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Original Article

Morphoanatomical and physicochemical profile of *Piper callosum*: valuable assessment for its quality control

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ABSTRACT

Piper callosum Ruiz & Pav., Piperaceae, popularly known as "elixir-paregórico" and "matricá" in Brazil, is used in folk medicine to treat gonorrhea, general pain, and digestive disorders, and has repellent, astringent, diuretic, depurative, and haemostatic properties. Despite the fact that this plant is sold as a traditional phytotherapeutic product, we did not find reports on its quality control. We, therefore, performed macroscopic, microscopic, histochemical, and physicochemical analyses using standard methods to establish botanical authentication and purity degree parameters for leaves and stem of this species in two forms: medicinal plant and herbal drug. We observed the size, shape, color, texture, fracture surface and transection characteristics, leaf venation patterns, and calluses are valuable diagnostic characters to identify the herbal drugs when they are not ground or powdered. Since medicinal plants and herbal drugs did not differ anatomically, the following key anatomical characters for *P. callosum* can be used for diagnostic purposes of both types raw plant materials: epicuticular wax and cuticular flanges patterns; collenchyma features; fibers in the midrib; arrangement pattern of the vascular bundles of the midrib and petiole; shape of the midrib, leaf margin, petiole, and stem; occurrence of raphides; and morphology of the starch grains. Acid lipids, essential oils, oleoresins, steroids, tannins and flavonoids were histochemically identified. Total ash (leaves: 11.25%; stem: 5.25%), sulphated ash (leaves: 68.02%; stem: 12.50%), acid-insoluble ash (leaves: 2.82%; stem: 0.27%), moisture (leaves: 8.60%; stem: 6.10%), loss on drying (leaves: 11.08%; stem: 8.58%), and pH (leaves: 5.57, stem: 5.28) values were determined. The order of analyzed metal levels in leaf and stem herbal drugs was Al > V > Cu > Mn > Cr > Ni. Similar levels of Cd and Co and low levels of Hg were found. The results obtained can be used as quality control parameters for medicinal plants and herbal drugs of *P. callosum*.

Introduction

Piper callosum Ruiz & Pav., Piperaceae, popularly known as "elixir-paregórico," "óleo-elétrico," "ventre-livre," "erva-de-soldado," "panquilé," "matricá" and "joão-brandin" in Brazil (Andrade et al., 2009), is a shrub native to Bolivia, Brazil, Peru, and Colombia. In Brazil, it occurs in Acre, Amazonas, Amapá, Pará, Rondônia, Distrito Federal, Mato Grosso, Espírito Santo, Rio de Janeiro, and Paraná States (Guimarães et al., 2014).

In Brazilian folk medicine, *P. callosum* leaves and young stem are used in the form of infusion or poultice to treat dysmenorrhea, intestinal colic, diarrhea, nausea, toothache, rheumatic and muscular pain, mosquito bites, and gonorrhea, and have repellent, astringent, haemostatic, digestive, diuretic, and depurative properties (Andrade et al., 2009). At open-air markets in northern Brazil, vegetative aerial parts of *P. callosum* are sold fresh, dried, ground, and rarely powdered or as an ingredient in artisanal preparations called "garrafadas" for medicinal purposes. The plant is also cultivated in backyards and medicinal gardens (authors' observations).

A number of volatile and fixed phytoconstituents have been isolated from *P. callosum*, including alkaloid amides; terpenes, such as hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes, and steroids; and phenolics, such as oxygenated flavonoids and phenylpropanoids (Parmar et al., 1997; Facundo et al., 2004; Andrade et al., 2009). Studies of essential oils obtained from *P. callosum* have demonstrated antifungal, insecticidal, and larvicidal activities (Andrade et al., 2009).

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P. callosum, currently being traded as a traditional phytotherapeutic product, represents a promising medicinal plant for phytopharmaceutical development due to the ethnopharmacological evidence for the numerous popular medicinal uses attributed to this plant and of the pharmacological potential of its phytoconstituents. Despite this, we did not find any systematic reports of its quality control parameters. The quality of raw plant materials represents the first step for the establishment of minimum criteria of acceptance and is a pre-requisite for the production and registration of phytomedicines (Couto et al., 2013; Anvisa, 2014). Hence, the present work aimed to establish parameters of botanical authentication and purity degree for the quality control of *P. callosum* leaves and stem as raw plant materials in forms of medicinal plant and herbal drug.

Materials and methods

Plant material

Fertile samples ($n = 14$ specimens; 7 specimens per sampled area) of *Piper callosum* Ruiz & Pav., Piperaceae, were collected from natural populations of two Brazilian states: Manaus-AM, and Belém-PA. A voucher specimen (MG 206892) was deposited at the João Murça Pires (MG) Herbarium of the Emílio Goeldi Paraense Museum. The taxonomic identity was confirmed by Elsie Franklin Guimarães, specialist in Piperaceae (Rio de Janeiro Botanical Garden Research Institute).

Preparation of the herbal drugs

Aerial parts of *P. callosum* (leaves from the 1st to 4th nodes and stem up to the 4th internode) were washed in 70% (v/v) ethanol and dried at 40 °C in a hot-air oven (Sterilifer SX 1.5 DTMS) until reaching a constant weight (Silva et al., 2016). Part of the leaf and stem herbal drugs were ground to a powder in a knife mill (Marconi MA580). The whole and powdered herbal drugs were stored at room temperature in airtight, light-resistant containers (WHO, 1998).

Pharmacobotanical analysis

Macroscopic and organoleptic characterization was performed on the whole and powdered herbal drugs using standard methods (WHO, 1998; Oliveira and Akisue, 2003; Farmacopeia Brasileira, 2010). The leaf herbal drugs were rehydrated, clarified, and stained for observation of the leaf venation (Silva et al., 2016). The photomacrographs were obtained using a digital camera (Nikon D 3100). The stereoscopic photomicrographs by reflective light (RL) and by differential interference contrast (DIC) were captured with a digital camera (Motic 2500) attached to a stereoscopic microscope (Motic SMZ-168) using Motic Images Plus 2.0 software.

Microscopic characterization was performed on the herbal drugs and fresh plant materials. For the latter, leaf (fully expanded mature leaves from the 4th node) and stem (from the 1st to 4th internodes) samples were obtained according to Silva et al. (2014), fixed in NBF-neutral buffered formalin (Lillie, 1965) and buffered glutaraldehyde/osmium tetroxide (Potiguara et al., 2013), and preserved (Johansen, 1940). NBF and glutaraldehyde/osmium tetroxide-fixed samples were used for light microscopy (LM) and scanning electron microscopy (SEM) observations, respectively.

Epidermal peels of the leaf blade were obtained through maceration in Jeffrey's solution, stained with astra blue, and mounted on glass slides with glycerol jelly (Johansen, 1940). Samples were infiltrated and embedded in methacrylate resin (Historesin, Leica®), and sectioned in a rotary, auto-advance microtome (Leica® RM 2245). The histological sections (transverse and longitudinal, 1.5–3.5 μm thick) were stained with citrate-buffered toluidine blue, pH 4.7 (O'Brien et al., 1964), and mounted on glass slides with synthetic resin (Permount-Fisher®) for structural characterization. Histological sections from fresh plant materials were made by hand with a steel razor and used for histochemical screening (Table 1). For all tests, standard control procedures were carried out simultaneously using the same procedures, and untreated sections were used to verify the natural coloration of the analyzed tissues (white). The photomicrographs by transmitted and polarized light were obtained with a digital camera (Motic 2500) attached to an optical microscope (Motic BA 310) equipped with an epifluorescence unit.

The SEM analysis followed the procedures described by Silva et al. (2014). Samples boiled in chloroform for one hour for partial or total removal of waxy deposits were also used. A Leo 1450 VP scanning electron microscope was used for the observations and capture of images.

Microscopic characterization of the herbal drugs was performed by LM and SEM. The whole herbal drugs were rehydrated and submitted to the above-mentioned methods, apart from histochemical screening. The powdered herbal drugs were processed according to WHO (1998) and Farmacopeia Brasileira (2010) for LM observations. For SEM observations, samples were mounted on SEM metal stubs, following procedures described by Silva et al. (2014).

Pharmacognostical analysis

Pooled samples of the herbal drugs were used for the physicochemical analysis. The total ash, acid-insoluble ash, sulphated ash, pH, moisture (Azeotropic method) and loss on drying (INFRATEST) were determined using standard procedures (WHO, 1998; Farmacopeia Brasileira, 2010). The analytical method to determine the selected metals (Al, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Ti, V, Hg and As) followed Pratsmoya et al. (1997), and the measurements were performed by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Varian model VISTA-MPX spectrometer for Al, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Ti and V, and a Thermo model ICAP 6000 spectrometer for Hg and As. Standard Reference Material (SRM 1547: peach leaves) from the National Institute of Standard and Technology (NIST) was used for validation of the applied analytical method using the same procedures.

All reagents were of analytical grade. Ultrapure water (18.2 MΩ-cm at 25 °C) from a Milli-Q system (Merck Millipore) was used. All determinations were performed in triplicate, and the results were expressed as mean ± standard deviation (mean ± S.D.).

Results

Pharmacobotanical characterization

The herbal drugs of whole leaves are complete, *i.e.* with leaf blade, petiole, and leaf sheath; *ca.* 3.4–8.7 cm long and 1.2–4.6 cm wide; wrinkled or folded; friable in texture; greenish in color on both faces, somewhat bright on the adaxial face; characteristic aromatic odor; taste predominantly characteristic aromatic, turning slightly bitter, and ending slightly spicy. The herbal drugs of powdered leaves are dark-green in color and have the same odor and taste as the whole herbal drugs (Fig. 1A and Q).

