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Original Article

Anatomical characterization of ultra-structures, biominerals and histolocalization of metabolites in leaves of *Genipa americana*

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ABSTRACT

Inserted in one of the largest families among the Angiosperms, *Genipa americana* L., Rubiaceae, can be found in all Brazilian territory, presenting great medicinal importance, where several uses have been attributed. In view of this, this work has the purpose of analyzing the ultrastructural, biomineral, phytochemical and histochemical characteristics of the leaves of this native species from Brazil. For this, light microscopy, polarization and scanning electron microscopy techniques were used with X-ray scattering energy, associated to chromatographic and histochemical tests. The anatomical ultrastructural characteristics of the leaves detailed information about the type and arrangement of the cuticle, trichomes, surface and arrangement of the tissues that determine the botanical identity of this species. The phytochemical tests allowed determining their chromatographic pattern and histochemistry to determine the exact storage site of these substances in the leaf. It was observed that the characterization of the crystalline macro-pattern present in the analyzed species, as well as its exact elemental composition, can be considered an important differential diagnosis factor. The results characterize the leaves of this species in different aspects, being a native species and pharmacologically promising, with different popular uses and proven pharmacological activities, and more in depth studies is needed.

Introduction

Rubiaceae is considered as one of the largest families among Angiosperms, it comprises the fourth largest number of species, with approximately 13,000 species distributed in 550 genera. The *Genipa* genus is characterized by two species: *Genipa americana* L. and *G. infundibuliformis* Zappi & Semir (Delprete et al., 2005). Among the studies carried out on the genus, the researches are concentrated on the *G. americana* species.

Popularly known as jenipapo, this species can be found all over Brazilian territory. It is a native plant of Brazil, of arboreal size, reaching up to 30 m in height (Corrêa, 1984; Delprete et al., 2005; Zappi, 2015). Naturalists, who visited Brazil in the colonial period, have long described the use of *G. americana* as a body dye (Alves, 2010, 2013).

Several medicinal uses have traditionally been attributed to this species. The leaf decoction has an antidiarrheal or antisyphilitic action, whereas the leaf infusion is used against liver diseases (Corrêa, 1984). Based on ethnobotanical surveys, Souza et al. (2013) point out the traditional use of this species in the treatment of cough, anemia, contusions, dislocations; as depurative and associated with popular beliefs. Among the secondary metabolites found in this species, it highlights the presence of tannins (Revilla, 2001), monoterpenes (Ono et al., 2007), steroids (Conceição et al., 2011) and mostly iridoids which are characteristic of the Rubiaceae family (Jensen, 1983; Ueda and Iwahashi, 1991; Ono et al., 2005).

Different studies have indicated the potential of *G. americana* for use in the treatment of asthma (Deng et al., 2013), ophthalmic diseases (Koriyama et al., 2013; Song et al., 2013), antiviral action (Lin et al., 2013), cardiovascular disorders (Zhang et al., 2013), antidepressant activity (Tian et al., 2010), in the treatment of Alzheimer's disease (Nam et al., 2013; Gao et al., 2014; Wang et al., 2012; Zhang et al., 2012), anti-inflammatory (Fu et al., 2012; Li et al., 2012) (Kim et al., 2012; Dando et al., 2013; Yang et al., 2013).

In view of the recognized popular use of this species and distinct pharmacological studies demonstrating its therapeutic potential, this work aims at analysing the anatomical ultrastructural, biomineral, phytochemical and histochemical characteristics of *G. americana*, native to Brazil.

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Materials and methods

Plant material

Genipa americana L., Rubiaceae, mature leaves were collected from adult species on the campus of the Universidade Federal de Pernambuco (−8,0464168, −34,9451315), Recife, Northeast Brazil, at October 12, 2015, in the fruiting season. The voucher specimen was deposited at the Herbarium Dárdano de Andrade Lima, for botanical legitimation, by the number 89999.

Anatomical characterization

Mature leaves were collected from second or third node, from up to five individuals. The samples were fixed in FAA50 (Johansen, 1940). Cross and paradermic sections of the middle portion of the leaflets were freehand obtained, submitted to double staining process with astra blue and safranin (Johansen, 1940; Kraus and Arduin, 1997) and mounted in glycerine on semi-permanent slides. Analyze of the structures was performed using optical and polarized light microscopy.

