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Pharmacognostical Comparison of Three Species of *Himatanthus*

[1,4]José Luiz Pinto Ferreira, [1]Ana Cláudia Fernandes Amaral, [1]Renata Bastos de Araújo,
[2]Júnior Ribeiro Carvalho, [3]Carolyn Elinore Barnes Proença,
[1]Sandra Aparecida Padilha Magalhães Fraga and [2]Jefferson Rocha de Andrade Silva
[1]Laboratório de Plantas Medicinais e Derivados, Depto de Produtos Naturais,
Farmanguinhos-FIOCRUZ-Rua Sizenando Nabuco, 100-Manguinhos/RJ, 21041-250, Brasil
[2]Laboratório de Cromatografia, Departamento de Química,
Universidade Federal do Amazonas/Campus Universitário,
Mini-Campus/Estrada do Contorno, 3000-Japiim, Manaus/AM, 69077-000, Brasil
[3]Departamento de Botânica, Universidade de Brasília, Brasília/DF, 70919-970, Caixa Postal 4457, Brasil
[4]Laboratório de Farmacognosia, Departamento de Farmácia e Administração Farmacêutica, Faculdade de Farmácia-UFF, Rua Mário Viana, 523, Santa Rosa, Niterói/RJ, 24241-000, Brasil

Abstract: The species *Himatanthus sucuuba* (Spruce ex Müll. Arg.) Woodson shows a wide range of use in folk medicine and other Amazonian species of this genus*: H. bracteatus* (A.DC.) Woodson and *H. stenophyllus* Plumel are also used by the great similarity between them. This study describes the macroscopic and microscopic morphological variation of leaves and stem bark of these species collected in the Amazonas state (Brazil). The contour of the leaf lamina, apex and the petiole aspects and the venation pattern were important features. The barks of *H. bracteatus*, differently from the other two species, did not present prismatic calcium oxalate crystals. Additionally, the extracts of the leaves, barks and latex of these species and the iridoids, plumieride (major in aqueous extracts of the leaves and latex from *H. bracteatus* and *H. sucuuba*) and isoplumieride (minor in all samples), were analyzed by HPTLC. The chromatographic profiles and the morphological analyses provided data for differentiation among the species.

Key words: Apocynaceae, *Himatanthus*, iridoids, morphological analysis, HPTLC

INTRODUCTION

Himatanthus is a small Apocynaceae genus composed of 14 species (Plumel, 1991) with alternate leaves congested at the tips of the terminal branches. Its flowers are in terminal dichasia that are subtended by two large, usually white deciduous floral bracts. There are colleters in the leaf axils and at the base of the floral bracts. The hypocrateriform corolla has a convolute, sinistrorse estivation and the stamens are adnate to the base of the corolla tube (Spina, 2004). The anthers are completely fertile and free from the stigma. The gynoecium has two carpels that are free at the base and united at the apex and a semi-inferior ovary without a nectariferous disk. The cylindrical stigma has two roundish appendicles. The *Himatanthus* flowers are visited by several *Lepidoptera* species and the winged seeds are wind dispersed (Ribeiro *et al*., 1999). The importance of the genus *Himatanthus* in popular medicine is supported by reports from various different local communities in Brazil where preparations of the bark and latex are used to treat mainly tumours, inflammations and ulcer (Silva *et al*., 1998; Amaral *et al*., 2007), while the leaves are used to treat hypertension (Coelho and Azevedo, 2000). As to the chemical composition of the three species studied in this study, *H. sucuuba*, *H. bracteatus* and *H. stenophyllus*, only the first one was investigated. Terpenes (Silva *et al*., 1998; Miranda *et al*., 2000), depsides (Endo *et al*., 1994) and iridoids (Perdue and Blomster, 1978; Silva *et al*., 1998; Wood *et al*., 2001) were already isolated and a recent work describes the quantification of iridoids in the bark and latex of *H. sucuuba* (Silva *et al*., 2007). The biological activity of some of these substances has been supported by literature, which validates certain folk use attributed to the genus species (Miranda *et al*., 2000; Wood *et al*., 2001; Deharo *et al*., 2001; Huaylas *et al*., 2002; Castillo *et al*., 2007). The objective of this study was to compare macroscopic and microscopic morphological variation of leaves and secondary stem bark of three Amazonian

Corresponding Author: Jefferson Rocha de Andrade Silva, Laboratório de Plantas Medicinais e Derivados, Depto de Produtos Naturais, Farmanguinhos-FIOCRUZ- Rua Sizenando Nabuco, 100-Manguinhos/RJ, 21041-250, Brasil Tel/Fax: 55 + 92 36474031

species of *Himatanthus*: *H. sucuuba* (Spruce ex Müll. Arg.) Woodson, *H. bracteatus* (A.DC.) and *H. stenophyllus* Plumel. In addition, the comparative chromatographic profiles of these species were established on the basis of colour reactions in visible and co-chromatography with isolated iridoids.