Leaf blade is *ca.* 5–9.6 cm long and 2.5–4.9 cm wide; symmetric; ovate-elliptical; entire margin; acuminate apex; cuneate base with callus in basilaminar position on each side of the adaxial face; surface rough to the touch on abaxial face; surface glabrous to the eye on both faces; smooth-granular fracture surface; prominent veins on both faces, mainly on the abaxial face; eucamptodromous major

Table 1
Histochemical screening performed in secretory structures of *Piper callosum*.

Compound groups	Metabolic classes	Reagents (Authors)
Lipids	Total lipids	Sudan black B (Pearse, 1980)
	Acid and neutral lipids	Nile blue A (Cain, 1947)
	Unsaturated lipids	Osmium tetroxide (Ganter and Jollés, 1969, 1970)
	Fatty acids	Copper acetate/rubeanic acid (Ganter and Jollés, 1969, 1970)
Terpene compounds	Essential oils and oleoresins	NADI reagent (David and Carde, 1964)
	Steroids	Antimony trichloride (Hardman and Sofowora, 1972; Mace et al., 1974)
	Sesquiterpene lactones	Abraham reaction (Caniato et al., 1989)
	Terpenoids with carbonyl group	2,4-dinitrophenylhydrazine (Ganter and Jollés, 1969, 1970)
Phenolic compounds	Total phenolics	Ferric trichloride (Johansen, 1940)
	Tannins	Vanillin–hydrochloric acid (Mace and Howell, 1974)
	Flavonoids	Aluminum trichloride[a] (Charrière-Ladreix, 1976)
Polysaccharides	Neutral polysaccharides	Periodic acid–Schiff (PAS) reagent (Feder and O'Brien, 1968)
	Pectins	Ruthenium red (Johansen, 1940)
	Mucins	Mayer's tannic acid-ferric chloride stain (Pizzolato and Lillie, 1973)
	Acid mucopolysaccharides	Alcian blue (Pearse, 1980)
Alkaloids	Total alkaloids	Dragendorff reagent (Svendsen and Verpoorte, 1983)

[a] Observed under UV light.

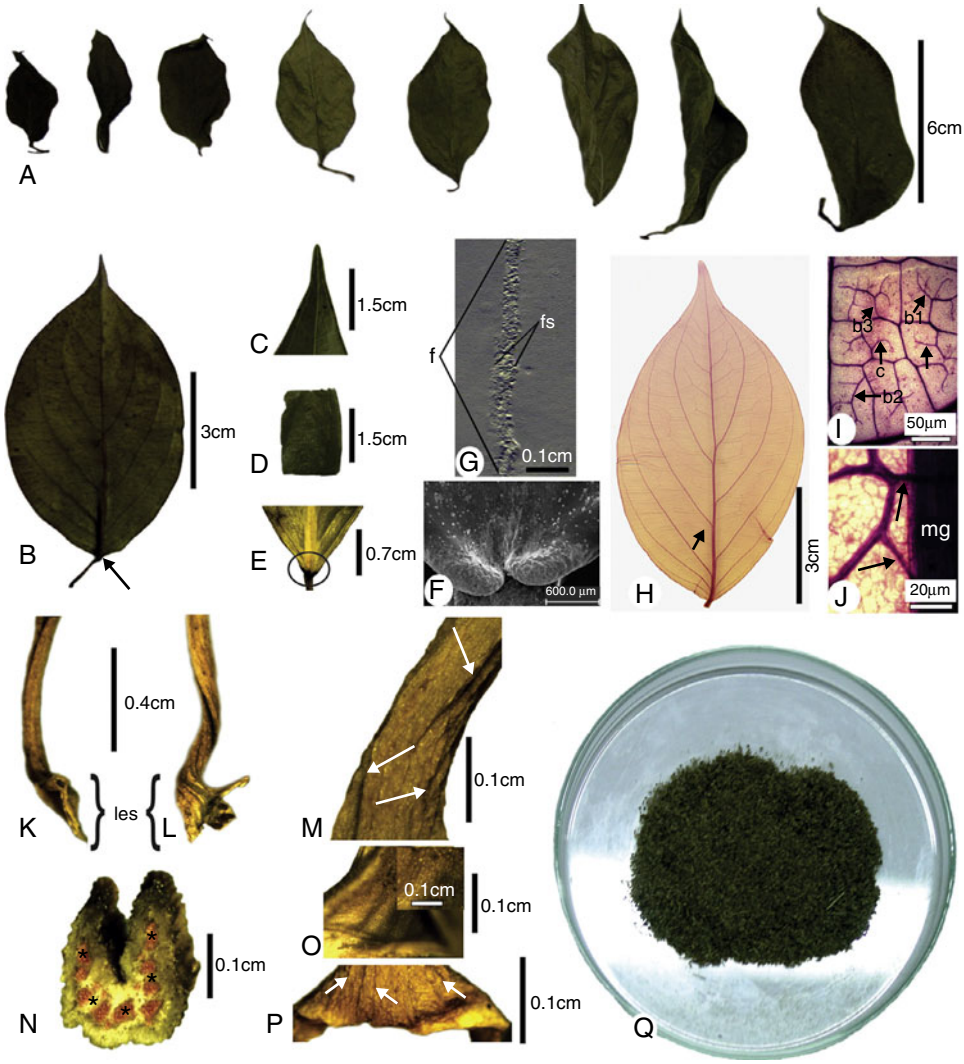

Fig. 1. Macroscopic features of the leaf herbal drugs of *Piper callosum* Ruiz & Pav., Piperaceae. Photomacrographs (A–E; H; Q). Stereoscopic photomicrographs by differential interference contrast (G) and by reflective light (K–P). Photomicrographs by transmitted light (I; J). Scanning electron micrograph (F). A. General morphological aspect. B–E. Hydrated herbal drugs. B. General view of the leaf. Note insertion of the petiole (arrow). C. Apex. D. Margin. E. Base. Note the calluses (ellipse). F. Detail of the calluses. G. Fracture surface of the leaf blade. H–J. Leaf venation patterns. H. Major venation pattern. Note intersecondary vein (arrow). I. Detail of areoles and veinlets. J. Marginal ultimate venation. Note free veinlets (arrows). K and L. General view of the petiole and leaf sheath. Curved (K) and twisted (L) petioles. M and N. Details of the petiole. M. Surface on the adaxial face. Note longitudinal striae (arrows). N. Transection. Note lignified elements (asterisks). O and P. Details of the leaf sheath. O. Adaxial face. Note surface (inset). P. Abaxial face. Note longitudinal striae (arrows). Q. Powdered herbal drug. Fracture (f). Fracture surface (fs). Leaf sheath (les). Linear simple veinlet (l). Curved simple veinlet (c). Veinlet branched once (b1), twice (b2), and three times (b3). Margin (mg).

venation; veins branched until 6° order; incomplete marginal ultimate venation; linear or curved simple veinlets; veinlets branched x1-3; irregular areoles with imperfect development and random arrangement (Fig. 1B–J).

Petiole is *ca.* 0.1–0.7 cm long and 0.05–0.1 cm wide; curved or twisted; inserted laterally; surface longitudinally striate to the eye on both faces; concave–convex in transection, with lignified elements in U-shaped pattern (Fig. 1B, K–N). Leaf sheath is *ca.* 0.025–0.3 cm long and 0.05–0.4 cm wide; concave-convex; surface smooth on the adaxial face and longitudinally striate on the abaxial face to the eye (Fig. 1K, L, O and P).

The herbal drugs of whole stem have evident nodes; surface smooth to the touch; surface glabrous and longitudinally finely striate to the eye; greenish in color; characteristic aromatic odor; taste predominantly characteristic aromatic and ending slightly spicy. The herbal drugs of powdered stem are somewhat fibrous, mixed in color, ranging from yellowish green to gray with black spots, and present the same odor and taste as the whole herbal drugs (Fig. 2A, B and I).

First and 2nd internodes easily broken, with smooth-granular outer fracture surface, and predominantly granular inner fracture surface. In transection, they show primary growth; irregular shape; outer region of the section greenish brown in color; inner region of the section green in color, with yellowish spots; outer and inner lignified elements with no defined arrangement (Fig. 2C–E).

Third and 4th internodes easily broken, with smooth outer fracture surface, and predominantly granular inner fracture surface. In transection, they show initial secondary growth; circular shape; outer region of the section dark-green in color; inner region of the section mixed in color, with brownish yellow central area, and greenish brown elliptical peripheral areas with yellowish spots separated by yellowish strands; inner lignified elements arranged in two concentric circles (Fig. 2F–H).

Fresh leaves and stem and their herbal drugs did not differ anatomically. In frontal view, the anticlinal epidermal cell walls of the leaf blade are straight to wavy on the adaxial face and sinuous on the abaxial face. The cuticle is smooth, with continuous plate of granular epicuticular wax parallel to the epidermal surface on both faces (Fig. 3A–C).

The leaves are hypostomatic and possess tetracytic and cyclocytic (with four or five subsidiary cells) stomata (Fig. 3C–E). Sunken, sac-like glandular trichomes are coated with smooth cuticle and occur randomly spread on both faces of the leaf epidermis. They are bicellular with a chalice-like lignified short basal cell encircled by epidermal cells and a sac-like secretory apical cell, which lies upon the epidermal surface (Fig. 3F).