Phytochemistry determination

The methanolic extract from fresh leaves was processed by infusion (0.1%) and subsequently analyzed by thin-layer chromatography (TLC) (Kieselgel 60, 0.2 mm, Merck), with different eluents systems and reagents to search alkaloids, mono- and sesquiterpenes, triterpenes and steroids, saponins, flavonoids, cinnamic derivatives and phenylpropanoid glycosides, hydrolyzable and condensed tannins. Phytochemical screening of the extract was carried out using a conventional protocol (Harborne, 1998; Randau et al., 2004).

Histochemical analyses

Freehand cross sections from the middle portion of fresh leaves were subjected to reaction with specific reagents, according to the different groups of metabolites to be investigated: Lugol reagent (Johansen, 1940) for starch, Sudan IV (Kraus and Arduin, 1997) for detection of epicuticle wax, acid phloroglucin (Jensen, 1962) for lignins, potassium dichromate (Gabe, 1968) for phenolic compounds, NADI reagent (David and Carde, 1964) for essential oils and oil-resins, vanillin-sulphuric acid (Wagner and Bladt, 1996) for iridoids, antimony trichloride (Mace and Howell, 1974) for steroids, vanillin-hydrochloric acid (Mace and Howell, 1974) for tannins, Dragendorff reagent (Yoder and Mahlberg, 1976) for alkaloids and hydrochloric acid (Johansen, 1940) for calcium oxalate crystals. For each procedure employed on the samples slides without any treatment (blanks) were also mounted. Images documentation was performed with a light microscope (Alltion) equipped with a digital camera.

Scanning electron microscopy

Cross sections obtained by hand were fixed with 2.5% glutaraldehyde (GA) + 4% formaldehyde buffer. After fixation and washing, the dehydrated samples by increasing ethanolic series (Johansen, 1940) were submitted to CO_2 Critical Point (HCP-2). Afterwards, the samples were mounted on metal brackets and coated with a gold layer metalizer Quorum Q150T ES (Haddad et al., 1998). The images obtained were used in the anatomical characterization of ultrastructures with a QUANTA 200 FEG scanning electron microscope (SEM).

Morphological analyze and elemental composition of crystals

The chemical microanalyses by EDS were done with X-ray detector attached to the Zeiss-EVO-LS15 scanning electron microscope, under the same operational conditions. Absorbance peaks indicating relative elemental chemical concentration $\geq$0.5% were considered significant.

Results

Anatomical characterization

The epidermal cells, which constitute the adaxial face, present polygonal contours containing amorphous inclusions within them that vary in size (Fig. 1A). On the abaxial side the cells are smaller and it can be noticed the presence of paracytic and anomocytic stomata, restricted to this face, characterizing the leaf as hypostomatic. Several inclusions are also observed on the abaxial face of the leaf blade, inside the epidermal cells and on stomatal guard cells (Fig. 1B). The images obtained by Scanning Electron Microscopy (SEM) address that the cuticle presents in the format of crystalline projections, such as an extensive layer of nanofilaments that cover the entire epidermal surface on both faces (Fig. 1C and D), as well as on the stomata (Fig. 1E).

In cross-section the epidermis is covered by a thick cuticular layer. The epidermis is unstratified, formed by larger cells on the adaxial side, more elongated in the anticlinal direction, as opposed to the abaxial epidermal cells, smaller and more elongated in the periclinal direction. The mesophyll has a dorsiventral organization and presents a palisade parenchyma facing the adaxial face, constituted by two-three layers of elongated cells (Fig. 1F). The lacunar parenchyma is multi-stratified composed of loosely organized rounded cells, with small intercellular spaces between them, comprising the abaxial region of the mesophyll. Some secondary vascular bundles can be seen dipped into the mesophyll. The presence of scarce multicellular and glandular tector trichomes (Fig. 1G and H) can be observed on the abaxial surface. Furthermore, the presence of these trichomes was also detected by electron microscopy, revealing small and scarce glandular trichomes on the abaxial side of the blade that are inserted between the stomata and the epicuticular layer (Fig. 1I).

