Rodriguésia 73: e02302020. 2022
http://rodriguesia.jbrj.gov.br
DOI: http://dx.doi.org/10.1590/2175-7860202273032

Original Paper

Pharmacobotanical study of *Croton floribundus* stem bark

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Abstract

Croton floribundus, Euphorbiaceae, popularly known as "capixingui" is a native tree of the Atlantic Forest. In folk medicine, the tea of *C. floribundus* stem bark is used for the treatment of leukemia, tumors, and syphilis. The aim of this work was to describe the morphological and anatomical characteristics of *C. floribundus* stem bark and to establish parameters for its quality control. Accordingly, different analyses were performed, including organoleptic, morphological, anatomical, and histochemical analysis of the samples, plus the characterization of druse crystals by energy-dispersive X-ray spectroscopy. The sample showed menthol and camphor odor, and bitter taste. The main macroscopic characteristic was the presence of a thin periderm with striations. The main distinctive microscopic features of the species were: dense gelatinous fibers, phenolic idioblasts, sclereids, numerous crystalline idioblasts with druses located in the cortex and abundantly in the parenchymal rays of the phloem, and absence of laticifers in the mature stem bark. These analyses made possible to describe the morpho-anatomy of that species, contributing to phytochemical and pharmacognostic future studies of *C. floribundus*.

Key words: energy-dispersive X-ray spectroscopy, Euphorbiaceae, histochemistry, polyphenols, quality control.

Resumo

Croton floribundus, Euphorbiaceae, conhecida popularmente como "capixingui" é uma árvore nativa da Mata Atlântica. Na medicina popular é relatada a atividade do chá da casca caulinar de *C. floribundus* para o tratamento de leucemia, tumores e sífilis. O objetivo deste trabalho foi descrever as características morfoanatômicas e estabelecer parâmetros para o controle de qualidade da casca caulinar de *C. floribundus*. Para isso, diferentes ensaios foram realizados, incluindo a análise organoléptica, morfológica, anatômica e histoquímica das amostras, além da caracterização dos cristais do tipo drusa por espectroscopia por energia dispersiva de raios-X. A amostra apresentou odor de mentol, cânfora e sabor amargo. A principal característica macroscópica foi a presença de periderme delgada com estriações. As principais características microscópicas distintivas da espécie foram: fibras gelatinosas calibrosas, idioblastos fenólicos, esclereides, numerosos idioblastos cristalíferos contendo drusa localizados no córtex e abundantemente nos raios parenquimáticos do floema, e ausência de laticíferos na casca caulinar. Com os resultados das análises foi possível descrever a morfoanatomia dessa espécie, contribuindo com futuros estudos fitoquímicos e farmacognósticos de *C. floribundus*.

Palavras-chave: espectroscopia por energia dispersiva de raios-X, Euphorbiaceae, histoquímica, polifenóis, controle de qualidade.

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Introduction

Croton L. is a large, diverse and very important genus of the Euphorbiaceae, a family of great economic value, which is found in the tropics and subtropics. *Croton* means "tick" in the indigenous language, because of the similarity of its seeds to this arthropod. The genus was described by Carl Linnaeus in 1753 and comprises over 1,100 species with a total of 73 species registered as synonyms (POWO 2021). According to Caruzo *et al*. (2020a), there are 300 accepted species and 230 endemic species in the country. Many of these species have been shown to have medicinal potential, but there are few pharmacological studies in the scientific literature, despite their wide use in folk medicine (Webster 1993; Hiruma-Lima *et al.* 2002; Trindade & Lameira 2014).

Croton floribundus Spreng., Euphorbiaceae, popularly known as "capixingui", is a tree native but not endemic to Brazil, present in the phytogeographic domain of the Atlantic rainforest and widely distributed throughout Brazil (Caruzo *et al*. 2020b). Until now, the main chemical constituent of *C. floribundus* is caur-16-en-19-oic acid (kaurenoic acid), which makes up 2% of the dry plant (w/w), and it has been isolated from the stem bark of the tree (Medina *et al.* 2009).

Several biological activities have been reported in the literature for kaurenoic acid, including antidiabetic, smooth muscle relaxant, analgesic, anti-inflammatory, diuretic, antioxidant, antitumor (leukemia, breast, and colon cancer), useful in neurological diseases (anticonvulsant, anti-Alzheimer), antibacterial, antifungal, antiprotozoal, antiviral, insecticide, anthelmintic, and molluscicidal activities (Villa-Ruano *et al.* 2016). Compounds present in *Croton* species have demonstrated cytotoxicity in different cell lines, such as labdanes isolated from *Croton oblongifolius* Delile (Sommit *et al.* 2003), and the dichloromethane extract of *Croton macrobothrys* Baill. (Motta *et al.* 2011). In addition, it is necessary to be mindful of the toxicity of *Croton* species, such as the seed oil of *Croton penduliflorus* Hutch., demonstrated by Ojokuku *et al.* (2015) to be toxic to the kidney and liver of mice. On the other hand, few studies have reported on the quality control of *C. floribundus* stem bark, such as its botanical analysis. Accordingly, the aim of this work was to describe the morpho-anatomical characteristics of *C. floribundus* stem bark, contributing to future studies on the quality control and collaborating with the pharmacognostic identification of this species.

