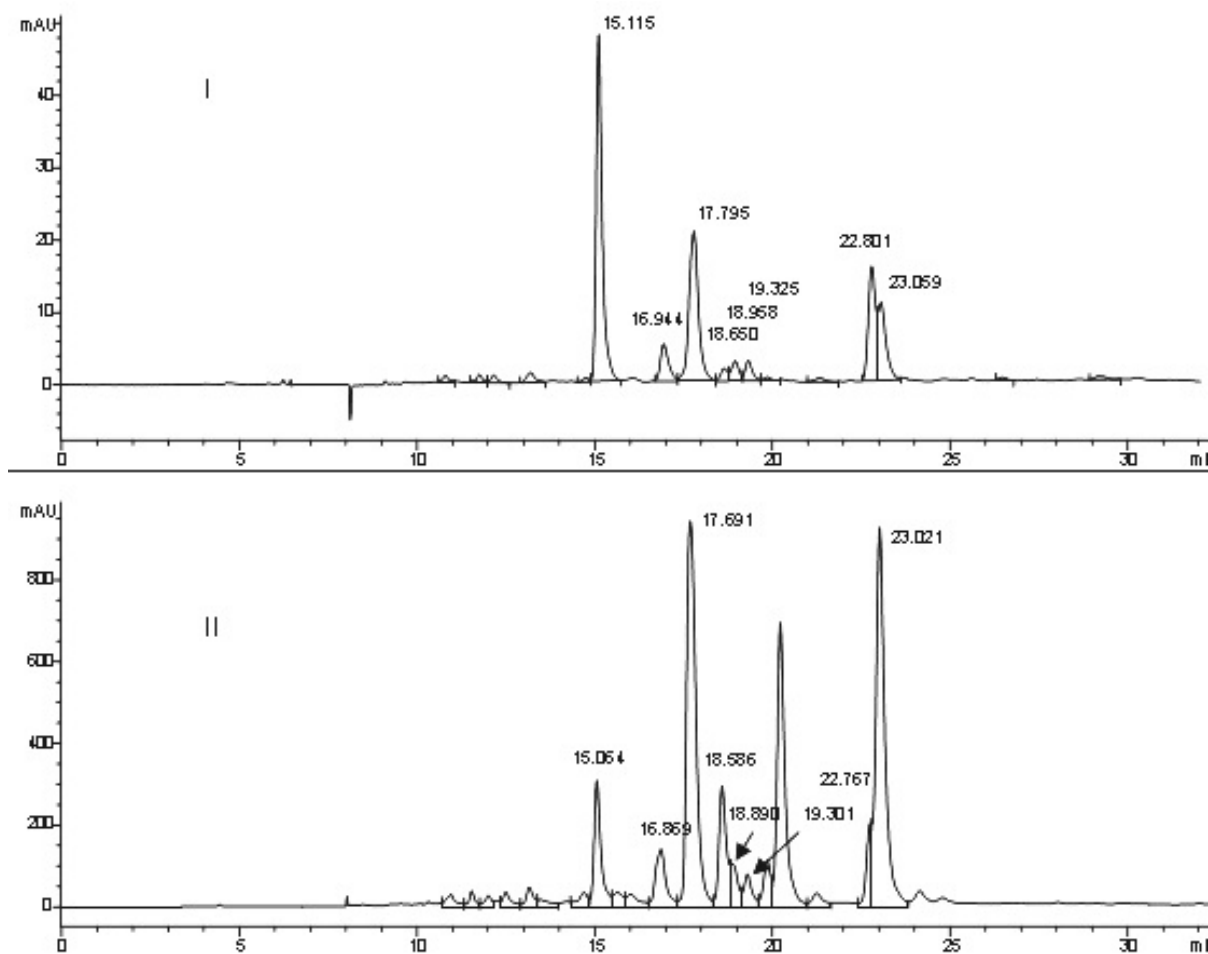


Fig. 17. Chromatograms at 350 nm of *Cissus* (I) and Passionflower (II) (reference) tincture.

The HPLC system described above was interfaced with atmospheric pressure chemical ionization (APCI)-electrospray mass-selective detector (MSD). The interface geometry with orthogonal positioning of the nebulizer relative to the capillary inlet, enabled the use of analytical conditions similar to those used for HPLC-DAD analysis. The conditions used for mass spectrometry (gas temperature 350°C at a flow rate of 10Lmin-1, nebulizer pressure 30psi, quadrupole temperature of 30°C and capillary voltage 3500 V) were optimized to achieve the maximum sensitivity of ESI values. The column, time period, and flow rate used were the same as those described above, without appreciable variation of the chromatographic profile. Full scan spectra from m/z 100 to 800 were obtained in positive-ion mode; the scan time was 1s. The volume of sample solution injected was 10µL.