The midrib presents biconvex symmetry, being prominent on the abaxial surface, with vascular bundles of concentric arrangement with no perivascular sclerenchymal fibers (Fig. 1J and K). At the center of the main vascular cylinder, in the medullary parenchyma region, there are accessory vascular caps, of which the presence of druses can be confirmed (Fig. 1L and M). Underlying the epidermal monolayer there are several strata of angular-type collenchyma cells where the presence of crystalline inclusions of druse type is recognized (Fig. 1N). Through polarized light microscopy, the presence of these crystals throughout the leaf blade can be observed, presenting a higher incidence in the cortical parenchyma of the midrib (Fig. 1O).

Phytochemistry determination and histochemical characterization

According to the results, the presence of iridoids, mono and sesquiterpenes and of triterpenes and steroids in the extract obtained from the leaves was observed. The tests also revealed the presence of hydrolyzable tannins, proanthocyanidins, cinnamic derivatives and phenylpropanoglycosides. The analyzes for alkaloids and saponins were negative. The results obtained formed the basis for performing the histochemical tests.

These tests were performed by comparing the sections after reaction with specific reagents under the negative control,

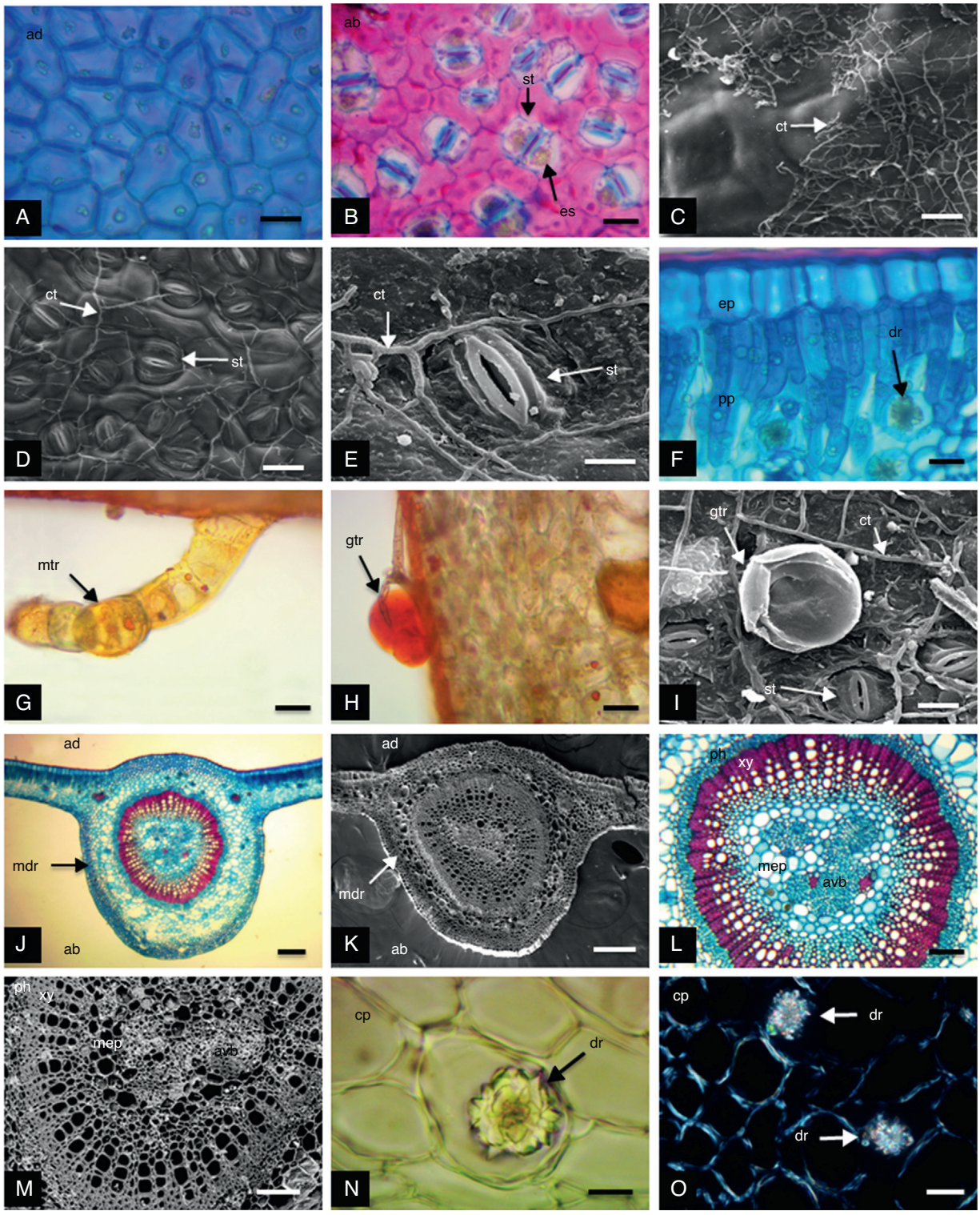

Fig. 1. Anatomical structures of leaves of *Genipa americana*. Leaf blade. (A) Adaxial epidermal cells. (B) Abaxial epidermal cells. (C) Adaxial epidermis covered by tubular cuticle (SEM). (D) Abaxial epidermis covered by tubular cuticle and presence of stomata (SEM). (E) Stoma in the abaxial face (SEM). (F) Dorsiventral mesophyll with druses. (G) Multi-serrated trichome. (H) Glandular trichome. (I) Detail of the glandular trichoma in the abaxial face (SEM). (J) Biconvex midrib. (K) Detail of the midrib (SEM). (L) Vascular cylinder with concentric arrangement. (M) Detail of the vascular cylinder (SEM). (N) Crystalline inclusion of druse type. (O) Druses observed through polarized light microscopy. ad, adaxial suface; ab, abaxial surface; avb, accessory vascular bundles; cp, cortex parenchyma; co, collenchyma, ct, cuticle; dr, druse; ep, epidermis; es, ergastic substance; gtr, glandular trichome; mdr, midrib; mep, medullary parenchyma; mtr, multiseriate trichome; pp, palisade parenchyma; sp, spongy parenchyma; st, stomatum; vb, vascular bundle; xy, xylem; ph, phloem. Bars: A, B, F, H, I, K, M, O = 25 μm. C, D, J = 25 μm. E = 10 μm. G, N = 40 μm. L = 200 μm.