MATERIALS AND METHODS

Plant material: Botanical specimens were collected (2003) in the Adolpho Ducke Reserve, Amazonas, Brazil, by Silo Soares. Vouchers are deposited in the INPA Herbarium under accession numbers 200263 (*H. bracteatus*), 180485 (*H. stenophyllus*) and 209858 (*H. sucuuba*).

Morphological methods: Fresh specimens used for macroscopic descriptions were studied with a hand lenses or through the naked eye inspection. Anatomical studies were made on histological transverse sections from the lower mid-third portion of the leaf lamina and from the bark pieces. The leaf blades were sectioned with a Ranvier microtome and then diaphanized by the sodium hypochlorite and stained by the double coloration method (Dop and Guatié, 1928), using Iodine Green and Congo Red. Slides were mounted in glycerin, examined and photographed with a scale on an Olympus BX40 microscope equipped with microphotographic system.

Chromatographic analysis: The ground leaves and barks of each *Himatanthus* species were extracted by infusion and decoction, respectively (2:10, plant: H_2O, w/v). After 30 min, the mixtures were filtered and lyophilized. The latex of each species was centrifuged for 5 min (3000 rpm) and the aqueous fraction was lyophilized. From aqueous extract of latex of *H. sucuuba*, the iridoids, plumieride and isoplumieride, could be isolated in pure form by medium-pressure liquid chromatography and HPLC analyses and identified by NMR spectroscopy. The solutions of the extracts and the iridoids (10 and 3 mg mL^{-1} (plumieride) and 1 mg mL^{-1} (isoplumieride), respectively) were prepared using methanol as solvent. Five microliter (5 μL) of the extracts and iridoids were loaded as 4 mm streak on the plate at 4 mm distance between two streaks, using Linomat IV, a Camag semi-automatic spotter.

The analysis were carried out on precoated silica gel 60 F_{254} (Merck) HPTLC plate (0.2 mm of layer thickness and 10 × 10 cm size) using $CHCl_3$:MeOH (8:2) as developing system. Plate was dried and the spots were visualized by spraying with detection reagent (vanillin-H_2SO_4), followed by heating at 110°C.

RESULTS AND DISCUSSION

The leaves from three species of *Himatanthus* from the Ducke Reserve showed the typical morphological characteristics of both this genus and the Apocynaceae´s family, as cited by Barros (1988) and Larrosa and Duarte (2005a). Being confirmed by microscopic analysis, it showed that the mesophyll was dorsiventral (Fig. 1a, c e), the midvein had bicollateral vascular bundles and non-articulated laticifer ducts that sometimes accompanied the vascular bundles. The mesophyll varied from 1-2 layers of palisade parenchyma, with predominance of the 2-layered type (Fig. 1a, c, e). Solereder (1908), Newcombe and Patel (1966) and Fjell (1983) described idioblasts with crystals in the leaves of various species of Apocynaceae and Larrosa and Duarte (2005a) noted their occurrence in *H. sucuuba*, however crystals with a specific, well-defined origin were not observed in the mesophyll and midvein of any of the species of *Himatanthus* being studied. The existence of isolated groups of phloem in the medullar parenchyma, observed by Barros (1988) in *H. lancifolius*, was noted in the three species.

As to the macroscopic aspects, the leaves of the three species were coriaceous in texture. The shape of the laminas varies, being usually spatulate or elliptic in *H. sucuuba* (Fig. 2a); oblong-elliptic in *H. bracteatus* (Fig. 2b) and often larger in size in this latter species than in the other two species. *H. stenophyllus* showed lanceolate laminas (Fig. 2c), yet the three species had attenuate leaf bases. The leaf apices were more acute or rounded in *H. sucuuba* (never obtuse) (Fig. 2a) and cuspidate in *H. bracteatus* (Fig. 2b) and in *H. stenophyllus* (Fig. 2c). Important differences were observed in leaf architecture: in *H. sucuuba*, the secondary veins present the midvein at an acute angle, arched and unite to form a submarginal vein 2-3 mm from the margin; in *H. bracteatus* the secondary, veins present the midvein at a less acute angle than in *H. sucuuba* and are straight or only slightly arched; in *H. stenophyllus*, the secondary veins present the midvein nearly at right angles and unite into a submarginal vein close to the margin (Ribeiro *et al*., 1999). The study of the petiole showed that this is canaliculate in *H. sucuuba* and *H. stenophyllus*, plane or somewhat plane on the upper surface in this latter species. *H. bracteatus* has a cylindrical petiole which is flattish on the upper surface and rounded-convex on the lower surface.