Identification of all constituents was performed by HPLC-MS analysis and/or by comparison of the retention times of peaks in the extracts with those of the authentic reference samples. Peak purity was checked by examination of the mass spectra and/or by use of HPLC with diode-array detection (UV spectra of the peaks were compared with those of authentic reference samples).

Table 5 shows the comparison of LC-MS data obtained from *Cissus verticillata* tincture to those from *Passiflora incarnata* which allows the characterization of the compounds listed therein (BILIA et al, 2002). Peaks A, B, C, D, G and H show very similar retention time in both chromatograms (Figure 17).

Cissus verticillata				Passiflora incarnata		
	[M+H] ⁺	Rt	Substance		Rt	Substance
A	595	15.11	Vicenin-2 (apigenin 6,8-di-C-glucoside)	A'	15.06	Apigenin 6,8 di-C-glycoside (probably vicenin-2)
B	565	16.94	Isoschaftoside (apigenin 6-C-arabino-8-C glucoside)	B'	16.87	Isoschaftoside
C	565	17.79	Schaftoside (apigenin 6-C-glucosyl-8-C arabinoside)	C'	17.69	Schaftoside
D	-	18.65	Homoorientin (luteolin 8-C-glucoside)	D'	18.64	Homoorientin
E	565	18.96	structural isomer of isoschaftoside	E'	18.97	Not reported
F	565	19.32	structural isomer of schaftoside	F'	19.30	Not reported
G	433	22.80	Vitexin (apigenin 8-C-glucoside)	G'	22.72	Vitexin
H	433	23.06	Isovitexin (apigenin 6-C-glucoside)	H'	23.02	Isovitexin

Table 5. LC-MS data of *C. verticillata* compared to *P. Incarnata*

Comparing mass spectra of these substances to those obtained from *P. incarnata* and using the information available in the literature the flavonosides of *C. verticillata* could be characterized. The peaks A and A' presented analogous mass spectra exhibiting [M+H]⁺ and [M+Na]⁺ ions at m/z 595 and 617. These data suggest the presence of an apigenin nucleus bounded to two glucose moieties, and thus it could be characterized as vicenin-2. Peaks B/B' and C/C' exhibited [M+H]⁺ and [M+Na]⁺ ions at m/z 565 and 587. These data suggest the presence of an apigenin nucleus plus a pentose and glucose. However, the absence for both peaks spectra of the fragment ions [M+H-162]⁺, [M+H-132]⁺ or [M+H-(162+132)]⁺ at m/z 403, 433 and 271 (corresponding to the aglycone apigenin) suggest that peaks B, B', C and C' are 6,8-C-glycosides of apigenin. These suggestions were confirmed by the typical fragment ions [M+ H-H₂O]⁺ and [M+H-2H₂O]⁺ that were evidenced at m/z 547 and 529.

Another characteristic fragment ions [M+H- 2H₂O-CH₂O]⁺, [M+H-5H₂O-CH₂O]⁺ and [M+H- 6H₂O-CH₂O]⁺ were also evidenced at m/z 499, 445 and 427. From these data, peak B was identified as 6-C-arabinosyl-8-C-glucosylapigenin (isoschaftoside) and the peak C was identified as 6-C-glucosyl-8-C-arabinosylapigenin (schaftoside). Peaks D (minor constituent) and D' were identified as homoorientin, by comparison of chromatographic and spectroscopic data with authentic samples.

Peaks E (minor constituent) and F (minor constituent) exhibited [M+H]⁺ and [M+Na]⁺ ions at m/z 565 and 587. These data suggested the presence of an apigenin nucleus plus a pentose and glucose. However, the absence for both peaks spectra of the fragment ions [M+H-162]⁺, [M+H-132]⁺ or [M+H-(162+132)]⁺ at m/z 403, 433 and 271 (corresponding to the aglycone apigenin) suggested that peaks E and E' were 6,8-C-glycosides of apigenin. These suggestions were confirmed by the typical fragment ions [M+ H-H₂O]⁺ and [M+H-2H₂O]⁺ that were evidenced at m/z 547 and 529.

