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Characterizing Casca d'anta: an Apocynaceae used to treat tropical diseases in the Amazonian region

Caracterización de la Casca d'anta: una Apocynaceae utilizada para tratar enfermedades tropicales en la región Amazónica

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ABSTRACT

Aspidosperma macrocarpon Mart. (Apocynaceae) is used in popular medicine to treat malaria and dengue. The aim of the present study was to morphoanatomically, histochemistry and phytochemically characterize *A. macrocarpon* young branches and leaves, seeking to indicate secondary compounds presenting medicinal potential to treat these diseases. Aspidosperma macrocarpon young branches and leaves were collected in private properties located in municipality of Alta Floresta, Mato Grosso State, Brazil. Sectional and paradermic sections were set freehand using a steelblade, were doubly stained and assembled on histological slides. The fresh material was subjected to histochemical and phytochemical tests for detection of saponins, tannins, alkaloids, flavonoids, cardiotonic glycosides, anthraquinone, steroids and terpenes. Aspidosperma macrocarpon presented glandular trichomes, cavities and secretory cells. We detected the presence of tannins, flavonoid, phenolic compounds, cardiotonic glycosides and alkaloids, which may confer antiplasmodic activity and antiviral properties to the species. As far as it is known, the present study was the first to identify aspects of the histochemical and phytochemical composition of young branches and leaves *A. macrocarpon* which may be associated with the popular use of this plant to treat malaria and dengue in a transition region between the Cerrado and Amazon biomes, where malaria and dengue occur endemically.

Keywords: *Aspidosperma macrocarpon*, histochemistry, pharmacological potential, popular medicine.

RESUMEN

Aspidosperma macrocarpon *Mart. (Apocynaceae) es empleada en la medicina popular para el tratamiento de la malaria y el dengue. El objetivo de este trabajo fue caracterizar morfóanatomicamente, histoquímicamente y fitoquímicamente el tronco y la lámina foliar de* A. macrocarpon*, buscando identificar compuestos secundarios con potencial farmacológico para el tratamiento de ambas enfermedades. Muestras de tallos verdes y hojas de* A. macrocarpon *fueron recolectados en propiedades particulares en el municipio de Alta Floresta, Mato Grosso, Brasil. Las secciones transversales y paradérmicas en los órganos fueron obtenidas a mano libre, con ayuda de lámina de acero, doblemente coloreadas y montadas en láminas histológicas. El material fresco fue sometido a pruebas histoquímicas y fitoquímicas para detección de saponinas, taninos, alcaloides, flavonoides, glucósidos cardiotónicos, antraquinonas, esteroides y terpenos.* A. macrocarpon *presenta tricomas glandulares, células secretoras y cavidades. Detectamos la presencia de taninos, flavonoides, compuestos fenólicos, glucósidos cardiotónicos y alcaloides, que pueden conferir a esta especie actividad antiplasmódica y propiedades antivirales. El presente estudio fue el primero en identificar aspectos de la composición histoquímica y fitoquímica de ramas jóvenes y hojas de* A. macrocarpon *que pueden estar asociados al uso popular de esa planta para el tratamiento de la malaria y del dengue en una región de transición entre los biomas Cerrado y Amazonia, donde la malaria y el dengue ocurren endémicamente.*

Palabras clave: Aspidosperma macrocarpon*, histoquímica, potencial farmacológico, medicina popular.*

Introduction

Brazil has in its territory one of the largest biodiversity of plants in the world, where many traditional peoples live with great cultural diversity (Funari *et al.* 2013). The close relationship of traditional populations with their natural environment allowed the accumulation of knowledge about the

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local flora and fauna. Traditional populations tend to use plants for their food, religious and medication benefit (Almeida *et al.* 2009). The broad use of *Aspidosperma macrocarpon* Mart. (de Mesquita *et al.* 2007), *A. vargasii* A. DC. and *A. desmanthum* Benth. ex Müll. Arg. in popular medicine to treat malaria and dengue by traditional amazon communities is an example (Djènontin *et al.* 2010).