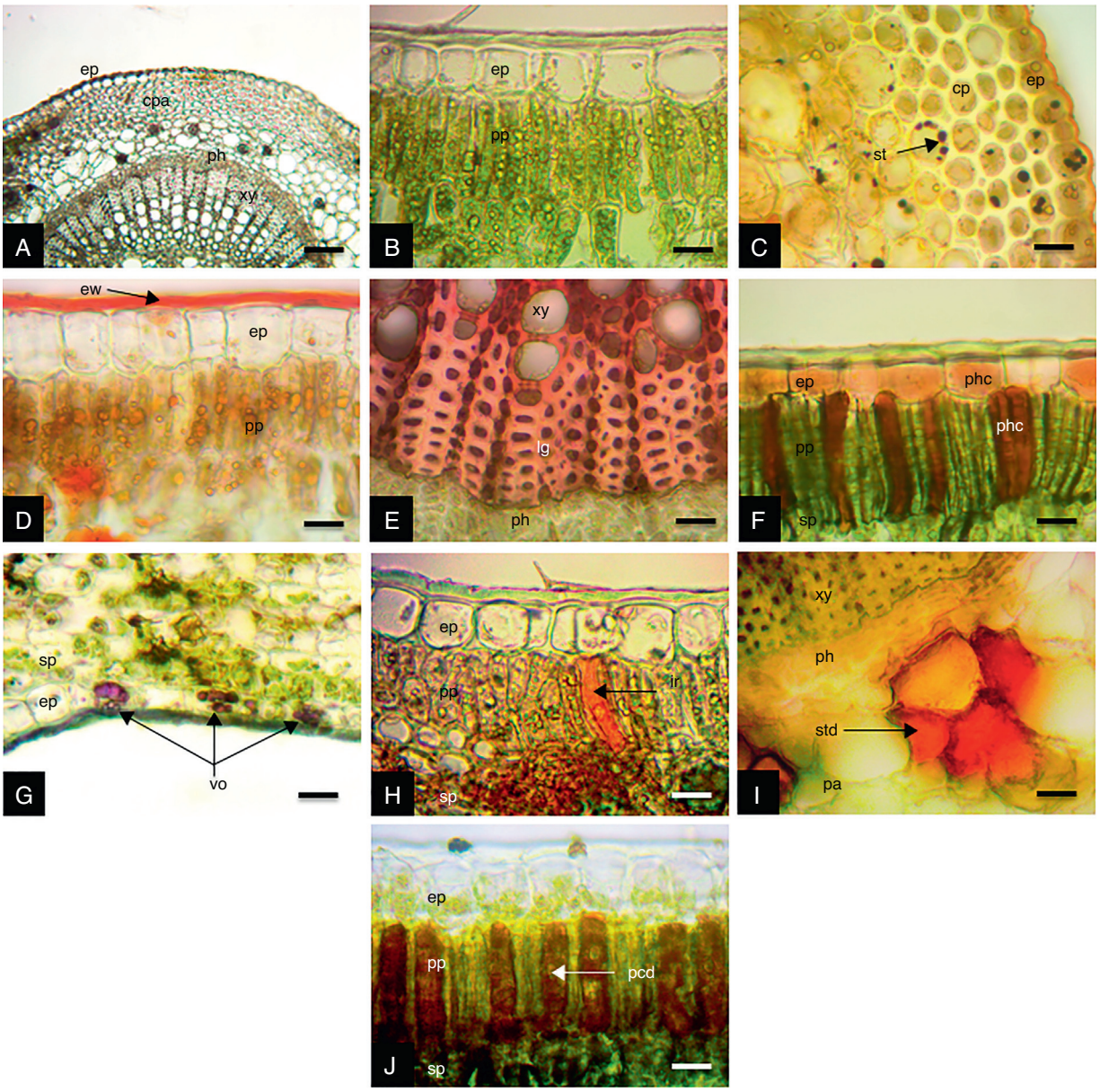

Fig. 2. Histolocalization of metabolites present in leaves of *Genipa americana*. Control sample: (A) Midrib. (B) Mesophyll. Histochemical tests: (C) Starch grains in parenchyma cells. (D) Covering cuticular epidermal cells. (E) Presence of lignins in woody vascular bundles. (F) Polyphenols present in epidermal cells and palisade parenchyma. (G) Volatile oils inside abaxial epidermal cells. (H) Iridoids inside the palisade parenchyma cells. (I) Steroids in perivascular idioblasts in the midrib. (J) Proanthocyanidins inside the palisade parenchyma cells. cp, cortex parenchyma; ep, epidermis; ew, epicuticular wax; ir, iridoids; lg, lignin; pa, parenchyma; pcd, proanthocyanidins; ph, phloem; phc, phenolic compounds; pp, palisade parenchyma; sp, spongy parenchyma; st, starch grain; std, steroids; vo, volatile oil; xy, xylem. Bars: A = 100 μm. B, C, D, E, F, G, H, I, J = 25 μm.

cross-sections of the midrib and mesophyll without treatment with the reagents (Fig. 2A and B). The results demonstrate the presence of starch granules present in the mesophyll, both in the palisade parenchyma and in the epidermal cells (Fig. 2C). The intense accumulation of amyloplasts can also be observed in the medullary parenchyma region, in the center of the vascular cylinder.

From the reaction with Sudam III, the presence of a thick epicuticular waxy layer can be observed, covering both sides of the blade. The cuticle presents as an orange layer (Fig. 2D). The presence of lignins can also be verified in the woody vascular bundles, characterized by the pink color conferred by floroglucinol (Fig. 2E).

After reaction with potassium dichromate, the presence of phenolic compounds can be verified, characterized by reddish coloration, distributed throughout the leaf blade region, mostly in the prismatic cells that make up the palisade parenchyma and epidermal cells of the adaxial face (Fig. 2F). The volatile oils present in the leaves of *G. americana* were evidenced by the reaction with the NADI reagent, conferring blue color to the spherical droplets described above, accumulated in the epidermal and subsidiary cells and abaxial epidermal cells (Fig. 2G).