Transections showed that the leaf epidermis is uniseriate on both faces. The cuticle is thickened, except in the leaf sheath, and forms V-shaped flanges on both faces of the petiole and on the abaxial face of the leaf sheath (Figs. 4A and E, 5D and 6C). Stomata are raised above the level of the other epidermal cells and have guard cells with piriformis lumen and horn-like outer ledges (Fig. 4D). Uniseriate hypodermis occurs on both faces of the leaf blade and is replaced by sclerenchyma in the leaf margin. Some hypodermal cells on the abaxial face contain raphides (Fig. 4A, B, E, H and I).

The mesophyll is dorsiventral with one-layered palisade parenchyma on the adaxial face and three-layered spongy parenchyma on the abaxial face. It is relatively undifferentiated in the leaf margin. Minor collateral vascular bundles are encircled by parenchymatic sheath. Druses are observed (Fig. 4A, B and H).

The midrib in transection is biconvex with 1–2 layers of fibers occurring immediately beneath the epidermis on both faces. The palisade parenchyma cells become gradually shorter toward the middle region. The hypodermal cells, present only on the adaxial face, are comparatively smaller than those of the inter-vein regions. Collateral vascular bundles in a straight line are centrally embedded in the ground parenchyma and encircled by parenchymatic sheath, with fibers in the xylem and phloem poles (Fig. 4E). Raphides are observed in the ground parenchyma (Fig. 4F). The leaf margin in transection is revolute. Sclerenchyma cells, mainly fibers, replace the mesophyll and occupy the distal region (Fig. 4G–I).

The petiole in frontal view possesses epidermis coated with smooth cuticle on both faces. Scattered non-ornamental epicuticular wax occurs in crusts on the adaxial face and in plates parallel to the epidermal surface on the abaxial face (Fig. 5A and B). The petiole in transection resembles an arch in shape (Fig. 5C). Continuous strata of lamellar collenchyma are located immediately beneath the epidermis (Fig. 5D). Collateral vascular bundles in an arch-shaped pattern are embedded in the peripheral ground parenchyma. One or two minor collateral vascular bundles encircled by parenchymatic sheath occur on the abaxial face, located externally to the other bundles (Fig. 5C and E). Prismatic crystals and raphides occur throughout the ground parenchyma (Fig. 5F).

Transections of the base of the leaf blade showed that calluses are structures of the petiole. The dermal, fundamental, and vascular tissues of calluses originate from the tissue systems of the petiole (Fig. 5G). The epidermis is coated with thick cuticle and has cell protuberances that consist of phenolic-containing cells in periclinal and anticlinal divisions, forming what somewhat resembles meristem at some sites (Fig. 5G–I). Continuous strata of lamellar collenchyma occur beneath the cell protuberances and coalesce into the enlarged distal end of the calluses, wholly occupying this region (Fig. 5G and H). The vascular system consists of xylem and phloem, either as transverse commissures or as inconspicuous collateral vascular bundles embedded in the ground parenchyma (Fig. 5J).

The leaf sheath in frontal view presents epidermis coated with smooth cuticle on both faces (Fig. 6A). The leaf sheath in transection is concave-convex (Fig. 6B). The epidermal cells are vertically elongated on the adaxial face (Fig. 6D). Continuous strata of lamellar collenchyma occur immediately beneath the epidermis on both faces (Fig. 6C–E). Collateral vascular bundles in an arc are embedded in the ground parenchyma. Two or three minor collateral vascular bundles may occur on the abaxial face, located externally to the other bundles (Fig. 6B and C). Prismatic crystals and raphides are observed in the ground parenchyma (Fig. 6F).

The stem epidermis in frontal view presents smooth cuticle. Aggregate crusts of non-ornamental epicuticular wax occur on epidermal surface (Fig. 7A). Sac-like glandular trichomes like those described for the leaf epidermis are rarely observed. The stem growth is predominantly primary from the 1st to 2nd internodes and inconspicuously secondary from the 3rd to 4th internodes (Fig. 7B–I).

The stem in transection is elliptical with wavy outline regardless of the type of growth (Fig. 7B). The uniseriate epidermis is coated with thick cuticle that forms V-shaped flanges (Fig. 7D). Lenticels in initial developmental stage protrude above the stem surface (Fig. 7C). The cortex consists of continuous strata of lamellar collenchyma, collenchymatous fibers, and ground parenchyma (Fig. 7C and D). The collenchyma occurs immediately beneath the epidermis, and the inner collenchyma cells are differentiated as fibers (Fig. 7D). The collateral vascular bundles are arranged in two concentric circles (Fig. 7C and E). The peripheral circle of bundles is bound internally and separated from the pith by a sinuous zone of fibers (Fig. 7C). The phloem pole may present fibers (Fig. 7C). The medullary bundles are encircled by parenchymatic sheath, and some may present a fiber cap next to the xylem (Fig. 7C and E). The pith is parenchymatic, in which amyloplasts with convex-biconcave aggregate starch grains occur (Fig. 7E and F).

Fascicular and interfascicular cambia occur from the 3rd internode (Fig. 7G and H). In the 4th internode, the secondary growth is restricted to the peripheral vascular region. Both xylem and

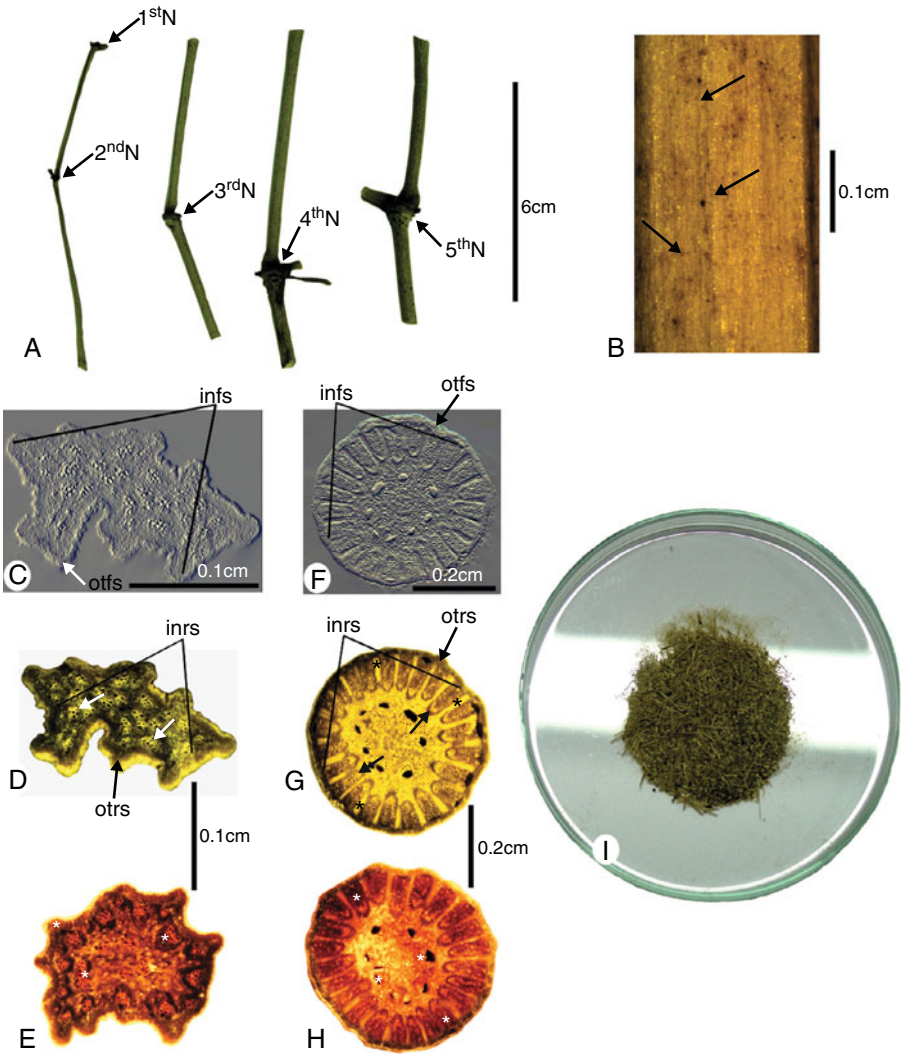

Fig. 2. Macroscopic features of the stem herbal drugs of *Piper callosum* Ruiz & Pav., Piperaceae. Photomacrographs (A; I). Stereoscopic photomicrographs by differential interference contrast (C and F) and by reflective light (B and D; E, G and H). A. General morphological aspect. B. Detail of the surface. Note longitudinal striae (arrows). C–E. First internode. C. Fracture surface. D and E. Transection. Note yellowish spots (arrows, figure D) and lignified elements (asterisks, figure E). F–H. Fourth internode. F. Fracture surface. G and H. Transection. G. Note elliptical areas (arrows) and yellowish strands (asterisks). H. Note lignified elements (asterisks). I. Powdered herbal drug. First node (1st N). Second node (2nd N). Third node (3rd N). Fourth node (4th N). Fifth node (5th N). Inner region of the section (inrs). Outer region of the section (otrs). Inner fracture surface (infs). Outer fracture surface (otfs).

phloem become radially elongated and remain organized as collateral bundles separated by parenchymatic rays. The fiber zone is interrupted, and the fiber cap next to the xylem is formed (Fig. 7I). The epidermal, cortical, and medullary features remain as previously described.