There is no scientific evidence to prove their pharmacological potential so far, but the applicability of the genus *Aspidosperma* in malaria and dengue treatment has been recently discussed (Henrique *et al.* 2010). Malaria remains a common disease among populations living in the Amazon, probably due to continuous colonization processes (Borges *et al.* 2015). In addition, the environmental conditions in the Amazon favor the faster completion of the reproductive cycle of malaria vectors such as mosquitoes belonging to the genus *Anopheles*, as well as the proliferation of protozoa belonging to the genus *Plasmodium,* mostly the species *Plasmodium falciparum, P. malaria* and *P. vivax* which are the causal agents of this disease (Monteiro *et al.* 2015). Nevertheless, dengue is caused by one of the most frequent arbovir uses belonging to family Flaviviridae and genus *Flavivirus* (serotypes: DEN-1, DEN-2, DEN-3 and DEN-4). The virus is transmitted the *Aedes aegypti* mosquito and has high incidence in the Amazon region throughout the rainy period (Borges *et al.* 2015). Therefore, studies are needed that point out ways to combat these diseases.

Morphoanatomical studies supports the correct identification of plant materials, as well as the identification of histological secretion structures, collaborating with chemical and pharmacological studies. The Histochemical analyses help visualizing the secondary metabolites produced by the plant *in situ*. The phytochemical studies allow quantifying the groups presenting the most relevant metabolites found in plants to complete these analyses (Barros *et al.* 2014). The scientific validation of the medicinal potential of a particular species popularly used for medical purposes is a long and multidisciplinary process, and is crucial to assure the species authenticity of its use.

Considering the lack of pharmacological studies researching the potential of the popular use of *Aspidosperma* along the geographical coverage of these diseases, studies that verify the potential of this popular use is necessary. Therefore, the main aim of the present study was to morphoanatomically and phytochemically characterize *Aspidosperma macrocarpon* Mart. young branches and leaf blades, searching to confirm the presence of secondary metabolites presenting potential for the treatment of both diseases. *A. macrocarpon* is an arboreal occurrence in the Amazonian and Cerrado South American, used in the treatment of malaria, dengue and viruses by populations of the portion south of the Brazilian Amazon, in infusions of bark and fresh leaves.

Materials and Methods

Aspidosperma macrocarpon plants are trees of a size that varies from 8-18 m in height. We collected vegetative parts of *A. Macrocarpon* in private properties located in the rural area of the municipality of Alta Floresta, Mato Grosso State (09º53‘37“S, 056º09‘52“W), in the transition zone between the Amazon and Cerrado biomes. Part of the collected botanical material was taken to HERBAM (Southern Amazon Herbarium, Universidade do Estado de Mato Grosso - UNEMAT) and was deposited under *voucher* number 11692. The method by Radford *et al.* (1974) was used to diagnose the studied material. Morphoanatomical and phytochemical characterization of young brunches and leaf blade was maked because these parts are frequently used in popular medicine. We added the morphological study to the research due to the peculiarities of the specimens found in the Amazon. The medicinal species described by the popular name Casca d‘anta presents arboreal size and larger leaves than the *A. macrocarpon* specimens described in the literature. The remaining part of the material was used for anatomical and hystochemical analyses. One fraction was used in cool temperature, whereas the other was subjected to a fixation process in FAA_{50} and stored in 70% ethanol (Kraus and Arduin 1997).

The median regions of young branches and leaves were selected for anatomical analysis. Transversal and longitudinal sections were performed freehand by using a steelblade. The sections were stained in Astra Blue and in basic fuchsin, and were assembled on histological semi-permanent slides containing glycerinated gelatin (Kraus and Arduin 1997).

Parademic sections were made on the adaxial and abaxial surface of leaves for epidermis analysis and dissected according to the modified Jeffrey method (Kraus and Arduin 1997). The modifications comprised longer leaf portion storage time (oven-driedat 60 ºC for 48 hours), hydrogen peroxide (30%) and glacial acetic acid (1:1). Images were obtained through an image-capture equipment coupled to a light microscope (Leica Microsystems, Wetzlar, Germany), and analyzed in Leica IM50 software in the Plant Biology Laboratory (UNEMAT).