The fragment ions $[M+H-2H_2O-CH_2O]^+$ and $[M+H-5H_2O-CH_2O]^+$ were also evidenced at m/z 499 and 445. No differentiation was found between mass spectra of isoschaftoside and compound E and between mass spectra of schaftoside and compound F. From these data, the minor constituents, peaks E and F, were identified as apigenin-6,8-di-C-glycoside (probably structural isomers of isoschaftoside and schaftoside respectively).

The peaks at 23,06min and 22,80 min in the LC-DAD chromatograms of *Cissus*, suggest the presence of isomers vitexin and isovitexin when compared with the respective standards, therefore the mass spectra shows the fragmentation pattern. These mass spectra show important fragmentations that help to differentiate the position of the pyranose unit in the ring of flavone apigenin characterizing the detection of vitexin and isovitexin in the analyzed samples.

The signal registered at 22.8 min in the *Cissus* chromatogram was characterized by comparison to that observed for commercial *Passiflora incarnata*. The mass spectra showed the same fragmentation pattern. Base peak coincided with molecular ion (433 $[M+H]^+$), the spectra also showed fragmentation of the pyranose ($[M+H-120]^+$) and the loss of two molecules of water ($[M+H-18]^+$, $[M+H-36]^+$), characterizing the structure of the vitexin. Similarly, the signal at 23,06 min, corresponds to a mass spectrum which shows the loss of 120 amu ($[M+H-120]^+$), that can be attributed to the fragmentation of part of the glucose molecule; the spectrum also showed three typical peaks of losses of three molecules of water from the glucose unit ($[M+H-18]^+$, $[M+H-36]^+$ e $[M+H-54]^+$, indicating to be due to isovitexin.

Peaks G and G' exhibit $[M+H]^+$ and $[M+Na]^+$ ions at m/z 433 and 455. Peak G and H were identified as vitexin and isovitexin respectively also by comparing their chromatographic and spectroscopic data with that provided by authentic samples.

On basis of these results it can be stated that the ethanol tincture at 98°GL of *Cissus verticillata* (L) Nicolson & C. E. Jarvis) contains at least eight flavonoid glycosides which can be used as markers to monitor the quality of plant material, its derivatives and products. The detection and characterization of these substances in pharmacopoeial preparations, using LC-DAD and LC-MS, can be very useful in developing phytomedicines, once the isolation and characterization of these substances by chromatographic and spectrometric usual methods are expensive and demand more time.

5. Characterization of a metabolic class as potential marker by UV-spectroscopy: Validation of a new quantification method according to Brazilian regulation

The standardization of plant extracts, which contain chemical substances without the necessary chemical characteristics to be used as quality markers, can be accomplished using chemical groups, or metabolic classes, found in the intermediary as active pharmaceutical constituent. This is the case, for example, of an Apocynaceae that composes a phytomedicine widely used in Brazil and has as its active principle, alkaloids. The complexity of the alkaloid fraction present in the intermediate and the instability of the substances when isolated indicated that the development of a method based on the alkaloid fraction, using ultraviolet spectroscopy to standardize this extract would be the best solution.

A species from the genus *Himatanthus*, popularly known as "agoniada", has its bark commonly used in the form of decoction to produce a phytomedicine indicated to treat uterine congestion; irregular, difficult and painful periods and uterine cramps, besides other associated symptoms (CRUZ, 1985).

The aqueous extract prepared with the barks of the plant, after concentration and drying, was treated with 1% aqueous HCl and filtered, then the solution was made alkaline with concentrated ammonium hydroxide to pH 10. The alkalinised solution was partitioned with chloroform. The chloroform fraction filtered through anhydrous sodium sulphate and concentrated without heating under reduced pressure, was used to prepare a methanol solution at 30 mg/mL which was used to quantify the total alkaloids fraction.

The observation of an absorption maximum at 281nm by the UV analysis of the alkaloid fraction allowed to choose Yohimbine as external standard for the quantification process, because of its absorption maxima at 281nm (OLIVEIRA, 1994). In addition the substance is an indole alkaloid, which is also present in Apocynaceae species (SOUZA, 2008), and is able to be acquired in the market as chemical reference substance (CRS). This substance was also utilized to produce a series of dilutions in methanol, which could be utilized to determine linearity of the method and to compose the calibration curve.

5.1 Brazilian guidelines to define validation procedures

Despite of the technique used to characterize markers, and the fact that they are isolated or in the form of a chemical group - metabolic class, the method developed for their quantification must be validated in order to ensure that this analytical method generates reliable information about the analysed sample (RIBANI, et al. 2004). Analytical method validation is the confirmation by examination and provision of objective evidence that the specific requirements for an intended specific use are met (NBR ISO/IEC/17025, 2001). Validation is intended to demonstrate that the method is appropriate for qualitative, semi-quantitative and/or quantitative determination of drugs and other substances in pharmaceuticals (BRASIL, 2003).