Material and Methods

Plant Material

The stem bark of *C. floribundus* was collected at the Campus of the Universidade Estadual de Maringá (23º24'W, 51º56'S), Maringá, Paraná, Brazil, in April 2016 by N.C. Gancedo. The collection of the plant material was registered with IBAMA-SISBIO No. 11995-6, under the responsibility of João Carlos Palazzo de Mello. Access to genetic heritage was registered by the Brazilian Biodiversity System, SisGen, *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* under Nº. A22A035. The plant material containing inflorescences was used to prepare a voucher specimen and stored at the Universidade Estadual de Maringá Herbarium (HUEM 30778). The collected material was compared and identified with exsiccate of the species deposited in HUEM.

Morpho-anatomical Analysis

The macroscopic characterization of *C. floribundus* stem bark was based on the notes of Oliveira *et al.* (2014). For the morpho-anatomical description, young branches (1st to 3rd internode, thickness less than 1 mm), bark of mature branches (thickness over 1 mm), and mature stem bark (thickness ≥ 4 mm) of *C. floribundus* were used. For analysis by light microscopy (LM) and scanning electron microscopy (SEM), the samples were sectioned freehand in the transverse, longitudinal radial, and tangential longitudinal planes. The samples were fixed for 48 h in FAA50 (5% formaldehyde, 5% acetic acid and 90% ethanol:water mixture; 50:50, v/v) (Johansen 1940) and 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (Kraus & Arduin 1997) for anatomical analysis under LM and SEM, respectively, after storage in 70% ethanol (Johansen 1940).

For LM, the sections were bleached with sodium hypochlorite solution (30%) for 10 min, washed with distilled water, double-stained with Astra blue (1%) and safranin (1%) in 0.1 M phosphate buffer, pH 7.2), and mounted on semi-permanent slides with glycerin jelly (Kraus & Arduin 1997). For SEM analysis, the sections were dehydrated in an ascending ethanol series, ending in absolute ethanol for 10 min twice, and critical-point dried with CO_2 (Balzers CPD 30 critical-point dryer) (Horridge & Tamm 1969). The dried samples were positioned on the different

anatomical planes on metal stubs, attached with double-sided carbon tape, and sputter-coated with gold in a Shimadzu IC-50 unit. A FEI Quanta 250 microscope (15 kV) was used for ultrastructural analysis. A morphological and chemical analysis of the crystals present in the bark of *C. floribundus* was performed using energy-dispersive X-ray spectroscopy (OXFORD AZtec) coupled with the electron microscope mentioned.

Histochemical tests were done in transverse sections, which were stained with the following: Lugol's iodine solution to reveal the presence of starch grains (Johansen 1940); iodinated zinc chloride for lignin (Jensen 1962); Sudan IV for lipophilic substances (Kraus & Arduin 1997); ferric chloride for polyphenols (Johansen 1940); chloral hydrate (60%) with sulfuric acid (25%) for calcium oxalate crystals (Sass 1951) and 2-4-dinitrophenylhydrazine for terpenoids with a carbonyl group (Ganter & Jolles 1969-1970).

The powdered plant material was rehydration, and semi-permanent slides were prepared for LM, as described above. The images of semi-permanent and histochemical test slides were obtained with a Nikon® Eclipse 80i light microscope equipped with a Nikon DS-FI1C cooled digital camera and analyzed using the program D-DA simple analyzer.

The color, texture, odor, and flavor of the *C. floribundus* stem bark were determined for organoleptic characteristics, according to the notes of Oliveira *et al.* (2014).