The presence of iridoids was confirmed by the sulfuric vanillin reaction, showing reddish staining, determining the accumulation site of these substances in the xylem cells and some cells of the palisade parenchyma close to the phloem (Fig. 2H). The presence of steroids is observed in idioblasts located in the perivascular regions, close to the phloem, along the entire midrib, showing a characteristic orange color after reaction with antimony trichloride (Fig. 2I).

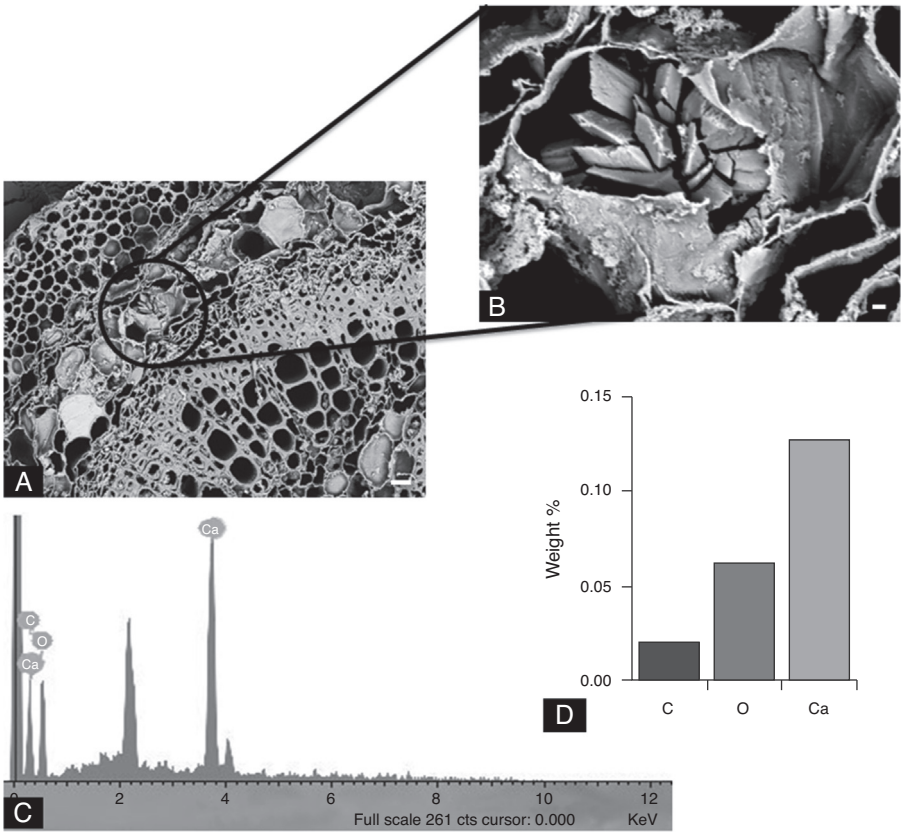

Fig. 3. Scanning electron micrograph and elemental composition of the crystals observed in *Genipa americana*. (A) Presence of druse in the midrib of the leaf in cross-section. (B) Detail of the rosette-like druse (whewellite type) covered by the membrane of the idioblast. (C) Analysis of elemental composition of the crystal. (D) Percentage of the chemical constituents of the crystal. Bars: A = 20 μm. B = 2 μm.

The presence of proanthocyanidins was characterized by poor red coloration as well as the results found in phytochemistry. The accumulation of these substances was verified in some cells of the palisade parenchyma, after reaction with sulfuric vanillin (Fig. 2J). The reaction with hydrochloric acid demonstrated the soluble nature of the crystalline inclusions, confirming the chemical composition of calcium oxalate of these crystals.

Morphological analyze and elemental composition of crystals

The dominant crystalline macro-pattern in the leaves of *G. americana* is druse type of crystals in rosette. This type of crystal can be found along the mesophyll, in the parenchymal cortex and in the medullary parenchyma of the midrib (Fig. 3A and B).