The histological sections of fresh young branches and leaf blades were obtained freehand by using a steel blade. Next, they were subjected to histochemical tests presented in Table 1.

The young branches and leaf blades was washed in distilled water for phytochemical analyses. The water excess was removed with a paper towel. We obtained a fresh mass of 293 g of young branches and 93 g of leaves. The material was left to dry in a circulating-air oven (40 ºC, for 72 hours). The dry weight yield was 284 g of young branches and 45 g of leaves. Subsequently, the plant material was ground in a Willey type mill, packed in paper bags, and stored in a refrigerator (6 to 10 ºC). The phytochemical screening methodology followed the protocols by Mouco *et al.* (2003). The protocols were modified for anthraquinonic compounds, cardiotonic compounds and alkaloids compounds. The following reactions were performed for compound identification: (a) tannin: Ferric chloride in 2% aqueous alkaloid solution, 10% neutral lead acetate, 5% copper acetate solution, 10% lead acetate, 10% glacial acetic acid and 2% gelatin; (b) alkaloids: modified Borchardt, Bertrand, Mayer and Dragendorff reagents, with reactions performed in a test tube; (c) flavonoids: Shinoda reactions, 5% aluminum chloride, ferric chloride at 3%and sodium hydroxide; (d) saponins: foam test (15 minutes); (e) anthraquinones: Borntrãeger reactions and sodium hydroxide0,5%; (f) cardiotonic glycosides: Liebermann-Burchard and Keller-Killiani modified reactions (with the addition of 2g of the weighed drug, 20 ml of 70% ethanol) warmed for two minutes in stand-by and filtered. The filtering results were added with 20 ml distilled water. The mixture was brought to the separating funnel in order to get to the organic phase extraction by using chloroform (16 ml); (g) steroids and terpenoids: Liebermann-Burchard reactions.

Results

Morphology

Aspidosperma macrocarpon Mart., Nov. Gen. Sp. Pl. 1: 59. 1824.

Popular names: Peroba mica, Casca d'anta, Guatambu and Pau-pereiro

Trees, 4.5-6 m height, young rough branches, latescent. Simple leaf, alternating, accumulated or sparse; petiole 1.5-2.0 cm length, rough, canaliculate; leaf blades 8.1-10.7 x 5-5.9 cm, elliptical, acute or rarely obtuse apex, obtuse base, rarely oblique, prominent and fuzzy midrib, adaxial and abaxial faces sparsely fuzzy, margin slightly sinuous and paper-like appearance, deciduous. Side inflorescences, pluri flowering, composed dichasium, floral rachis1, 7-2.5 cm, densely fuzzy, hermaphrodite flowers. Small flowers, spiral prefloration with floral symmetry; calyx 5 Mera, 2.7-3 mm length, gamosepalous, greenish, triangular

Table 1. Histochemical tests performed in Aspidosperma macrocarpon for the detection of secondary metabolites.

Secondary Metabolites	Reagent/Test
Alkaloids	Dragendorff (SVENDSEN & VERPOORTE, 1983)
Starch	Lugol (JOHANSEN, 1940)
Phenolic compounds	Ferric chloride III (JOHANSEN, 1940)
Steroid	Antimony trichloride (HARDMAN & SOFOWORA, 1972)
Sesquiterpene lactones	Sulphuric Acid (GEISSMAN & GRIFFIN, 1971)
Lipid	Sudan IV (PEARCE, 1980)
Mucilage	Tannic acid and ferric chloride (PIZZOLATO & LILLIE, 1973)
Essential oil	Nadi (DAVID & CARDE, 1964)
Tannins	Vanillin hydrochloric (MACE & HOWELL, 1974)
Terpenoids	2-4-dinitrophenylhydrazine (GANTER & JOLLÉS, 1970)

Details and method references can be accessed at https://drive.google.com/open?id=1uR3vForweWgTpYm79ciNie4c9nw-QG-p.

lacinea, densely fuzzy; 5 Mera, corolla tube 6-7 mm length, greenish, lacinea 14-18 mm length, white, fuzzy; androecium with 5 stamens, epipetals, anthers with rimosa dehiscence; ovary with 3 mm max length, midmedial, superior, glabrous, present nectariferous disk, obsolete stylet, capitate stigma. Follicles, 16-17 x 9-9.5 cm, obovate, woody, slightly wrinkled, attenuated base, rounded Apex, hairy 18-25 seeds, 6.5-7.2 x 6-7.4 cm, oblong, winged and papyraceous.