Results and Discussion

Croton is a diverse and complex genus. The diversity of species, including new and synonymous species, justifies the need to update the taxonomy and morphology of this plant group. According to Farias *et al.* (2009), there are few anatomical studies of the stem bark of *Croton* species. Even in official monographs, such as *Croton cajucara* Benth., there is no anatomical description of the stem bark of this species, despite the medicinal use of this organ (ANVISA 2015). The botanical and organoleptic description of plant species with medicinal potential serves as parameters for quality control evaluation. An analysis to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials, and should be carried out before any further tests are undertaken. In addition, when used in association with other analytical methods, these descriptions supply extremely important information that helps the accurate authentication of plant species (Shinde *et al.* 2009; WHO 2011; Efferth & Greten 2012; Drouet *et al*. 2018). When thinking about herbal drug production, adulteration of plant species is considered a big problem. This question can be solved by pharmacognostic studies that basically deal with standardization, authentication, and analysis of medicinal plants and herbal drugs (Chanda 2014). Furthermore, Zafar *et al.* (2019) observed that macroscopic and microscopic studies using LM and SEM as quality control tools, provided the basis for the authentication of plant species. In this context, the identity of *C. floribundus* can be determined after the analysis of the morpho-anatomical characteristics of the stem bark of this species. It is understood that the stem bark consists of all tissues located outside the stem vascular cambium (Oliveira *et al.* 2014).

The young branches of *C. floribundus* (Fig. 1a) have outer surface rich in trichomes, which when cross-sectioned secrete slightly whitish transparent latex in abundance, showing no oxidation (Fig. 1b). Trichomes are structures that can contribute to grouping *Croton* species. Two major trichomes types are found in the genus: stellate and lepidote. However, within both stellate and lepidote types, there is much variation. Stellate trichomes include variations such as stellate-porrect, stellate-dendritic, and stellate-lepidotes, as in *Croton palanostigma* Klotzsch and *Croton pullei* Lanj. Lepidote-type trichomes are found in a smaller number of species, as in *Croton cuneatus* Klotzsch and *Croton sacaquinha* Croizat (Webster 1996; Secco 2008). The mature branches have an outer surface with anastomosed longitudinal striations and lignified lenticels (Fig. 1c), characteristics also present in *Croton draco* Schltdl. & Cham. (Farias *et al.* 2009). The mature branches with a diameter greater than 0.5 cm, secrete orange latex in the regions of the pith and bark, which oxidizes, acquiring an orange-brown color (Fig. 1d). The mature stem bark has a rough outer surface, with deep transverse fissures, delicate longitudinal striae, and a grayish color. The presence of lichens on the outer surface is common (Fig. 1e-f).

The inner surface of mature stem bark is finely striated in the direction perpendicular to the largest axis of the organ and has a pinkish color (Fig. 1e), highlighted by the marbled white and pink color when fresh (Fig. 1f). It is observed that the bark of the trunk of *C. floribundus* does not exude latex after incision, differing from other species of *Croton* that release reddish latex after incisions,

such as *Croton lechleri* Müll. Arg., *Croton draconoides* Müll. Arg. and *Croton lanjouwensis* Jabl. (Amaral *et al.* 2005). According to Webster (1994), latex can be present or absent in *Croton* species. When present, the latex is usually colored. After drying, the stem bark acquires a curved shape and relatively homogeneous pinkish-brown color. The fracture is fibrous, as observed in *C. lechleri*, and differing from *C. lanjouwensis*, which presents a fibrous fracture in the inner surface and granular in the outer surface of the stem bark (Amaral *et al.* 2005). The stem bark has a characteristic odor of menthol and camphor and a bitter, astringent taste, which is slightly spicy.

In the anatomical analysis of young branches, the epidermis is unilayered, with an abundance of stellate non-glandular trichomes (Fig. 2a-c) and few bicellular glandular trichomes, with on short-stalked cell in the insertion region and a large secretory head (Fig. 2d) as observed in leaves of eleven Brazilian species of Crotoneae (Vitarelli *et al.* 2015). Subepidermally, there are four layers of angular collenchyma, followed by five to six layers of chlorophyll parenchyma (Fig. 2a-c). Throughout the cortex and especially in the phloem, long, large-caliber, unbranched articulated laticifers are abundant (Fig. 2e), as well as amyloplasts and idioblasts with druses. The vascular bundles are collateral. The stem pith is composed of flattened to isodiametric cells in the tangential longitudinal section and idioblasts with druses. Centrally, there is a wide secretory channel of the lysogenic type, with type of secretion not identify (Fig. 2f).

The stem bark of mature branches has a thin periderm, consisting of two to three layers of compacted cells, similar to that observed by Farias *et al.* (2009) in *C. draco*, maintaining this pattern also in mature stem bark. In *C. floribundus*, the periderm cells have reddish content and react positively with ferric chloride, showing the presence of phenolic compounds. In the cortical region, there are approximately twenty layers of chlorenchyma, responsible for the green coloration of the young and mature branches, and which remain in the trunk.