The microanalysis of druses, performed by scanning electron microscopy (SEM), by energy dispersive X-ray analyze (SEM), revealed three peaks of absorbance for calcium (0.37, 3.7 and 4.0 keV), a peak for oxygen (0.37 keV) and one for carbon (0.25 keV). According to the results above, we can state that the druse is composed mainly of calcium (0.13%), followed by oxygen (0.06%) and carbon (0.02%) (Fig. 3C and D).

Discussion

Anatomical characterization

Characteristics such as hypoestomatic leaf, epidermal cells with straight anticline walls, paracytic and anomocytic stomata, uni and multicellular tector trichomes and dorsiventral mesophyll are common occurrences for the family, among the representatives of Rubiaceae (Metcalfe and Chalk, 1979), as verified in *Tocoyena formosa* (Cham and Schlitd.) K. Schum. (Coelho et al., 2006) and *Psychotria viridis* Ruiz & Pav. (Quinteiro et al., 2006).

The scarce presence of uni and multicellular tector trichomes can be verified, besides glandular trichomes. The presence of glandular trichomes is a characteristic that differs from the results achieved by Santos et al. (2006) and Erbano and Duarte (2010) in *G. americana* leaves. Some studies have indicated that exogenous factors such as herbivory, temperature, luminosity, water availability and seasonality are able to influence the formation and functioning of glandular trichomes in different plant groups (Viljoen et al., 2005; Gonzáles et al., 2008; Martínez-Natarén et al., 2011). The presence of idioblasts is detected in the parenchymatic region, external and internally to the vascular cylinder, which present crystalline inclusions in the form of druses. Superior plants commonly present crystals stored in their tissues, being related to physical protection, removal of oxalate from the metabolic system, storage of calcium, and regulation of light during photosynthesis (Franceschi and Nakata, 2005).

The crystal type is an important diagnostic feature for genus and species of Rubiaceae. The presence of crystals like raphide, druse, styloid, prismatic or crystalline sand can be confirmed in species belonging to this family, being common the presence of druse and crystalline sand for *Genipa* genus (Metcalfe and Chalk, 1979). Raphides were noticed in *Palicourea longepedunculata* (Pereira et al., 2003), crystalline sand in some species of the genus *Rondeletia* L. (Kocsis et al., 2004) and druse in *T. formosa* (Coelho et al., 2006). Erbano and Duarte (2010) confirmed the presence of calcium oxalate druse on leaves and stem of *G. americana*. Moraes et al. (2011) report the presence of styloid prismatic and rusty crystals on leaves of some species of the genus *Psychotria*. The birefringence presented by druse in the analyzed species is considered

an anisotropic characteristic of crystals that have a chemical composition of a single element. Crystals that have mixed chemical composition are referred to as non-birefringent, making it impossible to evaluate the different refractive indexes that they possess, since they present alterations in optical properties in relation to pure crystals (Hammond, 2009; He et al., 2012).

Phytochemistry determination and histochemical characterization

The cuticle shape is dependent on the compounds present in the wax. Asymmetric secondary alcohols and β-diketones form wax spaces in the form of nanotubes, whereas primary alcohols and symmetrical secondary alcohols form flat plates (Hallam, 1967; Jeffree et al., 1975). Differences in cuticle patterns can be taxonomically useful in generic terms and even between species (Metcalfe and Chalk, 1979; Cutter, 1986). The fibers are associated with the vascular system, as cited by Metcalfe and Chalk (1979) for Rubiaceae.

Analyses revealed phenolic compounds intensely visible throughout the palisade parenchyma, being also observed in the adaxial epidermal cells of less intense form. Studies have also reported the presence of phenolic compounds in different species of the Rubiaceae family, such as *Chomelia obtusa* Cham. & Schltdl. (Barros et al., 2008), *Palicourea rigida* Kunth (Rosa et al., 2010) and *G. americana* (Porto et al., 2014).

Most studies on essential oils in this species are limited to fruits only. Some studies indicate that these metabolites are composed mainly of carboxylic acids, esters and alcohols (Borges and Rezende, 2000; Pino et al., 2005; Pinto et al., 2006). In the analyzed leaves, the essential oils present a greater incidence of distribution in the abaxial face, being found in the form of granules with storage site in the epidermal and subsidiary cells.