All regulatory agencies in Brazil and in other countries require the validation of the analytical methodology for the registration of new products (RIBANI, et al. 2004). In Brazil, there are two certification agencies, ANVISA (National Agency for Sanitary Surveillance) and INMETRO (National Institute of Metrology, Standardization and Industrial Quality) which verify the competence of analytical laboratories. These bureau provide guidelines to define the validation procedures of new analytical methods, respectively, the resolution No. 899, from 5/29/2003 (BRASIL, 2003) and the document INMETRO DOQ-CGCRE-008, June 2007, which aims at guiding the validation of analytical methods.

Here we adopted the criteria described by the resolution No. 899 of ANVISA, as the basis for the development of a quality control method for the aqueous extract prepared from *Himatanthus* sp. According to this resolution there are four set of tests for validation of a new method, as follows:

1. Quantitative assays for the determination of an active ingredient in pharmaceuticals or raw materials;
2. Quantitative tests or limit test for the determination of impurities and degradation products in pharmaceuticals and raw materials;

3. Performance tests (e.g. dissolution, active substance release);
4. Identification tests

A method that aims at the quantification of a marker in a phytomedicine fits category I and can be considered validated when the following parameters: selectivity/specificity, linearity, interval, precision, limit of detection (sensitivity), accuracy, robustness are determined and verified.

5.1.1 Selectivity/specificity

A segment of the UV spectrum obtained from the alkaloid fraction of *Himatanthus* sp aqueous extract (30 $\mu\text{g/mL}$), the Yohimbine hydrochloride (22 $\mu\text{g/mL}$) and methanol solvent in the range between 200nm and 400nm is shown in Figure 18 and discloses the absorption maximum at 281nm just to Yohimbine and the alkaloid fraction. Herewith, it could be confirmed that at this wavelength it is selectively possible to quantify the reference substance and the alkaloid fraction even in the presence of other components.

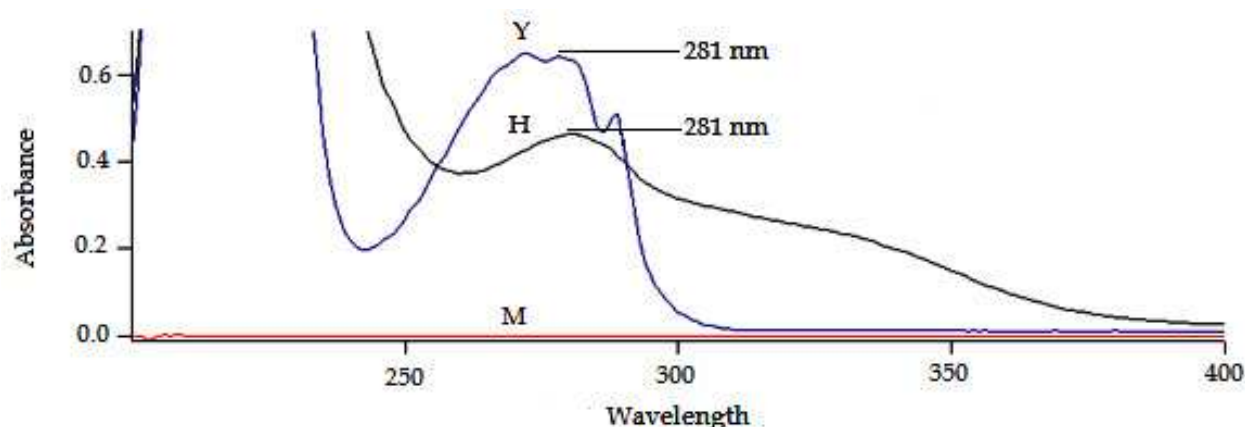


Fig. 18. Segment of UV spectra of Yohimbine (Y) and the alkaloid fraction of the aqueous extract of *Himatanthus* sp. (H), in comparison to the methanol solvent (M) showing the selectivity of the developed method.

5.1.2 Linearity

The spectrophotometric method presented linearity at 281nm for the concentrations tested. The linearity of the method, the coefficient of determination (R^2) and correlation (r) to Yohimbine are expressed in Figure 19.