In mature stem bark, the cortical parenchyma region undergoes an increase in the number of parenchymal cells, being divided into two regions. The first, underlying the epidermis, has about six to ten layers of cells of reduced diameter and circular shape, thin walls, and reduced intercellular spaces, but with some cells with a collenchymatous

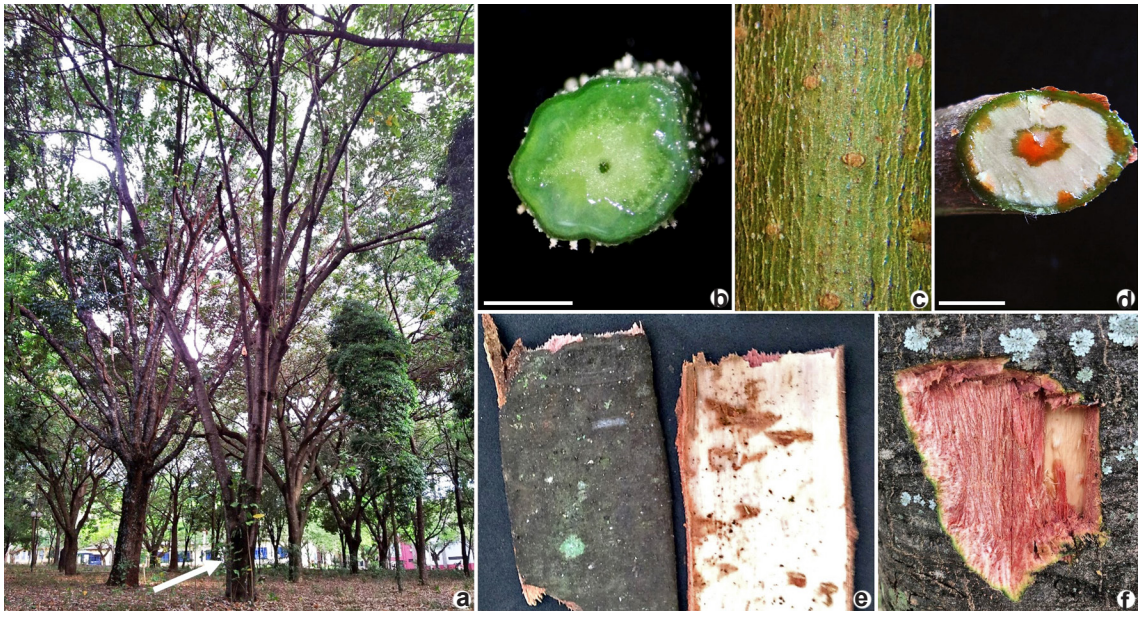

Figure 1 – a-f. Macroscopic analysis of *Croton floribundus* – a. the general appearance of the specimen grown at the Campus of the Universidade Estadual de Maringá (arrow); b. young branch with latex exudate, in cross-section; c. detail of anastomosed longitudinal striations of mature branches; d. mature branch over 0.5 cm in diameter, with latex exudate in the region of the bark and pith, in cross-section; e,f. appearance of the outer and inner side of the mature stem bark. Bar a: 0.2 cm and bar b: 0.4 cm

appearance, when in cross-section (Fig. 3a-c). Included in this parenchyma are few sclereids, isolated or in groups whose pits are simple or branched (Fig. 3d).

The second region of the cortex is characterized by the presence of larger-diameter parenchyma cells of various sizes and shapes, usually square or rectangular in radial longitudinal section. These cells are formed by thinner walls compared to cells of the external parenchymatic region, and there are few intercellular spaces, in cross-section (Fig. 3a-c). This region contains, in larger numbers, groups of six to twelve elements of large-caliber gelatinous fibers (40 to 100 µm in diameter), with abundant lamellations and simple or branched pits (Fig. 3a,b). Such groups of fibers were also observed in the samples of *C. floribundus* analyzed by Dias-Leme & Angyalossy-Alfonso (1998), being interrupted by parenchyma cells, forming a single arc near the phloem.

In the vascular cambium region, near the phloem, the gelatinous fibers show retracted inner layers (Fig. 4a,b), which was also observed in *Croton urucurana* Baill. by Luchi (2004), because the cellulosic lamellae ('G' layer) detach from the lignified layer. Gelatinous fibers are present in many genera of Euphorbiaceae, as reported by Mennega (2005), being common in *Croton* species, and abundant in the secondary xylem of young *C. echioides* Baillon stems (Novello *et al.* 2012). In this cortical portion, we also found amyloplasts (Fig. 4c) and idioblasts with druses (Fig. 4d) that reacted positively with 60% chloral hydrate with 25% sulfuric acid. Energy-dispersive X-ray spectroscopy analysis of these crystals showed calcium (20.3%), carbon (27.4%), and oxygen (41.3%) peaks (Fig. 5).