The iridoids are present in most of the species of Rubiaceae, constituting an important chemical and pharmacological marker in *G. americana* (Alves and Ming, 2015). According to the analyses, the accumulation site of these substances was observed in the phloem cells and in some palisade cells, nearby these vessels. The presence of these compounds is observed in *Eucommia ulmoides* Oliv. (Hirata et al., 2011; Nam et al., 2013; Zhang et al., 2013), *Bellia trixago* (L.) All. (Venditti et al., 2013), *Gardenia jasminoides* J. Ellis (Zhang et al., 2013), *Castilleja tenuiflora* Benth (Carrillo-Ocampo et al., 2013).

In fruits of *G. americana* were identified the presence of different phytosteroids (Barbosa, 2008). Conceição et al. (2011) report that the presence of steroids in extracts of *G. americana*, being related to anticancer activity. There are few studies that indicate the presence of these metabolites in the leaves. The assays performed in this work revealed that, in the leaves, these substances are stored in idioblasts located in the perivascular regions, close to the phloem.

The phytochemical, results in the search for proanthocyanidins, contributed with the histochemical tests, where little reddish coloration was observed inside some cells of the palisade parenchyma. According to Porto et al. (2014), there is a low accumulation of these substances in this species, corroborating the histochemical results described for the leaves analyzed in this study.

Morphological analyze and elemental composition of crystals

Scanning electron micrographs, coupled with spectrometric and histochemical assays, revealed the presence of crystalline inclusions of the druse type of rosette crystals (whewellite) throughout the leaf blade. From these tests, the chemical composition of calcium oxalate of these crystals was confirmed. The presence of these inclusions, the determination of their chemical nature and their morphology, as well as the location site within the medicinal plant, can help in the characterization of taxonomic groups, becoming relevant in the identification and purity of plant drugs (Metcalfe and Chalk, 1979; Monje and Baran, 2002; Anitha and Sandhiya, 2014).

In the last decades, many plant crystals made of calcium oxalate have not been accurately identified. This biomineral occurs in two hydration states in plants: monohydrate (whewellite) or dihydrate (weddellite) (Franceschi and Horner Junior, 1980; Frey-Wyssling, 1981; Arnott, 1982). Monje and Baran (2002), in their works distinguished the whewellite of the weddellite druse from its star-like forms. Different crystalline morphotypes can be observed in Rubiaceae as styloid crystals, raphide and druses (Metcalfe and Chalk, 1979). Zini et al. (2016) report the presence of druses in *Borreria latifolia* K. Schum., *B. orientalis* E.L. Cabral, R.M. Salas & L.M. Miguel, *B. palustris* (Cham. & Schltdl.) Bacigalupo & E.L. Cabral. Styloid crystals are observed in some species of the genus *Psychotria* L. Erbano and Duarte (2010) report in general the presence of druses into leaves and stem of *G. americana*, but details about the morphological analyze, elemental composition and histolocalization of these inclusions in medicinally important leaves are scarce in the literature referring to these species. These druses can be considered relevant structures to the ultrastructural anatomic characterization.

Conclusion

The anatomical characteristics commonly attributed to the representatives of Rubiaceae are not considered relevant in interspecific differentiation, since they are of general occurrence for the family. In response to this, the use of SEM becomes a useful tool in the anatomical characterization of the *G. americana* foliar ultrastructures. Besides the phytochemistry, the histochemistry allowed to obtain more specific data, determining the exact storage site of these substances in the leaf. The characterization of the crystalline macro-pattern, as well as its exact elemental composition, can be considered an important factor of differential diagnosis. The results presented allowed the characterization of this species in different aspects and point to *G. americana* as a promising native species with different popular uses and proven pharmacological activities, and further studies are needed.

Authors' contributions

ALV and AVS assisted in carrying out the laboratory work. RJRP and LCA helped in conducting the scanning electron microscopy (SEM) analyses. ALV wrote the paper and KPR provided a critical reading of the manuscript. ALV and KPR planned the project, collected the plant material and was responsible for its identification. KPR supervised the laboratory work. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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