The value of the correlation coefficient itself is not sufficient to ensure the adequacy of the linear adjustment to the calibration curve, because calibration models with high residue in the analytic signal, or unevenly distributed points along the calibration range can nevertheless offer a good correlation coefficient (RIBEIRO, et al., 2008). For that reason the analysis of residue of the data used for the determination of the method linearity was performed and could demonstrate a uniform data distribution, constant variance (homocedasticity), average zero and absence of atypical samples (Figure 20) observed in homogeneous distribution of points along the axis of the chart indicating that the curve is well adjusted.

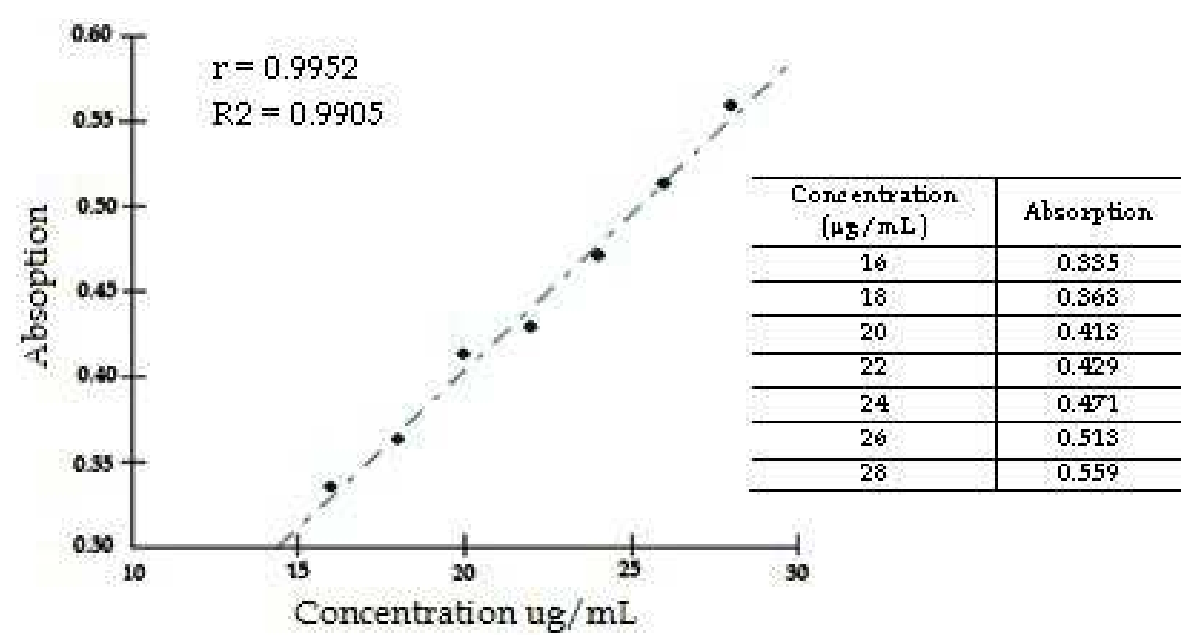


Fig. 19. Graphical representation of the linearity method for the series dilution of Yohimbine in methanol at 281nm, in detail, the used concentrations and their absorbance.