The secretory idioblasts have a spherical to elliptical shape, and their cell wall presents a trilamellar structural aspect with a lignified intermediary layer (Fig. 8K). They occur in the mesophyll (Fig. 4A, C and G), in the ground parenchyma of the midrib (Fig. 4E), among the sclerenchyma cells situated beneath the epidermis on the adaxial face of the leaf margin (Fig. 4H), in the ground parenchyma and collenchyma of the petiole and leaf sheath (Figs. 5D, 6E and 8M), in the ground parenchyma of the calluses (Fig. 5G), and in the cortical and medullary ground parenchyma of the stem (Fig. 7C and E). Secretory idioblasts are also observed in the phloem and xylem parenchyma of leaf and stem vascular bundles. In the xylem parenchyma, they have a generally wavy outline (Figs. 5E, 7C and G and 8I).

The histochemical tests indicated that the secretion is heterogeneous and rich in lipids, mainly acid lipids, in the glandular trichomes, secretory idioblasts, and calluses (Table 2, Fig. 9A–F). Essential oils, oleoresins, mixture of essentials and resins, and steroids (Table 2, Fig. 9G–M) as well as tannins and flavonoids (Table 2, Fig. 9N–S) were identified. The other histochemical tests rendered negative results (Table 2).

The herbal drugs of powdered leaves possess leaf blade fragments with straight to wavy (adaxial face) or sinuous (abaxial face) anticlinal epidermal cell walls, sac-like glandular trichomes encircled by epidermal cells, tetracytic and cyclocytic stomata (abaxial face), smooth cuticle, continuous plates of granular epicuticular wax parallel to the epidermal surface, raphides, and secretory idioblasts of acidophilic content. Isolated secretory idioblasts of acidophilic or yellowish-colored content and tracheary elements with annular wall thickening are also observed (Fig. 8A–G).

The herbal drugs of powdered stem have isolated fibers, fiber bundles, and blackish-colored particles as predominant elements. Also present are fragments with very thick and straight to wavy anticlinal epidermal cell walls, smooth cuticle, and aggregate crusts of non-ornamental epicuticular wax; tracheary elements with helical wall thickening; secretory idioblasts of acidophilic content; and non-aggregate convex-biconcave starch grains (Fig. 8H–L).

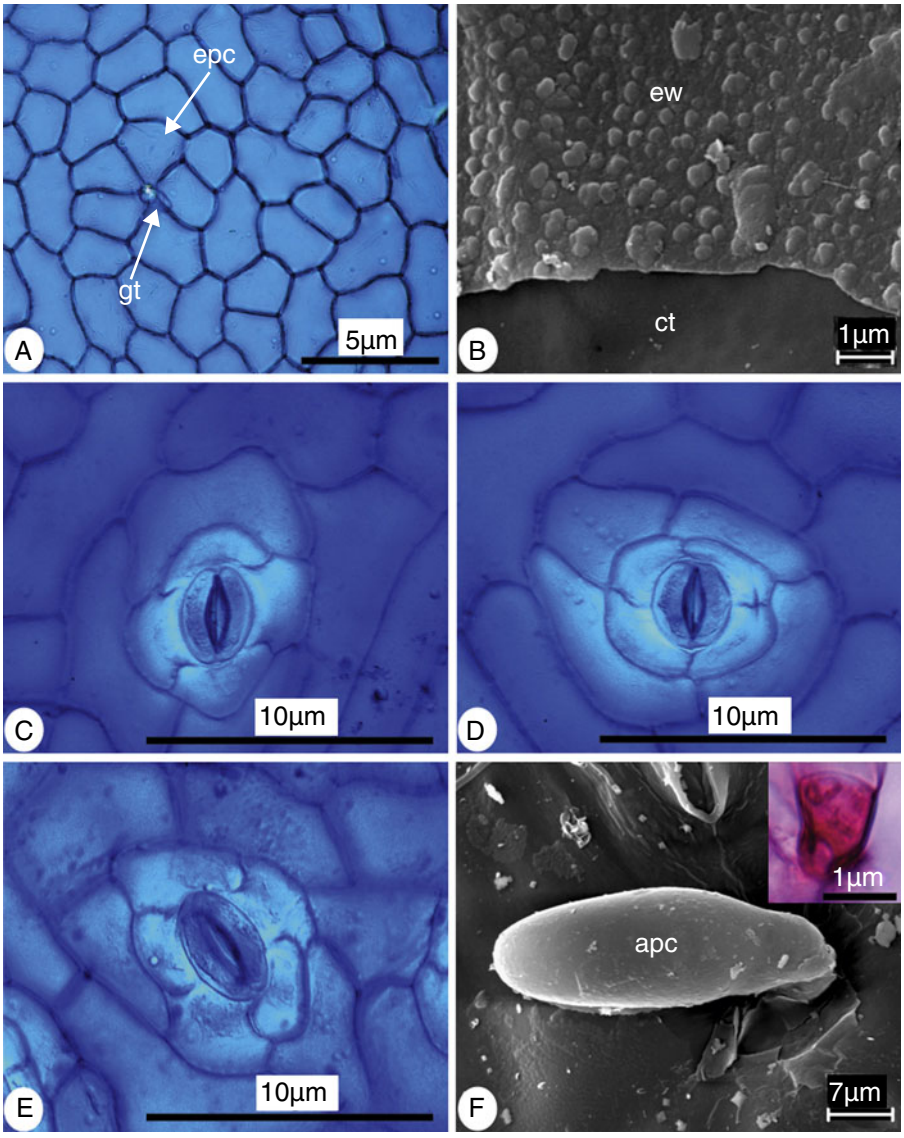

Fig. 3. Frontal view of the epidermis of the leaf blade of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs (A; C–E; inset at F). Scanning electron micrographs (B and F). A and B. Adaxial face. A. General view. B. Detail of the cuticle and epicuticular wax. C–F. Abaxial face. C–E. Tetracytic (C) and cyclocytic (D; E) stomata. F. Glandular trichome. Note basal cell (inset). Epidermal cells encircling the glandular trichome base (epc). Glandular trichome (gt). Cuticle (ct). Epicuticular wax (ew). Apical cell (apc).

Pharmacognostical characterization

The levels of volatile and inorganic matter were comparatively higher in the leaf herbal drugs than in the stem herbal drugs. The stem herbal drugs resulted in a high reduction of the pH value of the distilled water (Table 3). The distilled water used had a pH of 7.010 ± 0.032.

The highest concentrations of most of the examined metals, except for Cd and Cr, were observed in the leaf herbal drugs. For the leaf herbal drugs, the metals with high concentrations were Al, V, Cu and Mn, in order of increasing concentration. For the stem herbal drugs, the high metal concentrations were of Al, Cu, V and Mn, in order of increasing concentration. Al presented the highest concentration of all examined metals, and Hg and Co the lowest concentrations in both leaf and stem herbal drugs. Both leaf and stem herbal drugs contained Mo, Pb, Ti and As below the detection limit. The stem herbal drugs did not contain detectable amounts of Hg. The results are considered satisfactory, with recoveries being within the range 76.48–117.20% (Table 4).

Discussion

The general external morphological features observed in *Piper callosum* leaf and stem herbal drugs are in accordance with the morphological descriptions of the taxon (Yuncker, 1972). External and internal macroscopic characteristics, leaf venation patterns, and sensory characteristics are described herein for first time for the *P. callosum* herbal drugs. Since the macroscopic and organoleptic examinations serve as valuable pharmacognostical parameters to establish the botanical identity and the degree of purity of medicinal plant materials and should be carried out before any further tests are undertaken (WHO, 1998; Cheng et al., 2014), the organoleptic and morphological features reported here for the *P. callosum* herbal drugs are useful diagnostic characters for their quality control.

Regarding anatomical aspects of the leaf and stem, *P. callosum* displayed conservative and distinctive characteristics for *Piper* and Piperaceae. The following can be considered as conservative characteristics of *P. callosum* because they have been cited as common