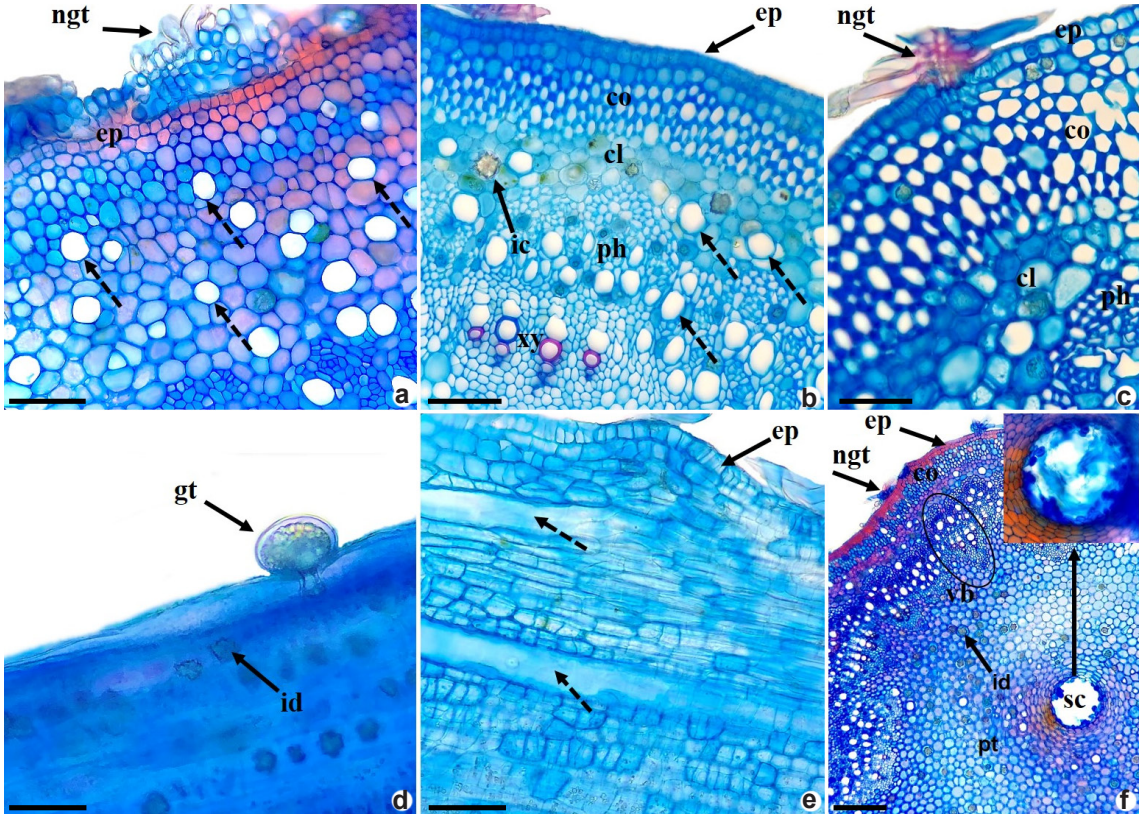

Figure 2 – a-f. Light microscopy of anatomical sections of young branches of *Croton floribundus* – a-c. epidermis (ep), collenchyma (co) and parenchyma cells present in the first, second and third internode, in cross-section, respectively; b. druse cristal idioblasts (id) and chloroplasts (cl); d. glandular trichome (gt), in tangential longitudinal section; e. articulated unbranched laticifers, in tangential longitudinal section (dotted arrows); f. the general appearance of the cortex, collateral vascular bundles (vb), pith (pt), and detail of the internal secretory channel (sc) in the third internode. ph = phloem; xy = xylem; ngt = non-gladular trichome. Bar a-f: 100 µm.