Examined Material BRAZIL. MATO GROSSO. Alta Floresta, Monte Alegre community, Nossa Senhora Aparecida Sector, Rural Zone, 26/X/2015, fl., *D. G. Larocca 003* (HERBAM). **Nova Bandeirantes**, Sítio do Anilton, 03/I/2016, fr., *Ribeiro, R. S. 134* (HERBAM) **(Figures 1A-H).**

Young branches anatomy

Epidermis of young branches *A. macrocarpon* with glandular trichomes (Figures 2A, 2C, 2D) are unstratified (Figures 2A, 2C, 2D). Epidermis cells are juxtaposed and present oval to irregular shape (Figure 2D) with uniseriate and multicellular tectonic trichomes, the glandular trichomes are unicular and

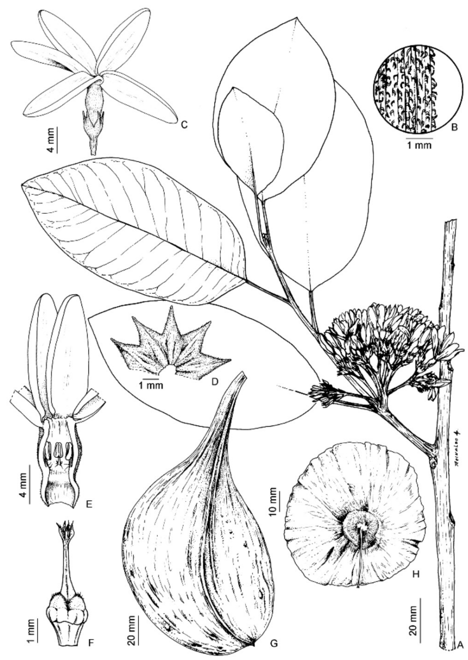

Figure 1. *Aspidosperma macrocarpon* (A-H): A- Young branch presenting leaves and inflorescence; B- Indument of the young branch; C- Open flower; D- Open chalice; E- Part of the open corolla with the epipetalous stamens; F- Gynoecium; G- Fruit; H- Seed.