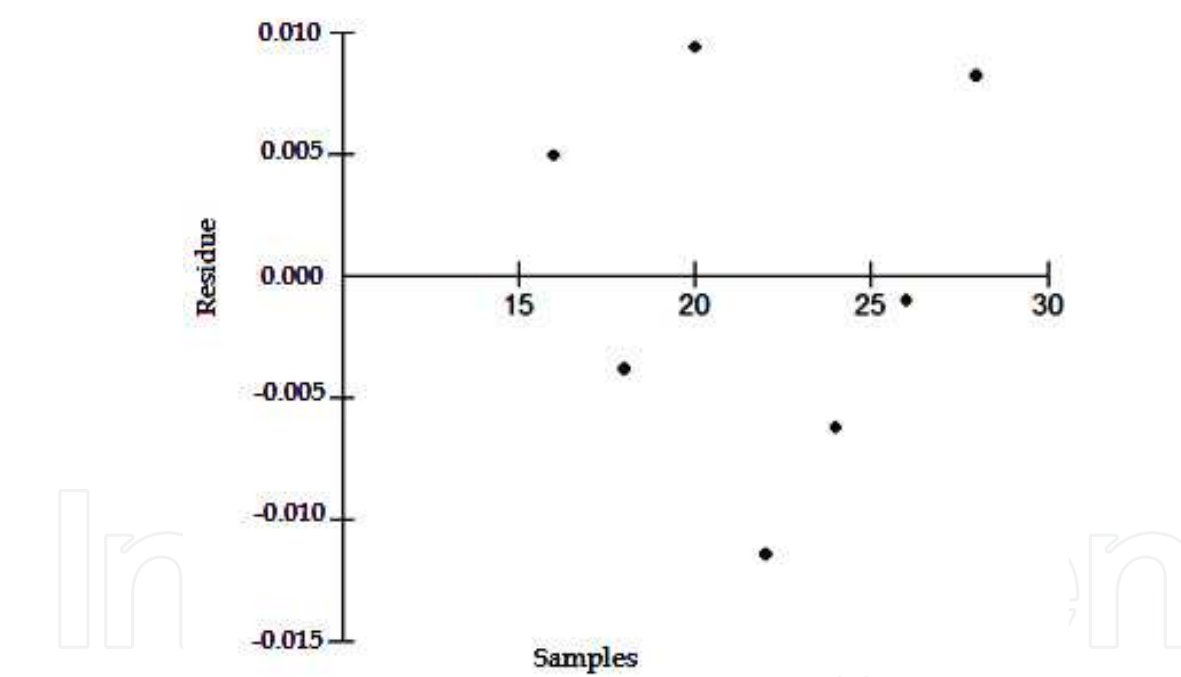


Fig. 20. Graphical representation of the residues of method's linearity data using the dilution series of Yohimbine.

5.1.3 Interval

Interval means the range between the maximal and minimal limits of quantification of an analytical method. Typically, it is derived from the study of linearity and depends on the intended application of the method (BRASIL, 2003). Thus, the range employed for the standardization of the aqueous extract of *Himatanthus sp* (16 to 28 µg/mL) was established in accordance with the average of absorptions found for Yohimbine (range of 80% to 120%).

5.1.4 Calibration curve

The analytical curve, with the respective line equation and Pearson correlation coefficient (r) for Yohimbine is demonstrated in Figure 21.

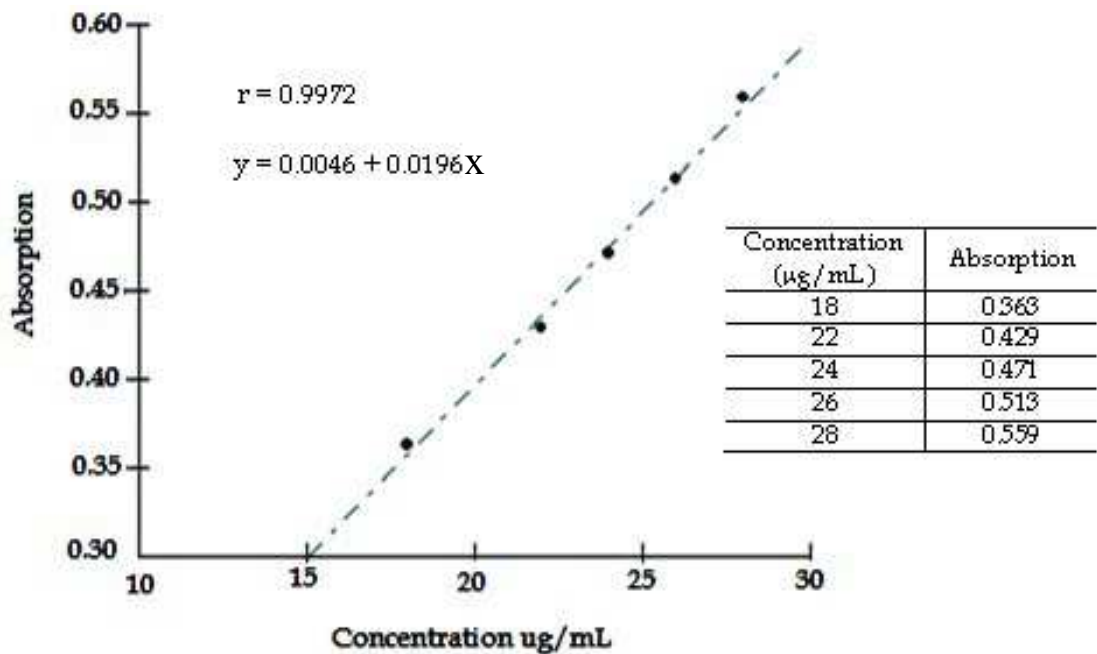


Fig. 21. Graphical representation of the calibration curve obtained using the dilution series with Yohimbine.