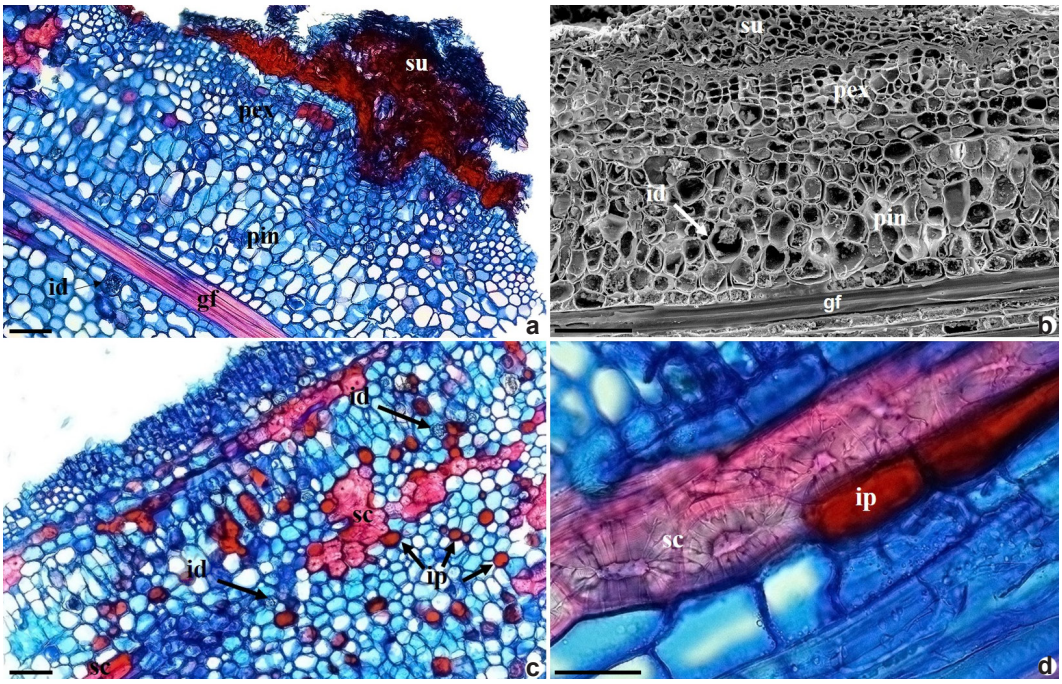

Figure 3 – a-d. *Croton floribundus* mature stem bark – a,b. suber (su), external (pex) and internal (pin) cortical parenchyma, crystalline idioblast (id) (arrows), and gelatinous fiber (gf), in longitudinal sections; c. general view of the cortex, in cross-section; d. detail of sclereids (sc), and idioblasts with polyphenols (ip), in longitudinal section. a, c, d: LM; b: SEM. Bar a and c: 100 µm; b: 150 µm; d: 50 µm.

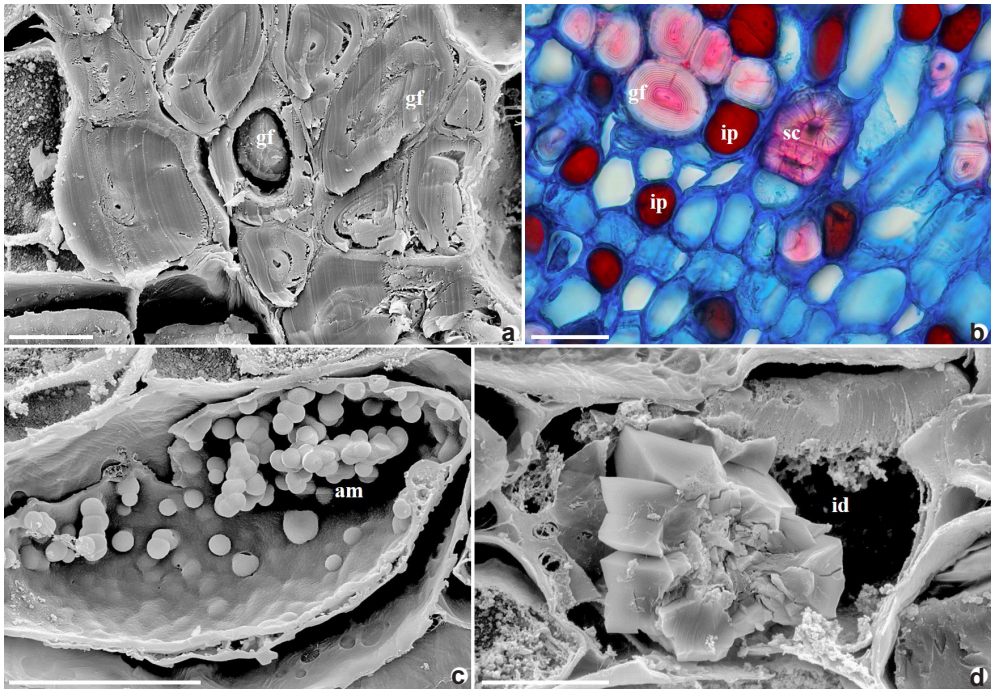

Figure 4 – a-d. *Croton floribundus* mature stem bark – a,b. groups of gelatinous fibers (gf) and sclereids (sc), in cross-section, respectively; c,d. detail of the amyloplast (am) and idioblast (id) with crystals, respectively. ip = idioblasts with polyphenols. a, c, d: SEM, b: LM. Bar a: 20 µm; b: 50 µm; c: 30 µm; d: 10 µm.