The barks of *Himatanthus stenophyllus* (Fig. 1f) and *H. sucuuba* (Fig. 1b) exhibited a large quantity of prismatic calcium oxalate crystals that had already been observed in this latter species (Costa *et al*., 2002; Larrosa and Duarte 2005b). Larrosa and Duarte (2005b) describe calcium oxalate druses in the stem cortex of *H. sucuuba*, however this crystalline form was not detected in the bark of this species from the ducke reserve specimens. The

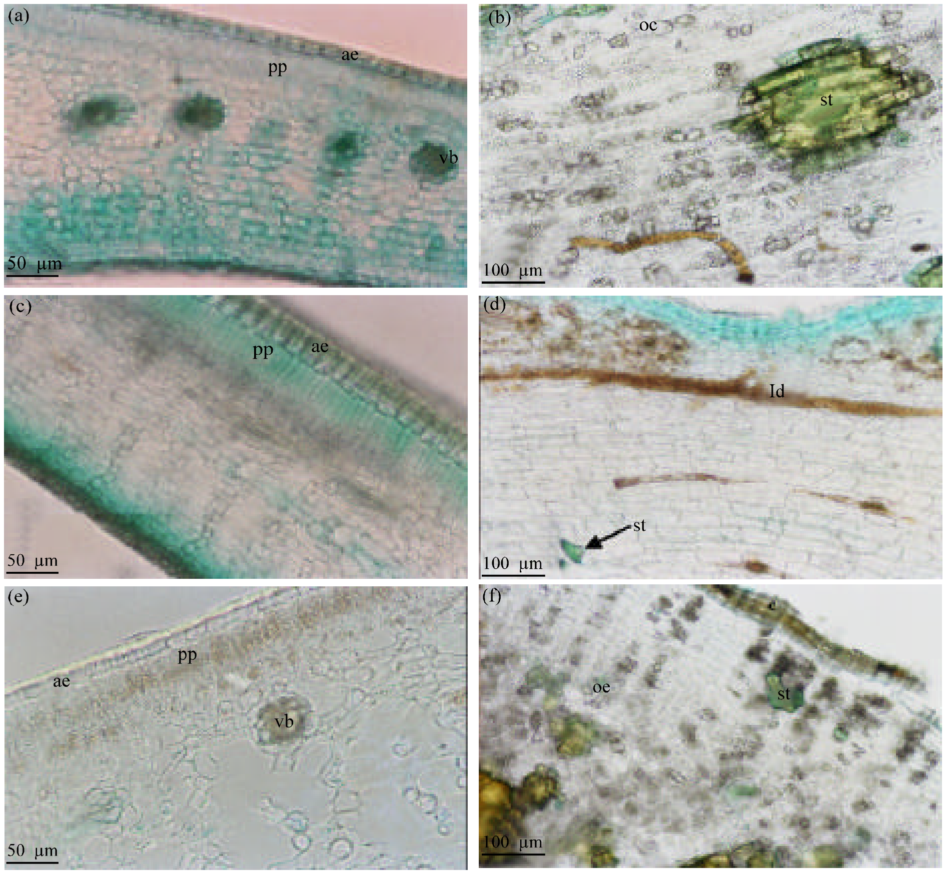

Fig. 1: *H. sucuuba*: (a) dorsiventral mesophyll with vascular bundles, (b) cortical and phloem regions with sclereids and prismatic calcium oxalate crystals; *H. bracteatus*: (c) dorsiventral mesophyll, (d) cortical region with laticifers and sclereids; *H. stenophyllus*: (e) dorsiventral mesophyll with vascular bundles and (f) cortical region with sclereids and prismatic calcium oxalate crystals. pp: Palisade parenchyma, vb: Vascular bundles, ae: Adaxial epiderm, oc: Prismatic calcium oxalate crystals, ld: Laticifer ducts, st: Sclerenchymatous tissue, c: Cork

cortex of the three species had starch grains as reserve substance as noted for *H. sucuuba* bark. Although the vascular system of *Himatanthus* is typically bicolateral, as typical characteristic observed in the Apocynaceae, this could not be verified in the bark fragment due to its thinness (the usual thickness of the commercial bark), which does not show internal phloem. Non-articulated laticifers were present in the cortical (Fig. 1b, d, f) and phloematic regions of the barks, a universal character of this family and abundant sclerenchymatous tissue was found in the cortical and phloematic parenchyma of the bark of the three species (Fig. 1b, d, f) (Larrosa and Duarte, 2005b).