5.1.5 Precision and accuracy

Data of repeatability (intra-run), intermediate precision (inter-run) and accuracy are demonstrated in Table 6 where it can be seen that the relative standard deviation values found vary from 0.19% to 2.09% and that the accuracy values found oscillate between 98% and 102.90%. ANVISA establishes that the precision variation may not exceed 5% and that the accuracy should not be less than 95% (BRASIL, 2003). These data confirm that the proposed method for the quantification of total alkaloids of aqueous extract of *Himatanthus* sp. by UV spectrophotometry is in accordance with current legislation and provides reliable results.

5.1.6 Detection limit

It is the lowest amount of analyte present in a sample which can be detected, but not necessarily quantified, under the established experimental conditions. In the case of instrumental methods the estimation of the detection limit can be determined by the equation:

$$DL = \frac{SDa \times 3}{IC}$$

Where: SDa is the standard deviation of the y-intercept of at least 3 calibration curves built containing concentrations of the drug near the supposed limits of quantification and IC is the inclination of the calibration curve. The value of the detection limit estimated by the equation for the here proposed method is 4.59 µg/mL.

$$DL=\frac{0.03 \times 3}{0.0196} = 4.59 \mu\text{g} / \text{mL}$$

Test	Calculated Concentration. (µg/ml)	N	C (µg/ml)	DP	DPR (%)	Accuracy (%)
Repeatability	16	3	15.698	0.030	0.19	98.00
	20	3	19.864	0.181	0.90	99.32
	28	3	27.724	0.392	1.41	99.01
Intermediate precision Day 1	16	3	16.448	0.205	1.25	102.80
	20	3	20.580	0.257	1.25	102.90
	28	3	28.373	0.245	0.86	101.33
Intermediate precision Day 2	16	3	16.213	0.339	2.09	101.33
	20	3	20.345	0.054	0.27	101.72
	28	3	28.391	0.271	0.96	101.39

(C) average concentration of (N) determinations; (SD) standard deviation; (RSD %) Relative Standard Deviation.

Table 6. Results of repeatability, intermediate precision and accuracy of the developed UV-Spectrophotometric method to determine the total alkaloids fraction contained in the aqueous extract of *Himatanthus* sp.

5.1.7 Quantification limit

It is the lowest amount of analyte in a sample, which can be determined with acceptable accuracy and precision under the established experimental conditions and can be expressed by the equation:

$$QL=\frac{SDa \times 10}{IC}$$

Where: SDa is the standard deviation of the y-intercept of at least 3 calibration curves built containing concentrations of the drug near the supposed limits of quantification and IC is the inclination of the calibration curve. The quantification limit of the reported method is 15.31µg/mL.

5.1.8 Robustness

The robustness of the proposed method was evaluated by changing the supplier of the solvent used to prepare the sample (Table 7). Three determinations were made in low (16 µg/mL), middle (20 µg/mL) and high (28 µg/mL) concentrations, with three replicates each. The absorption maxima at 281nm obtained for these Yohimbine solutions prepared with solvents from two different suppliers were compared and no variation in the obtained spectra could be observed. The data are shown in table 7 and were submitted to a statistical variance analysis, which provided a p value of 0.5694, evidencing no significant statistical difference, because p > 0.05, thus demonstrating a robust method.

Solvent supplier	Calculated Concentration (µg/mL)	N	Determined Concentration (µg/ml)	Absorption
1	16	3	16.448	0.293
	20	3	20.580	0.373
	28	3	28.373	0.523
2	16	3	16.217	0.261
	20	3	19.651	0.315
	28	3	28.141	0.449

(C) average concentration of (N) determinations

Table 7. Results of the evaluation of robustness of the developed UV-Spectrophotometric method for the determination of total alkaloids contained in the aqueous extract of *Himatanthus* sp.

5.2 Quantification of total alkaloids fraction present in the aqueous extract of a *Himatanthus* sp.

The analysis of a methanolic solution at 30µg/mL of an alkaloid fraction obtained from the aqueous extract of a *Himatanthus* species barks; performed in triplicate, in a spectrophotometer, at 281nm, provided an average absorption of 0.462. Using the equation deduced from the calibration curve ($y = 0.0046 + 0.0196x$), it is possible to calculate the amount of total alkaloids present in the sample; the results are condensed in Table 8.

Material	Herbal Drug	Dry aq. extract	Alkaloid Fraction	Total Alkaloid
Weight	25000mg	9549mg	26.74mg	20.80mg
I	100%		0.0832% (832ppm)
II		100%		0.218%
III			100%	77.79%

Table 8. Amount of total alkaloids on the powdered bark of *Himatanthus* sp employed for the preparation of dried herbal drug (I), in relation to dried aqueous extract (II) and in relation to total alkaloid fraction obtained (III).