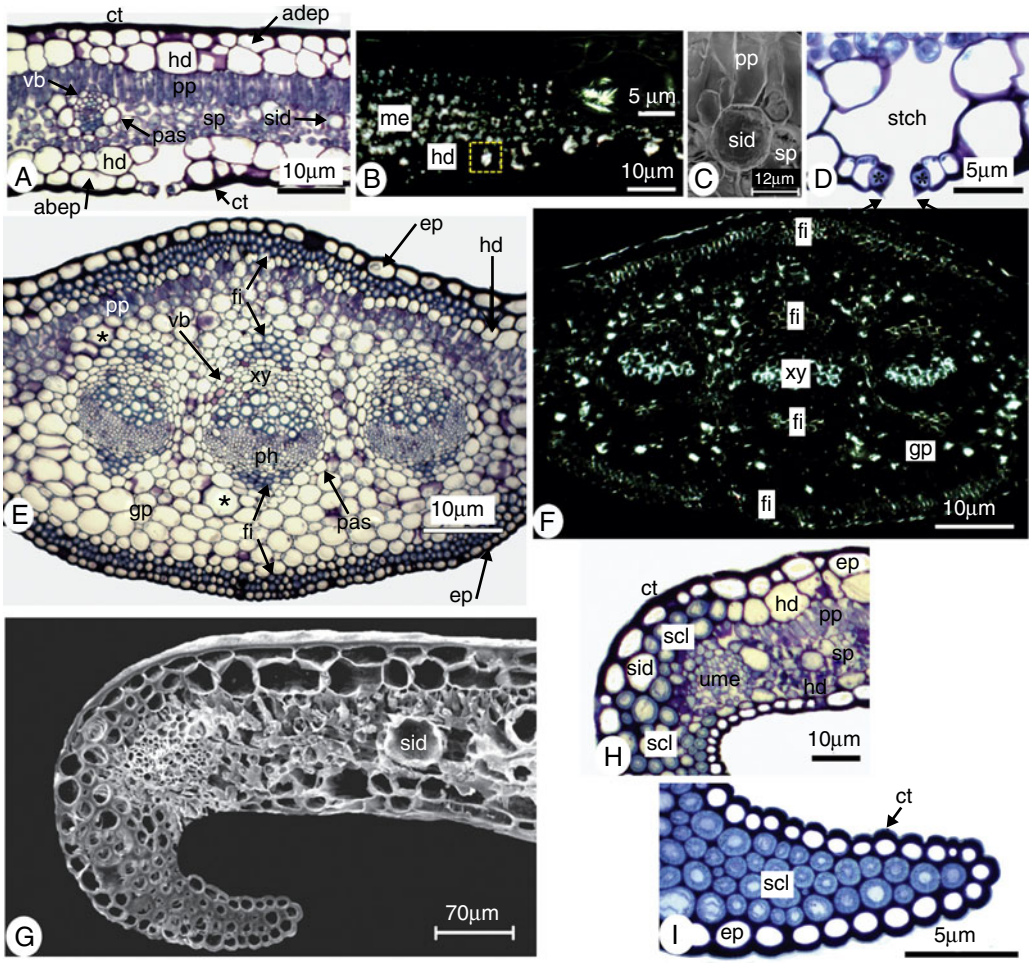

Fig. 4. Transections of the leaf blade of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs by transmitted light (A, D, E, H and I) and by polarized light (B and F). Scanning electron micrographs (C; G). A–C. Mesophyll. A. General view. B. Polarized view showing birefringent druses (mesophyll) and raphides (hypodermis). Note raphides (inset). C. Detail of the secretory idioblast. D. Stoma. Note lumen (asterisk) and outer ledges (arrows) of the guard cells. E and F. Midrib. E. General view. Note secretory idioblasts (asterisks). F. Polarized view showing birefringent raphides. G-I. Margin. G. General view. H. Region of the mesophyll. I. Distal region. Adaxial face of the epidermis (adep). Abaxial face of the epidermis (abep). Epidermis (ep). Cuticle (ct). Hypodermis (hd). Substomatal chamber (stch). Palisade parenchyma (pp). Spongy parenchyma (sp). Mesophyll (me). Undifferentiated mesophyll (ume). Fiber (fi). Sclerenchyma (scl). Ground parenchyma (gp). Parenchymatic sheath (pas). Secretory idioblast (sid). Vascular bundle (vb). Phloem (ph). Xylem (Xy).

Table 2
Results of the histochemical screening performed in secretory structures of *Piper callosum*.

Metabolic classes	Reagents	Secretory idioblasts		Callosus emergences	Glandular trichomes	
		Leaf	Stem	Petiole	Leaf	Stem
Total lipids	Sudan black B	++	+	++	++	+
Neutral lipids	Nile blue A	–	–	–	–	–
Acid lipids	Nile blue A	++	+	++	++	+
Unsaturated lipids	Osmium tetroxide	–	–	–	–	–
Fatty acids	Copper acetate/rubeanic acid	–	–	–	–	–
Essential oils	NADI reagent	–	–	++	–	–
Oleoresins	NADI reagent	++	+	–	–	–
Mixture of essentials and resins	NADI reagent	++	+	–	–	–
Steroids	Antimony trichloride	++	++	+	–	–
Sesquiterpene lactones	Abraham reaction	–	–	–	–	–
Terpenoids with carbonyl group	2,4-dinitrophenylhydrazine	–	–	–	–	–
Total phenolics	Ferric trichloride	++	–	++	++	+
Tannins	Vanillin–hydrochloric acid	–	–	++	–	–
Flavonoids	Aluminum trichloride[a]	++	–	–	++	–
Neutral polysaccharides	Periodic acid–Schiff (PAS) reagent	–	–	–	–	–
Pectins	Ruthenium red	–	–	–	–	–
Mucins	Mayer's tannic acid-ferric chloride stain	–	–	–	–	–
Acid mucopolysaccharides	Alcian blue	–	–	–	–	–
Total alkaloids	Dragendorff reagent	–	–	–	–	–

+, Positive; ++, Intense positive; –, Negative.
[a] Observed under UV light.

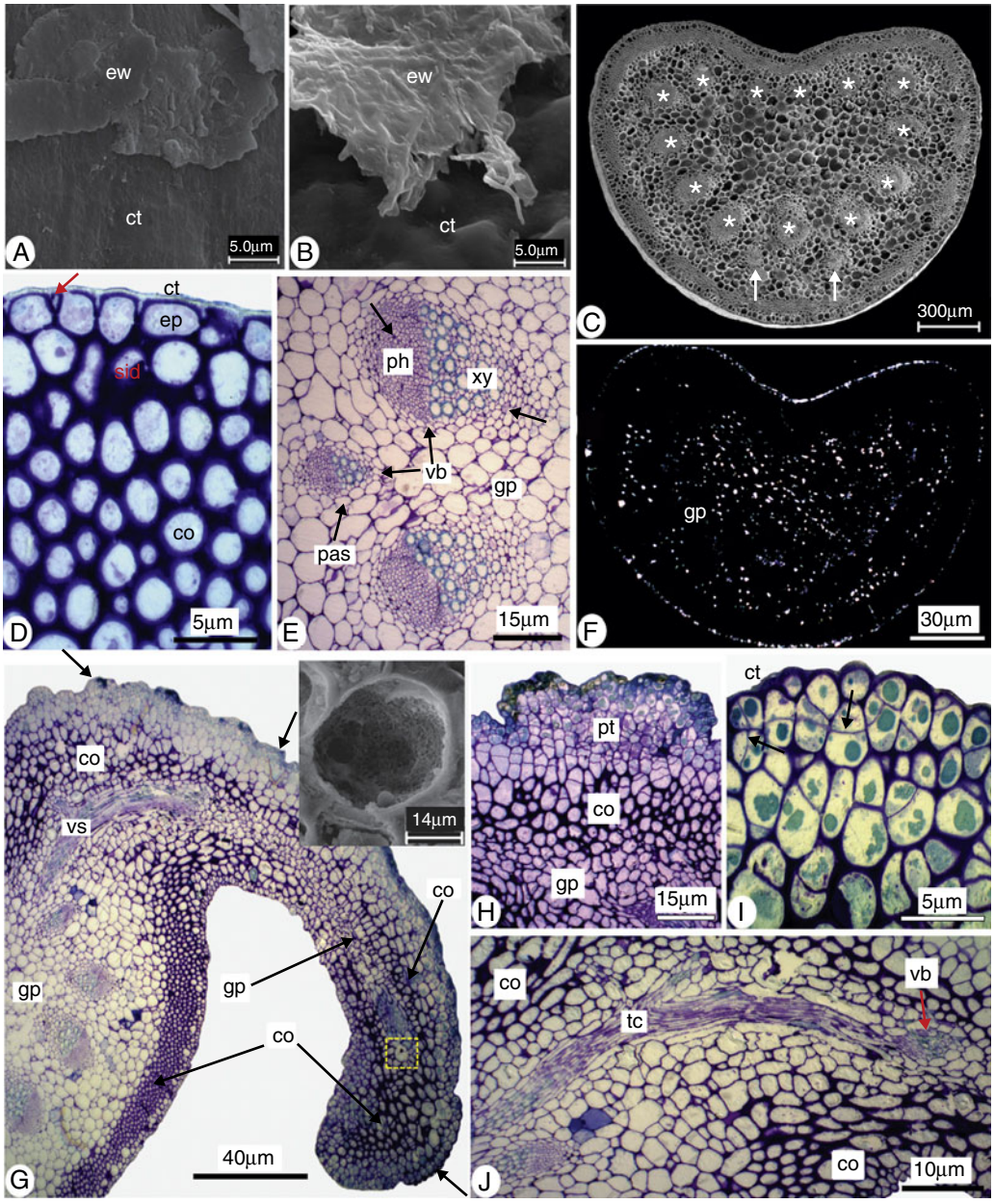

Fig. 5. Petiole of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs by transmitted light (D, E; G–J) and by polarized light (F). Scanning electron micrographs (A–C; inset at G). A and B. Frontal view of the epidermal surface. Adaxial (A) and abaxial (B) faces. C–J. Transections. C. General view. Note arrangement of the vascular bundles (asterisks) and minor bundles (arrows). D. Adaxial face detailing epidermis and collenchyma. Note cuticular flange (arrow). E. Vascular bundles. Note secretory idioblasts (arrows). F. Polarized view showing birefringent crystals. G–J. Callosus emergence. G. General view. Note cell protuberances (arrows) and secretory idioblast (inset). H and I. Cell protuberances. H. General view. I. Detail of the phenolic-containing cells. Note periclinal and anticlinal divisions (arrows). J. Vascular system. Cuticle (ct). Epicuticular wax (ew). Epidermis (ep). Collenchyma (co). Ground parenchyma (gp). Parenchymatic sheath (pas). Secretory idioblast (sid). Vascular system (*vs*). Vascular bundle (vb). Transverse commissure (tc). Phloem (ph). Xylem (xy).

Table 3
Results of physicochemical parameters determined in herbal drugs of *Piper callosum*.