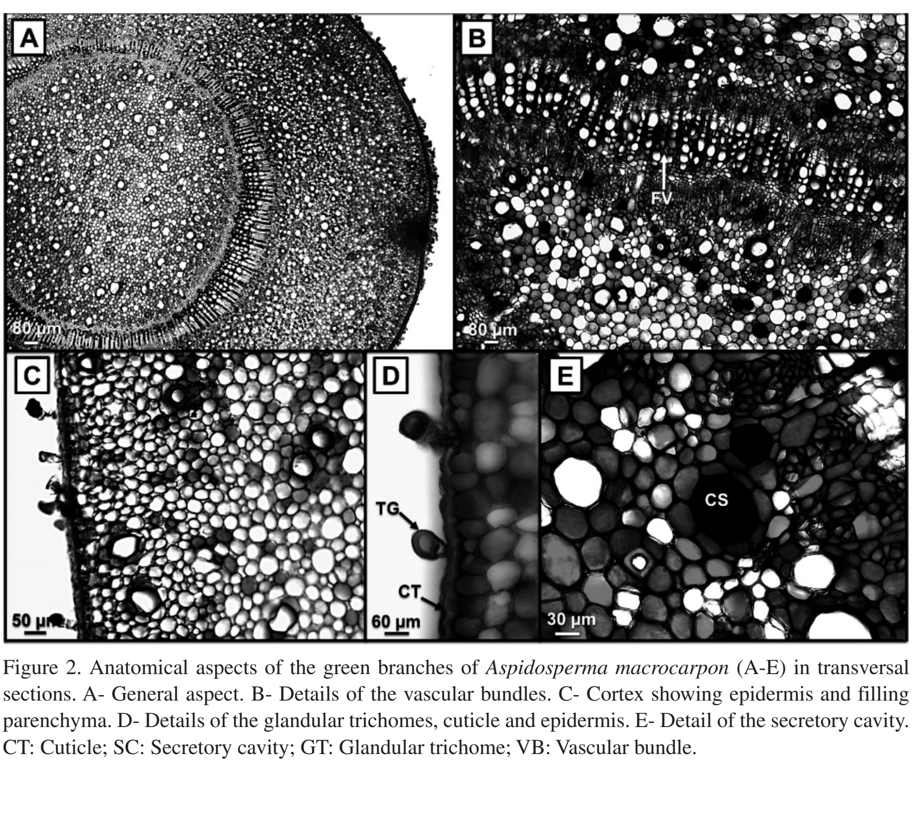

Figure 2. Anatomical aspects of the green branches of *Aspidosperma macrocarpon* (A-E) in transversal sections. A- General aspect. B- Details of the vascular bundles. C- Cortex showing epidermis and filling parenchyma. D- Details of the glandular trichomes, cuticle and epidermis. E- Detail of the secretory cavity. CT: Cuticle; SC: Secretory cavity; GT: Glandular trichome; VB: Vascular bundle.

bicellular. The young branches cortex is composed of parenchyma, which cover from 50% to 60% of the organ and presents secretory cavities, secretory cells and brachysclereids (Figures 2B, 2C, 2E). The secretory cavities are circular and of varying sizes; they are composed of a cellular epithelium that contains approximately 6 to 8 cells (Figure 2E). The vascular bundles are bicollateral and their exchange rate region is not well-defined (Figures 2A, 2B). The region of the midrib presents parenchyma of filling with secretory cavities of esquizogenic origin and brachysclereids (Figures 2A, 2B).

Leaf anatomy

Aspidosperma macrocarpon leaves are hypo-estomatic and have anomocitic stomata (Figures 3A, 3B). Cell disposition at the adaxial face forms a juxtaposed surface that has straight adhesion between the lateral walls, fact that stops water loss. Stomata on the adaxial face are protected by the trichomes that cover almost the entire foliar blade surface. It impairs visualizing the guard cells and the other epidermal cells on the same plane. Stomata are located on the same level of other epidermal cells within the cross section (Figure 3J). The epidermis is unstratified and isodiametric (Figure 3G). The adaxial face has a thick cuticle, which presents uniseriate and bicellular silica and glandular trichomes cells in the adaxial face (Figures 3H, 3J). Tector and glandular trichomes overlay the abaxial epidermis, which is papillous (Figure 3J). The little developed substomatal chamber was evidenced (Figure 3J). The slightly sinuous adaxial epidermal cells could be observed in front view (Figure 3A).

The leaf mesophyll is dorsiventral and presents approximately 2-3 palisade parenchyma layers and 9-11 lacunous parenchyma layers (Figures 3G, 3H). The presence of brachysclereids, secretory cells and secretory cavities (with content) is noticed in the midrib region (Figures 3E, 3F). The secretory cavities are constituted of 6-8 epithelium cells