Druses observed in the cortical parenchyma of *C. floribundus* were not reported in *C. echioides* (Novello *et al.* 2012), but were abundant in *C. lechleri* and *C. lanjouwensis* (Amaral *et al.* 2005). Idioblasts with dense content reacted positively with ferric chloride and Sudan IV, confirming the presence of polyphenols (more abundant) and lipid substances, respectively, which are more frequent in thicker stem bark. In this region, there are idioblasts with fine and spherical inclusions, which did not react with Lugol's solution or 2-4-dinitrophenylhydrazine. The sclereid and fiber groups were found to be responsible for the white color of the marbled appearance of the fresh stem bark.

The phloem rays are formed by one to three (rarely four) series of cells, and druses occur in practically all of them. In the samples analyzed, laticifers could not be differentiated in the cutting planes analyzed, and according to Rudall (1994), laticifers in *Croton* species are less abundant in older stems than in young stems, as observed in *Croton comosus* Müll. Arg.

Histochemical assays confirmed the presence of idioblasts with polyphenolic content in the phloem (Fig. 6a), starch (Fig. 6b), and calcium oxalate druse crystals (Fig. 6c).

The analysis of *C. floribundus* powder showed fragments containing thick fibers with evident pits (Fig. 7a), groups of sclereids with simple and branched pits (Fig. 7b), parenchyma fragments with square and rectangular cells together with starch grains and/or idioblasts with druses (Fig. 7c-e).

The botanical characteristics are useful for differentiation of *C. floribundus* stem bark from other species of genus described in the literature, and provide parameters to authentication and quality control of this species. The analysis of the morphological and anatomical characteristics of *C. floribundus* stem bark revealed a complex structure, formed by several cell types. The principal distinctive microscopic characteristics of *C. floribundus* stem bark are the absence of laticifers and the presence of druses as the only type of crystal. Furthermore, this study demonstrates the need to realize detailed morpho-anatomical studies of families with medicinal plants, such as Euphorbiaceae, establishing standardization parameters useful for authentication and quality control of plant species.

Acknowledgements

The authors thank Prof. Dr. Eneri Vieira de Souza Leite-Mello, for allowing the use of the light microscope with camera; Complex of Research Support Center at State University of Maringá, for allowing the use of the electron microscope; and Admir Arantes, for technical support. CNPq for the master's scholarship granted. Dr. A. Leyva (USA) provide English editing of the manuscript.

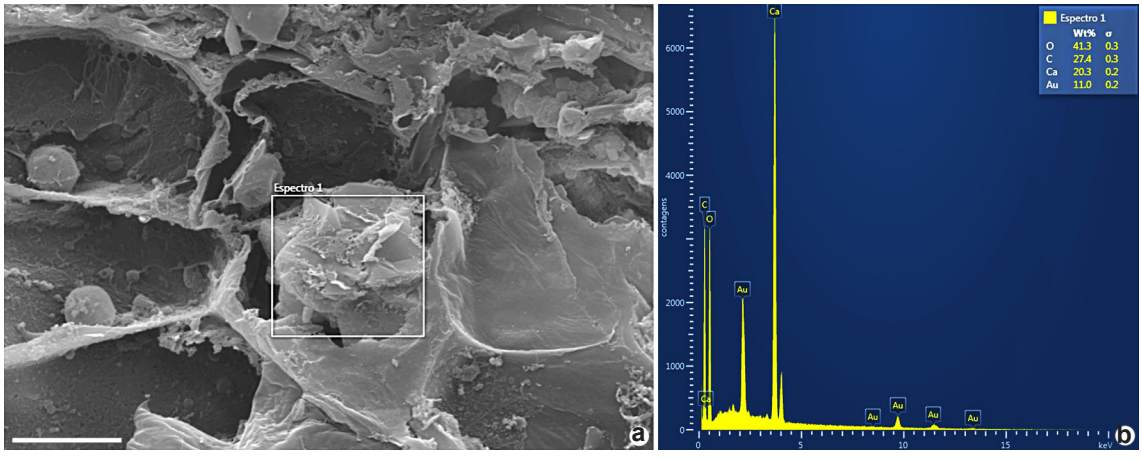

Figure 5 – Druse in *Croton floribundus* stem bark (a) and spectrum of the energy-dispersive X-ray spectroscopy analysis (b). Bar a: 10 µm.