Herbal drug	Parameters (mean ± S.D.; *n* = 3)					
	Loss on drying (INFRATEST)	Moisture content	Total ash	Sulphated ash	Acid-insoluble ash	pH
Leaf	11.08% ± 0.00	8.60% ± 0.30	11.25% ± 0.01	68.02% ± 0.03	2.82% ± 0.00	5.57 ± 0.01
Stem	8.58% ± 0.01	6.10% ± 0.20	5.25% ± 0.01	12.50% ± 0.05	0.27% ± 0.01	5.28 ± 0.14

SD, Standard deviation.
n is the number of independent determinations.

ones for Piperaceae (Metcalfe and Chalk, 1950) and mentioned for other *Piper* species (Marinho et al., 2011; Gogosz et al., 2012; Periyanayagam et al., 2012; Silva et al., 2014, 2016; Machado et al., 2015; Santos et al., 2015): epidermal cells of leaf blade with straight to wavy or sinuous anticlinal walls; smooth cuticle; hypostomatic leaves; tetracytic and cyclocytic stomata; sac-like glandular trichomes; uniseriate leaf and stem epidermis; leaf hypodermis; occurrence of hypodermis and palisade parenchyma into

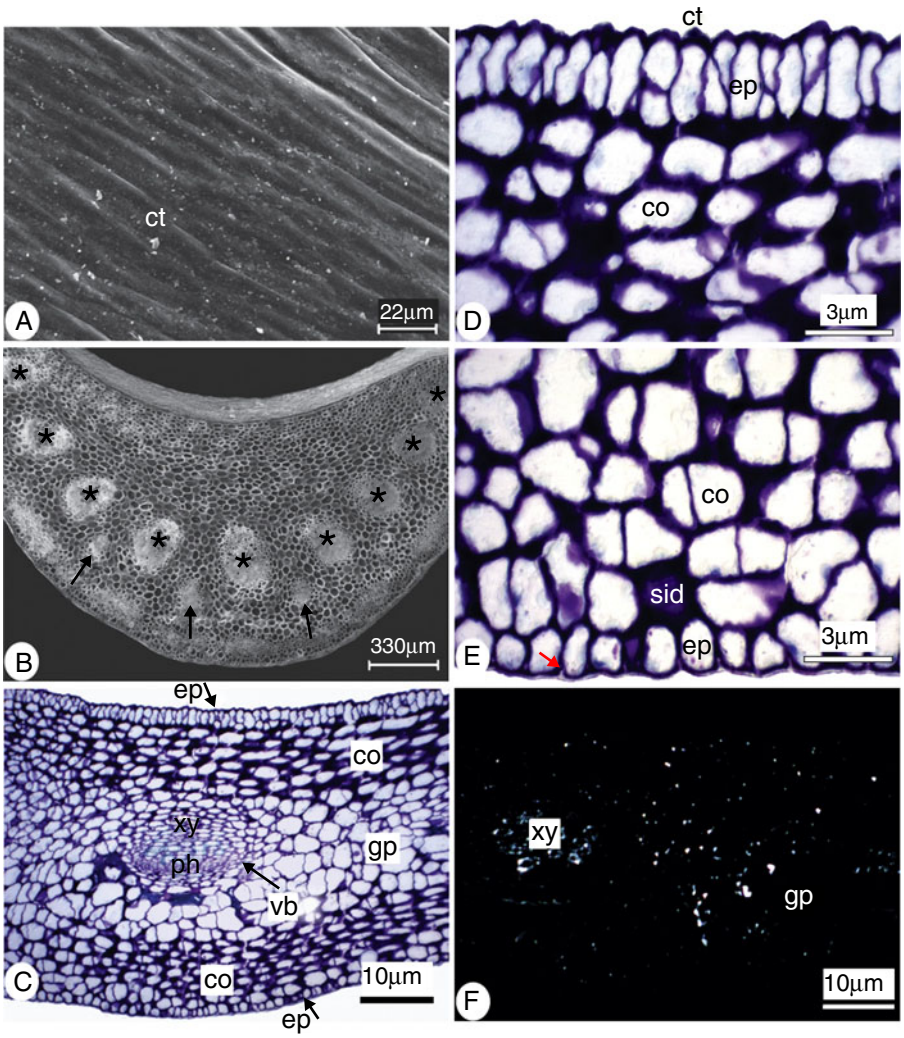

Fig. 6. Leaf sheath of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs by transmitted light (C–E) and by polarized light (F). Scanning electron micrographs (A and B). A. Frontal view of the adaxial face of the epidermal surface. (B–F) Transections. B. General view. Note arrangement of the vascular bundles (asterisks) and minor bundles (arrows). C. Detail of the structural organization. D and E. Detail of the adaxial (D) and abaxial (E) faces. Note cuticular flange (arrow, figure E). F. Polarized view showing birefringent crystals. Epidermis (ep). Cuticle (ct). Collenchyma (co). Ground parenchyma (gp). Secretory idioblast (sid). Vascular bundle (vb). Phloem (ph). Xylem (xy).

Table 4
Results of determination of metals in herbal drugs of *Piper callosum*.

Herbal drug	Metal content (mean ± S.D. mg kg^{-1}; n = 3)												
	Al	Cd	Co	Cr	Cu	Mn	Mo	Ni	Pb	Ti	V	Hg	As
Leaf	100.95 ± 8.37	0.03 ± 0.00	0.05 ± 0.03	0.81 ± 0.05	9.50 ± 0.60	6.03 ± 0.12	<LOD[b]	0.44 ± 0.28	<LOD[b]	<LOD[b]	19.02 ± 1.00	0.02 ± 0.01	<LOD[b]
Stem	32.75 ± 6.68	0.03 ± 0.00	0.01 ± 0.00	0.88 ± 0.14	8.52 ± 1.08	2.42 ± 0.35	<LOD[b]	0.18 ± 0.04	<LOD[b]	<LOD[b]	3.90 ± 0.36	<LOD[b]	<LOD[b]
SRM[a] 1547	233.67 ± 4.98	0.03 ± 0.01	0.06 ± 0.01	1.17 ± 0.02	3.10 ± 0.35	74.90 ± 3.47	<LOD[b]	0.62 ± 0.10	<LOD[b]	1.10 ± 0.28	0.40 ± 0.29	0.03 ± 0.01	0.06 ± 0.02
Recovery (%)	93.84	102.96	83.85	117.20	82.86	76.48	[c]	89.07	[c]	[d]	113.55	93.84	102.96

SD, Standard deviation.
n is the number of independent determinations.
[a] Standard Reference Material.
[b] LOD (Detection limit): Mo = 0.001 mg kg^{-1}; Pd = 0.005 mg kg^{-1}; Ti = 0.002 mg kg^{-1}; Hg = 0.00004 mg kg^{-1}; As = 0.00012 mg kg^{-1}.
[c] Non-satisfactory recovery.
[d] Value not certified.

the midrib; dorsiventral mesophyll; lamellar collenchyma; sclerification of the stem collenchyma; structure of the leaf collateral vascular bundles; arrangement pattern of the vascular bundles of the leaf sheath; organization pattern of the stem vascular system; shape in transection of the leaf sheath; prismatic crystals; druses; and presence of secretory idioblasts in the leaf and stem tissues.

Concerning the distinctive characteristics that were observed in *P. callosum*, we recorded for both leaves and stem the following: ornamentation and disposition patterns of the epicuticular wax; cuticular flanges patterns; type and position of the leaf and stem collenchyma; type of supporting tissue in the midrib; arrangement pattern of the vascular bundles of the midrib and petiole; shape in transection of the midrib, leaf margin, petiole, and stem; occurrence of raphides; and morphology of the starch grains. The crystal macropatterns described for *P. callosum* (Silva et al., 2014) must be added to these diagnostic microscopic characteristics as they are species specific.

The pharmacognostical and taxonomic significance of the above-cited characters as distinctive for *P. callosum* have been emphasized (Dickson, 2000; Oliveira and Akisue, 2003) and used