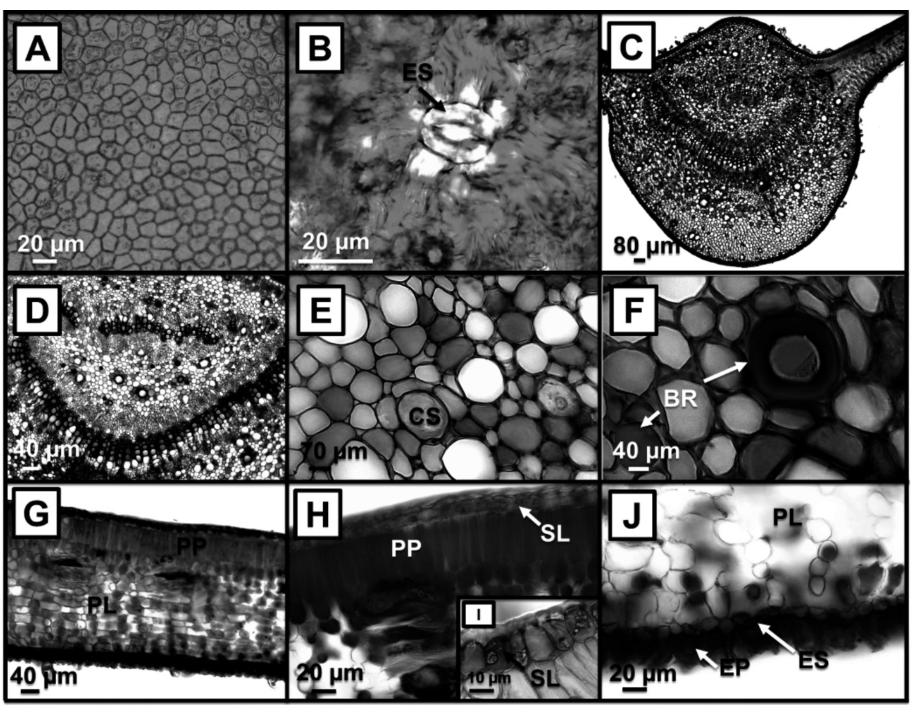

Figure 3. Anatomical aspects of the *Aspidosperma macrocarpon* foliar blade (A-J) in transversal and paradermic sections. A- Adaxial face. B- Abaxial face showing papillose epidermis and the stomata arrangement. C- General aspect of the midrib. D- Vascular bundle. E- Detail of the secretory cavity (with contents). F- Brachysclereids. G- General aspect of the mesophyll. G- Palisade parenchyma. H- Detail of the palisade parenchyma. I- Detail of the cuticle, epidermis and silicas. J- Detail of the stomata and substomatal camera on the abaxial surface. PE: Papillose epidermis; SP: Spongy parenchyma; PP: Palisade parenchyma; S: Stomata; SL: Silica; SC: Secretory cavity; BR: Brachysclereids.

(Fig. 3E). The vascular bundles are bicollateral (Figures 3C, 3D).

Histochemical analysis

We detected the presence of tannins in the trichomes of young branches and leaves (Figures 4A, 4B); the lipidic substance is concentrated in the cortex and near the vascular bundle in the young branches and leaves (Figures 4C, 4D). Phenolic compounds were present mainly in the young branches and foliar adaxial epidermis (Figures 4E, 4F); mucilage in secretory young branches and leaf cavities (Figures 4G, 4H); alkaloids in the secretory cavities of the young branches and leaves (Figures 4I, 4J); essential oils in the young branches and leaves (Figures 4K, 4L); starch in the young branches, throughout the cortex, and in the leaves, mainly near the secretory cavities and vascularized regions (Figures 4M, 4N); terpenes in the leaves, which may be found in the epithelium cells of the secretory cavities or close to the stone cells (Figure 4O). Results were negative in both young branches and leaves for the detection of sesquiterpene lactones using sulfuric acid, and the detection of steroids using antimony trichloride.

Phytochemistry

The phytochemical tests showed the presence of tannins, flavonoids, anthraquinonoid glycosides, saponins, and alkaloids both in young branches and leaves (Table 2). Cardiotonic glycosides, terpenoids and steroids were detected in the leaves only.

Discussion

The presence of the glandular trichomes, secretory structures in the leaf mesophyll, cortex and in the stem marrow could be noticed through the morphoanatomical diagnosis of *A. macrocarpon*. Secondary metabolites in plants are generally produced in specific structures such as trichomes, channels, cavities or secretion cells; thus, the presence of these structures can help confirming the medicinal potential of the plant material (Demarco 2014). In addition, the presence of latex in *A. macrocarpon* was noted only in the main stem of the plant. Laticiferous tubes were described