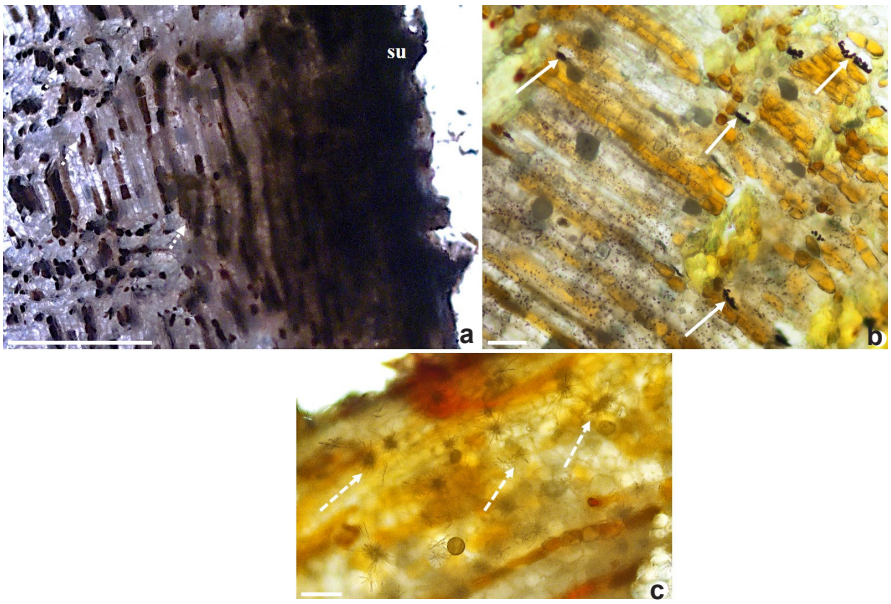

Figure 6 – a-c. Histochemical tests of *Croton floribundus* mature stem bark – a. the reaction of the suber (su) and idioblast polyphenolics with ferric chloride (dotted arrows); b. starch identification (compact arrows) with Lugol's solution; c. reorganization of calcium sulfate acicular crystals (dashed arrows) after exposure of calcium oxalate druse to chloral hydrate solution with sulfuric acid. Bar a: 500 µm; b-c: 100 µm.

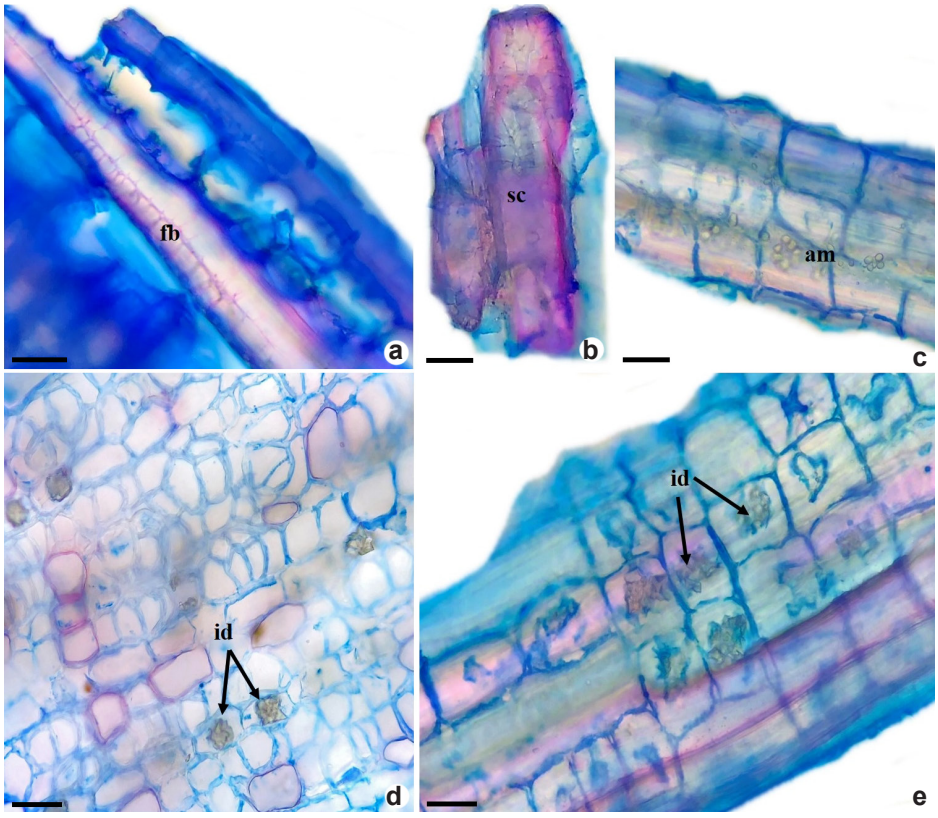

Figure 7 – a-e. Rehydrated powder fragments of *Croton floribundus* stem bark – a. fiber (fb); b. sclereids (sc); c. starch grains (sg); d,e. idioblast (id) crystals. Bar a-e: 100 µm.

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Area Editor: Dr. Leopoldo Baratto
Received in January 05, 2021. Accepted in April 28, 2021.