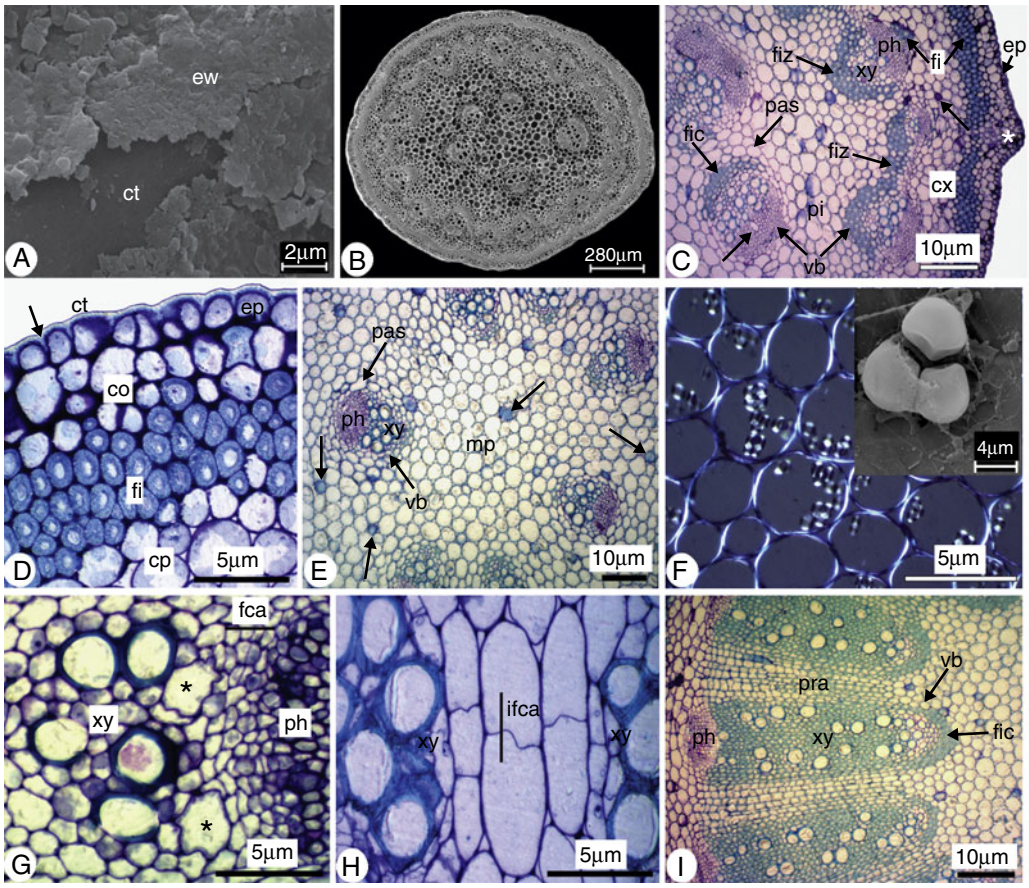

Fig. 7. Stem of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs by transmitted light (C–E; G–I) and by polarized light (F). Scanning electron micrographs (A; B; inset at F). (A–F) Primary growth. (G–I) Initial secondary growth. A. Frontal view of the epidermal surface. (B–I) Transections. B. General view. C. Primary stem structure. Note secretory idioblasts (arrows) and initial lenticel (asterisk). D. Detail of the epidermis and cortex. Note cuticular flange (arrow). E and F. Pith. E. General view. Note secretory idioblasts (arrows). F. Polarized view showing amyloplasts with birefringent starch grains. Note aggregate starch grains (inset). G. Vascular bundle. Note secretory idioblasts (asterisks). H. Interfascicular cambium. I. Initial secondary stem structure. Cuticle (ct). Epicuticular wax (ew). Epidermis (ep). Collenchyma (co). Fiber (fi). Fiber cap (fic). Fiber zone (fiz). Cortex (cx). Cortical parenchyma (cp). Medullary parenchyma (mp). Pith (pi). Parenchymatic ray (pra). Parenchymatic sheath (pas). Fascicular cambium (fca). Interfascicular cambium (ifca). Vascular bundle (vb). Phloem (ph). Xylem (xy).

successfully to separate closely related species, *e.g. Smilax* (Martins et al., 2013) and *Piper* (Gogosz et al., 2012; Horner et al., 2012; Silva et al., 2014, 2016).

We emphasize that the conservative and distinctive anatomical characteristics should be considered together for an accurate botanical authentication; the former are designed for general taxonomic delimitation and the latter for specific taxonomic diagnosis.

With regard to the powdered herbal drugs, the anatomical markers of authentication must be based on the distinctive characteristics of leaf and stem of *P. callosum*. For the leaf herbal drugs, the distinctive characteristics are the epicuticular wax features as well as the presence of raphides in the leaf fragments. For the stem herbal drugs, the anatomical markers are the parietal and epicuticular wax features of the stem fragments, blackish-colored particles, and form of the starch grains.

The types of wall thickening of the tracheary elements cannot be considered anatomical markers for *P. callosum* powdered herbal drugs because they also occur in other *Piper* species (Periyanayagam et al., 2012; Silva et al., 2016).

The small callosities present at the base of the *P. callosum* leaf blade constitute an important distinctive morphological character of this species to distinguish it from other *Piper* species (Yuncker, 1972). The anatomical features of the calluses are reported here for the first time. From an anatomical viewpoint, the calluses of *P. callosum* can be considered as complex glandular emergences of petiolar origin. The cell protuberances of the callus epidermis may be a periderm differentiated due to the arrangement of the cells in division, such as phellogen, and their phenolic (tannin) content. According to Beckman (2000), there is strong positional, biochemical and physiological evidence to suggest that phenolic-storing cells may be intimately involved in processes that result in periderm formation.

As callus is a general morphological term that describes excrescent or protuberant tissues without distinctive features (Gonçalves and Lorenzi, 2011), we suggest that the calluses of *P. callosum* be termed callosus emergences, denoting both morphological and anatomical aspects.

The mixture of hydrophobic and hydrophilic compounds identified in the secretion of the *P. callosum* secretory structures is in agreement with the phytoconstituents of this species (Parmar et al., 1997; Facundo et al., 2004; Andrade et al., 2009). The complexity of content has already been cited in secretory structures of *Piper* (Marinho et al., 2011; Gogosz et al., 2012), although the idioblasts are commonly characterized by the secretion of oil or mucilage (Evert, 2006). In Piperaceae, the idioblasts are considered to be oil-secreting cells (Judd et al., 2009), and anatomical studies in *Piper* generally describe such structures as oil cells (Marinho et al., 2011; Gogosz et al., 2012; Periyanayagam et al., 2012). According to Marinho et al. (2011), the term oil cell can be partially justified by the presence of an oily secretion or conspicuous oil drops in oil-secreting cells, mainly in fresh material, but it fails to describe accurately the chemical diversity of the content of these idioblasts.

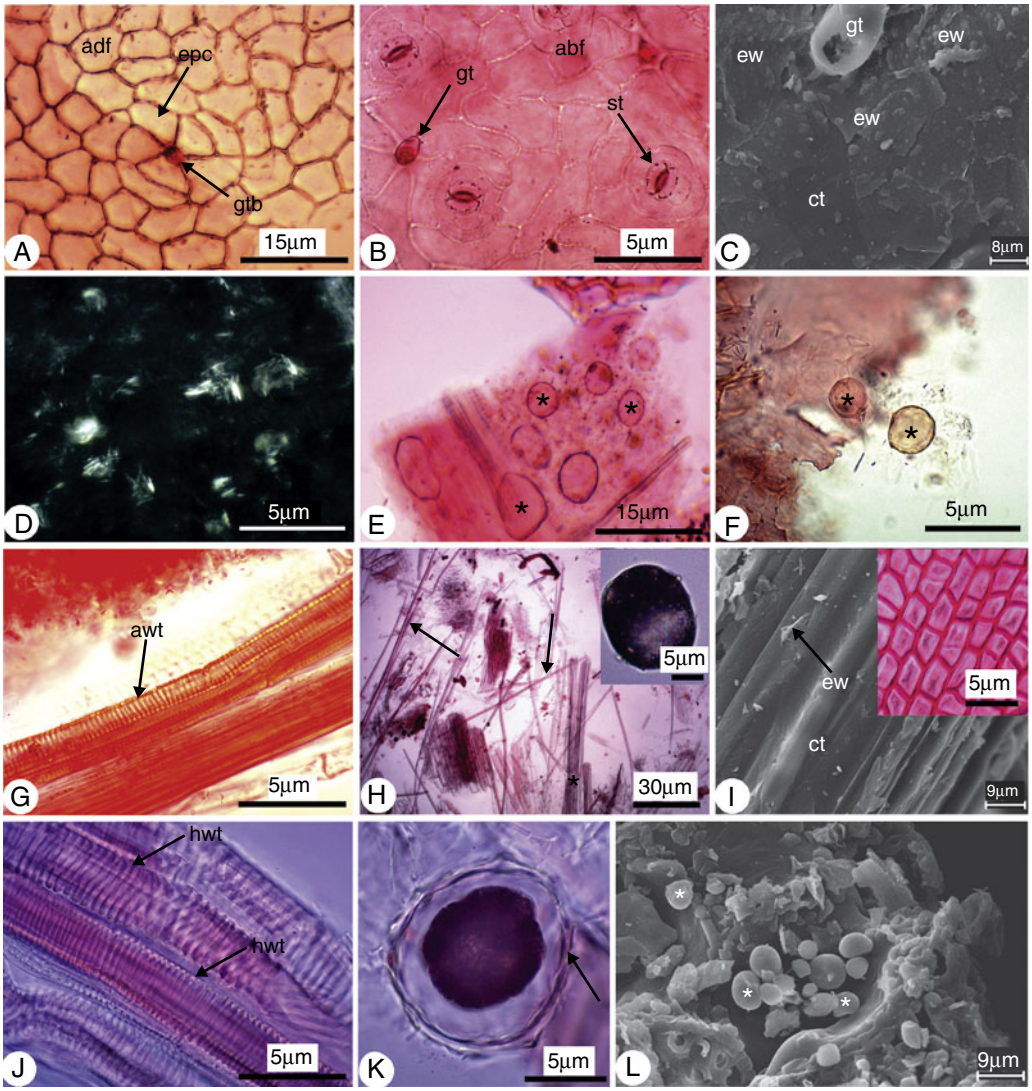

Fig. 8. Microscopic features of the powdered herbal drugs of *Piper callosum* Ruiz & Pav., Piperaceae. Photomicrographs by transmitted light (A, B; E–H; J; K; inset at I) and by polarized light (D). Scanning electron micrographs (C, I and L). (A–G) Leaf herbal drugs. (A–E) Leaf blade fragments. D. Polarized view showing birefringent raphides. E. Note secretory idioblasts (asterisks). F. Isolated secretory idioblasts (asterisks). G. Tracheary elements. (H–L) Stem herbal drugs. H. Note isolated fibers (arrows), fiber bundles (asterisk), and blackish-colored particle (inset). I. Stem fragment. Note epidermis (inset). J. Tracheary elements. K. Secretory idioblast. Note cell wall with trilamellar structural aspect and lignified intermediary layer (arrow). L. Non-aggregate starch grains (asterisks). Adaxial face (adf). Abaxial face (abf). Glandular trichome (gt). Glandular trichome base (gtb). Epidermal cells encircling the glandular trichome base (epc). Stoma (st). Cuticle (ct). Epicuticular wax (ew). Annular wall thickening (awt). Helical wall thickening (hwt).