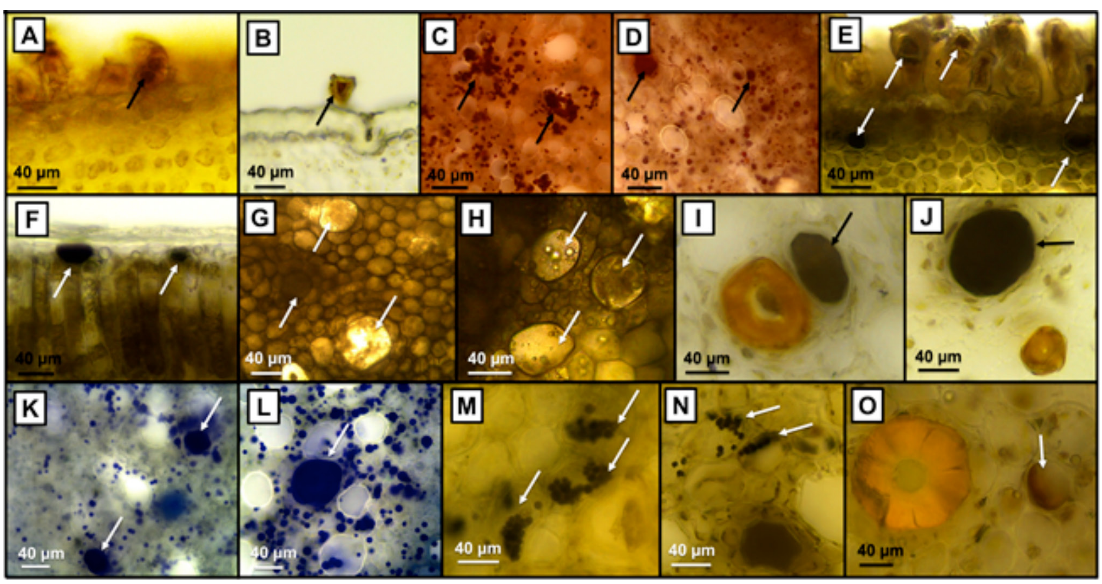

Figure 4. Transversal sections of the *Aspidosperma macrocarpon* green branches and leaf (A-O) showing the results of positive histochemical tests. Presence of tannins in the green branches (A) and the leaf (B) in reaction to hydrochloric vanillin; presence of lipids in the green branches (C) and leaf (D) in reaction to Sudan IV; presence of phenolic compounds in the green branches (E) and the leaf (F) in reaction to iron chloride III 10%; presence of mucilage in the green branches (G) and leaf (H) in reaction to tannic acid and ferric chloride at 3%; presence of alkaloids in the green branches (I) and leaf (J) in reaction to Dragendorff reagent; presence of essential oil in the green branches (K) and on the leaf (L) in reaction of NADI reagent; reaction of lugol in the green branches (M) and on the leaf (N); reaction of 2-4-Dinitrophenylhydrazine on the leaf (O). Arrows indicate the sites of reaction.

Table 2. Phytochemical screening of *Aspidosperma macrocarpon* vegetative organs used in popular medicine to treat malaria and dengue.

Secondary Metabolites	Reactive/Test	A. macrocarpon	
		Young branches	Leaf
Tannins	Ferric Chloride III 2%	+	+++
	Neutral lead acetate 10%	–	++
	Copper acetate 5%	–	+
	Glacial acetic acid 10%	–	+
Flavonoids	Magnesium + hydrochloric acid	+	++
	Ferric Chloride 2%	++	+++
	Aluminum chloride 5%	+	+
	Sodium hydroxide 5%	++	++
Anthraquinonic glycosides	Reactive Bortranger	+(a)	++(a)
	Sodium hydroxide 0,5%	+(a)	++(a)
Saponins	The foam test	+++	+
Cardiotonic glycosides	Liebermann-Burchard	–	+++
	Keller-Killiani	–	++
Terpenoids/Steroids	Liebermann-Burchard	–	++
Alkaloids	Mayer	++	+++
	Borchardt	++	+++
	Bertrand	+	+++
	Dragendorff	+++	+++

a - yellow color: reduced anthraquinone; (+++) Strong positive; (++) Moderate positive; (+) Weak positive; (–) Negative.

for the family Apocynaceae in association with the secondary xylem. The presence of latex and of these tubes is mainly associated with plant protection. Some traditional communities, besides using the peels or leaves from a large variety of species belonging to Apocynaceae considered important for the treatment of malaria, also use latex as medicine to treat other diseases such as depression and digestive tract diseases (Pereira *et al.* 2007). Despite its importance in the family, the occurrence of latex was not anatomically recorded on the studied leaves and young branches; however, it was possible to find latex on the thicker branches of the field plants.