The bark fragments showed a plane surface in *H. sucuuba* (Fig. 2d) and a curved surface in *H. bracteatus* and in *H. stenophyllus* (sometimes in scroll-like shape in this latter species) (Fig. 2e, f). The three species showed a lighter-colored internal surface. The surface of transversal fracture was granulose in the external portion and fibrous in the internal portion in *H. sucuuba*, shiny in *H. bracteatus* and slightly granulose in *H. stenophyllus*.

Therefore, the morphological analysis of the adult leaves added to the barks of *Himatanthus sucuuba*, *Himatanthus bracteatus* and *Himatanthus stenophyllus* provided the basis for differentiating the species. The contour of the leaf lamina, apex and the petiole aspects and the veination patterns were important features for the leaves. With regard to the barks, the inner, outer and transversal fracture surfaces must be conjointly analyzed. By the microscopic analysis of barks collected twice, the main difference among the species was the lack of the prismatic calcium oxalate crystals in the *H. bracteatus*.

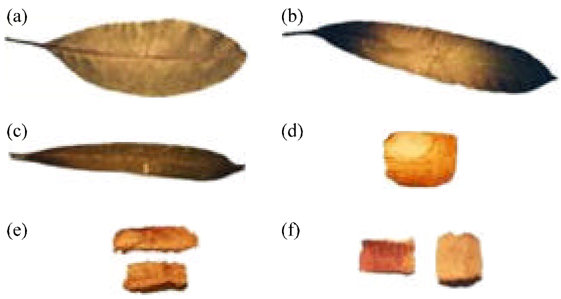

Fig. 2: *H. sucuuba*: (a) leaf abaxial face, (b) leaf adaxial face, (c) leaf abaxial face (d) bark inner surface, *H. bracteatus*: (e) bark inner surface, *H. stenophyllus*: and (f) bark outer and inner surface

Fig. 3: HPTLC fingerprint of *H. bracteatus* leaves, bark and latex extracts (1, 2, 5), *H. stenophyllus* leaves, bark and latex extracts (3, 4, 6), *H. sucuuba* latex, leaves and bark extracts (7, 8, 9). P: Plumieride, I: Isoplumieride. [CHCl3:MeOH (8:2)]

HPTLC chromatographic profile: Preliminary samples evaluations were made with silica gel and RP-18 TLC for the purpose of choosing the phase and solvents system, before the utilization of HPTLC. For the silica gel TLC, there were applied the solvents systems CH_2Cl_2:AcOEt (7:3), $CHCl_3$: AcOEt :MeOH (7:2:1), $CHCl_3$:MeOH (8:2) and for the RP-18 TLC, H_2O with 0,05% TFA: MeOH (6:4) and H_2O with HOAc 0,1%: CH_3CN (7:3). The best chromatographic condition used to develop the HPTLC fingerprint was silica gel 60 F_{254} (Merck) plate (0.2 mm of layer thickness and 10×10 cm size) using $CHCl_3$:MeOH (8:2) (Fig. 3). The chromatographic analysis of the extracts of the plants exhibited considerable differences in the profile. In relation to the revelation of the iridoids, the chromatogram showed in a first moment a characteristic green color and after 2 min under 110°C heat, the color of these substances has been intensified, resulting in brown or green-brown zones. In the chromatographic conditions mentioned, the R_f values found for these substances were 0.35 (plumieride) and 0.26 (isoplumieride). The spots present in the extracts at Rfs above showed quenching like that of the both iridoids. All samples loaded on the plate had plumieride presence. Under analysis conditions, this substance appeared in larger quantity in samples 1, 5, 7 and 8 and in smaller proportions in samples 2, 4 and 6 (Fig. 3). The isoplumieride was found in smaller proportions in all samples.

CONCLUSION

The analysis of the leaves, bark and latex of the three *Himatanthus* species studied by HPTLC presented different profiles for the samples and correlation with two important iridoids of this genus through simple and rapid procedure. Additionally, the morphological analyses of the leaves and bark provided data for differentiation among the species by means of the characteristics botanical.

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