The results achieved by the histochemical screening of *P. callosum* leaves and stem corroborate the arguments of the authors, and the term secretory idioblasts or cells adequately describes such structures because it does not make inferences from its chemical content.

The different therapeutic utilizations in folk medicine of the *P. callosum* leaves and stem (Andrade et al., 2009) are likely related to the chemical variety of the metabolic content identified histochemically in the idioblasts. The following pharmacological properties have been reported for phenolic compounds, such as tannins and flavonoids, steroids, and essential oils (Costa, 1994; Monteiro et al., 2005): antibacterial, antifungal, insecticidal, larvicidal, antispasmodic, carminative, choleretic, cholagogue, antidiarrheal, astringent, anti-inflammatory, anti-allergic, anesthetic, analgesic, antihemorrhagic, and diuretic.

From a pharmacognostical viewpoint, the *P. callosum* leaf and stem herbal drugs present excellent quality, which is ensured by preservation in the herbal drugs of morphoanatomical characteristics observed in the fresh plant materials, *e.g.* the occurrence of secretory idioblasts with content. Likely, the proper conditions of preparation of the herbal drugs, such as time and temperature of drying, contributed to this fact. Plant materials when subjected to high temperatures can undergo both morphological and cell content alterations of the tissues (Silva et al., 2016). On the other hand, the drying of plant materials until constant weight makes it impossible for portions to remain moist due to inefficient drying and prevents the degradation of chemical compounds by excessive drying (Hubinger et al., 2009).

The moisture content value of the *P. callosum* herbal drugs is below the maximum recommended limit of 14% (Fonseca et al., 2010), indicating that the conditions of drying, storage, and conservation of the herbal drugs was satisfactory. The water content is an important quality parameter for herbal drugs because residual water encourages microbial growth, insect infestation, deterioration, and enzymatic hydrolysis of the chemical compounds (Alves et al., 2010). The loss on drying values above the moisture values reveals the presence of other types of volatile matter in the *P. callosum* herbal drugs, which are likely thermo-unstable organic

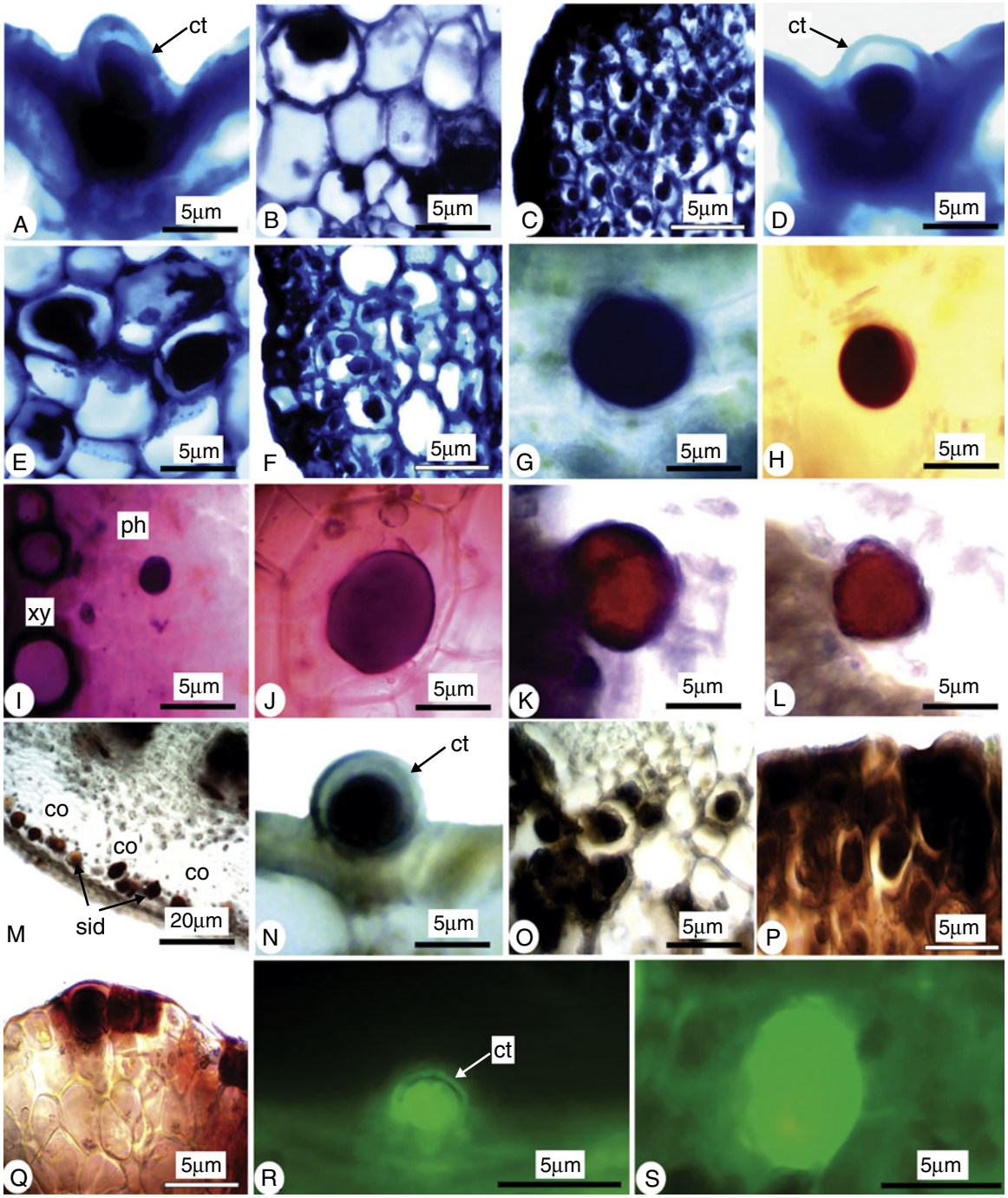

Fig. 9. Photomicrographs of transections of leaves and stem of *Piper callosum* Ruiz & Pav., Piperaceae, showing intense positive results from histochemical tests. Apical cell of the glandular trichomes (A, D, N and R). Secretory idioblasts: mesophyll (K; S); ground parenchyma of the midrib (H), petiole (B; E; J; O), callosus emergences (G), stem cortex (L), xylem and phloem (Ipe)*; collenchyma of the petiole (M). Cell protuberances of the callosus emergences (C, F, P, Q). (A–C) Total lipids. (D–F) Acid lipids. (G–J) Essential oil (G), oleoresin (H), and mixture of essentials and resins (I and J). (K–M) Steroids. (N–P) Total phenolic compounds. Q. Tannins. R and S. Flavonoids displaying yellowish green fluorescence under UV light. Collenchyma (co). Cuticle (ct). Secretory idioblast (sid). Phloem (ph). Xylem (xy). *The superscript indicates that the xylem and phloem belong to the vascular bundles of the petiole (pe).

compounds that occur in the content of its idioblasts. The analysis for loss on drying determines both water and volatile matter (WHO, 1998; Farmacopeia Brasileira, 2010); therefore, the difference between the loss on drying and moisture values may correspond to volatiles, such as essential oils. The total and acid-insoluble ash values in the *P. callosum* herbal drugs are in accordance with specifications of the British Herbal Pharmacopoeia (1990), in which the maximum limit of total and acid-insoluble ash is 15% (w/w) and 5% (w/w), respectively. The Brazilian Pharmacopeia does not establish limit values for ash. For sulphated ash, the higher values than that of total ash are due to (1) the addition of mass in the form of sulphuric acid in the samples and (2) the treatment of the samples with sulphuric acid, which transforms the oxides, carbonates, and halogen compounds into sulphates, forming firmer ash that is more stable at high temperatures (Evans, 2009). Regardless of the type of ash, the higher ash values for the leaf herbal drugs compared to the stem herbal drugs are likely due to the high crystal content of *P. callosum* leaves. Crystalline mineral inclusions in plants

contribute to high amounts of ash, mainly for the sulphated ash values (Mohamad et al., 2013).

The reduction observed in the pH value of the distillate water reveals the occurrence of acid compounds in the *P. callosum* herbal drugs, which is in agreement with the richness of acid metabolic classes identified histochemically in the content of the idioblasts of this species.

The metal concentrations found in the *P. callosum* herbal drugs are within safety baseline levels for human consumption (WHO, 1996, 1998, 2006; Farmacopeia Brasileira, 2010) and can be used as a quality criterion since the regulatory agencies do not establish permissible levels of metals in raw plant materials, except for arsenic, cadmium, and lead (WHO, 1998), and the increase of metal content in plants, mainly those that are potentially toxic or termed "heavy metals," can indicate external contamination by environmental factors and/or processing methods (Başgel and Erdemoğlu, 2006).

Conclusion

The results obtained in the present study concerning the macroscopic, microscopic, and physicochemical analysis of *P. callosum* leaves and stem can be used as safe parameters for quality control of raw plant materials in both forms: medicinal plant and herbal drug. The macro- and microscopic data will contribute to the botanical authentication, the physicochemical data will permit the evaluation of purity degree, and the organoleptic characteristics could be used for both botanical authentication and purity degree.

Authors' contributions

RJFS contributed in the collection and identification of plant samples, preparation of herbarium specimens, laboratory work, analysis of data, design of the study and the writing of the paper. ACAAD supervised the laboratory work. KCFF contributed to analysis by inductively coupled plasma optical emission spectrometry. ACAAD and MSM contributed to critical reading of the manuscript. 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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