The proposed quantification method could be validated, since it shows selectivity at 281nm for the alkaloid fraction from the *Himatanthus* species aqueous extract, giving reliability to the quantification of total alkaloids fraction in the plant material. In addition it is a robust method, according to the parameters established by the legislation in use in Brazil. The correlation between absorbance and concentration, according to the equation obtained, is linear, at a given wavelength; the quantification method is also exact and precise, as well as accessible and easy to be applied.

In Brazil, the National Health Surveillance Agency - ANVISA - responsible for regulating the use of medicinal plants and their derivatives, protects and promotes the health of the population, ensuring the safety of health products and services. This control seeks to deconstruct the idea that herbal medicines are products of less quality or without toxic potential risk, because it evaluates various parameters as quality, safety and efficacy, demanding requirements similar to those required for synthetic medicines. The Collegiate

Direction Resolution (RDC) No. 14/2010 from ANVISA regulates the registration of phytomedicines in Brazil and defines how to achieve standardization of intermediates used in the galenic development of herbaceous medicines. The developed methods, in this process, shall be validated according to the rules described in the Special Resolution (RE) No. 899/2003, also from ANVISA, which indicates as validation parameters for herbal extracts: selectivity of the method to detect the marker, calibration curve and linearity by the quantification of the marker, precision by the calculation of the content of the marker, limits of detection and quantification of the marker in the sample, and robustness of the method regarding modifications in its routine.

6. Quantification of isolated substances using HPLC

The method for the quantification of marker compounds isolated from active pharmaceutical ingredients (APhI) that will give rise to phytomedicines must have the same validation criteria presented in section 5.1. These substances should be ideally associated to the alleged activity of the medicinal plant although for the registration of the product, according to the Brazilian legislation, this fact is not decisive.

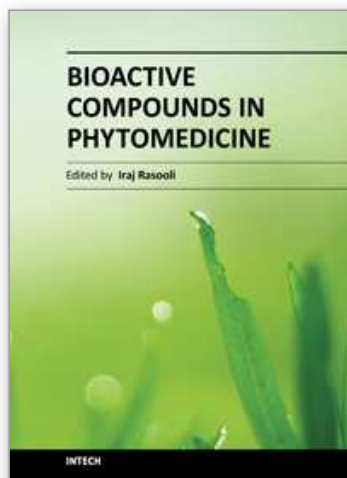
The presence of the marker shall be characterized in the matrix (extract from which the phytomedicine will be developed) as demonstrated in section 3, by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) data. The structure of the substance must be elucidated or characterized by spectroscopic techniques such as UV, infrared and nuclear magnetic resonance (NMR) as in 3.1 and 3.2 and by mass spectrometry as described in section 4, where the technique was applied hyphenated to HPLC. Once the structural characteristics of the candidate substance to be used as a marker are described and the analytical conditions by HPLC defined, a calibration curve using this technique and a dilution series containing the potential marker can be drawn, by correlating the peak area of the substance registered on the chromatogram, to the concentration of the solution employed to register it. Once the area of the substance in the chromatogram of the APhI is registered, this value shall be applied to the straight line equation defined by the calibration curve (as in section 5.1.4).

The validation of a quantification method based on isolated markers demands the same criteria previously described and exemplified in section 5.1. HPLC methods for the standardization of herbal extracts used to develop phytomedicine produces more accurate results, since the most common source of error, in this technique, is the preparation of samples and components of the eluent, steps prepared by human operators. These possible deviations can be minimized by obtaining data in replicate.

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Bioactive Compounds in Phytomedicine

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There are significant concerns regarding the potential side effects from the chronic use of conventional drugs such as corticosteroids, especially in children. Herbal therapy is less expensive, more readily available, and increasingly becoming common practice all over the world. Such practices have both their benefits and risks. However, herbal self-therapy might have serious health consequences due to incorrect self-diagnosis, inappropriate choice of herbal remedy or adulterated herbal product. In addition, absence of clinical trials and other traditional safety mechanisms before medicines are introduced to the wider market results in questionable safe dosage ranges which may produce adverse and unexpected outcomes. Therefore, the use of herbal remedies requires sufficient knowledge about the efficacy, safety and proper use of such products. Hence, it is necessary to have baseline data regarding the use of herbal remedies and to educate future health professionals about various aspects of herbal remedies.

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