The detection of essential oils in *A. macrocarpon* indicates a potential for antifungal, antiviral and antioxidant activitiy (Miranda *et al.* 2015), justifying its use in the traditional medicine knowledge about its use in the treat dengue and malaria. The presence of essential oils in certain families is associated with specific structures such as trichomes, cavities and secretion cells, which promote their synthesis and accumulation (Mendonça *et al.* 2009).

The presence of mucilage and starch is directly linked with ecological and physiological aspects of the plant. Mucilage is associated with mechanical protection, nutrient reserve and water retention processes (Bezerra *et al.* 2015). Starch is a fundamental substance for plant development processes, and can be saved for future use in stress periods (Rocha and Machado 2009). The presence of serum lipids was also described in other species of *Aspidosperma* (de Mesquita *et al.* 2007; Demarco 2014). This metabolites are fundamental for the maintenance of vital chemical growth and reproduction processes (Prata-Alonso *et al.* 2015).

The presence of tannins, flavonoids and cardiotonic glycosides has been described in other species of Apocynaceae (Santos *et al.* 2013), but no information was available so far for *A. macrocarpon*. Our positive results for the presence of tannins, flavonoids and cardiotonic glycosides in *A. macrocarpon* reinforce its potential for bactericide, fungicide, antiviral and antiplasmodic activity, as was described for other *Aspidosperma* species (Milliken 1997). The presence of these compounds are likely related to the use of this plant for the treatment of malaria and dengue in popular medicine. The presence of saponins and terpenes may also contribute to the medicinal use of the plant, since these compounds have anti-inflammatory and analgesic properties, respectively, and diseases such as malaria and dengue cause muscular pain and febris recurrens (Meira-Neto and Almeida 2015).

The phenolic compounds identified in young branches and leaves of *A. Macrocarpon* may be of economic interest, because these substances can be used as dyes in the food industry, besides having antioxidant, antimicrobial and antiviral potential (Barros *et al.* 2014). The compound has potential application as an antiviral drug to treat dengue (Hyacienth and Almeida 2015).

Currently, several studies have been performed using species to genus *Aspidosperma* in search of indole alkaloids in their representatives (Henrique *et al.* 2010). Approximately 100 types of indole alkaloids have already been isolated, fact that shows the great medicinal value of these compounds (Oliveira *et al.* 2009) and the presence of these compounds is related to their anti-malaria activity (Meneguetti *et al.* 2014). *Aspidosperma ramiflorum* Müll. Arg., for example, showed efficiency in the treatment against *P. falciparum*, from extracts of the stem and leaves (Aguiar *et al.* 2015). Ethanol extracts from the roots, stem bark and leaves of *A. macrocarpon* tested in vitro also showed promising results for inhibition of *P. falciparum* (de Mesquita *et al.* 2007), with better results for root extracts.

Aspidosperma macrocarpon is also used by traditional populations to treat cancer (de Mesquita *et al.* 2007). Aspidoscarpine and ellipticine are the main indole alkaloids responsible for the antibacterial, antiplasmodic and antitumor activity (Meneguetti *et al.* 2014). There are other biological activities attributed to *Aspidosperma* extracts such as antitumor, antiplasmodic, antibacterial and antidepressive (Henrique *et al.* 2010). Therefore, this species has great pharmacological potential that has yet to be investigated.

Conclusions

Aspidosperma macrocarpon young branches and leaves present specific secretory structures, such as glandular trichomes and secretory cavities. The histochemical and phytochemical analyses revealed the presence of tannins, flavonoids, phenolic compounds and cardiotonic glycosides, which are secondary metabolites related to antiviral biological activity, which suggests the species should be further evaluated for its pharmacological potential to treat dengue. The presence of alkaloids was also detected, which justifies the popular use of the plant as an antimalarial.

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