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Original article

Anatomical investigations of *Piper amalago* (jaborandi-manso) for the quality control

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ABSTRACT

Piper amalago L., Piperaceae, popularly known as jaborandi-manso, is a shrub that spans a height of 2–7 m. It can be found in the regions of Southern America downward up to the south of Brazil. Traditionally it is used to treat digestive problems, heart problems, and burns. This study aims to conduct an anatomical investigation and analysis of the leaves and stems of *P. amalago* through electron scanning and optical micro techniques. The analysis showed that *P. amalago* has a hypostomatic leaf, with a subepidermal layer on its surface. There are grandular trichomes that resemble sacs, conic non-glandular trichomes, dorsiventral mesophyll, and a plano-convex midrib having a single vascular bundle in the center. The petiole is short with irregularly shaped and adaxially grooved. The stem is circular in shape and contains two circles of vascular bundles and a sclerenchymatic sheath in the perimedular region. These anatomical features of the *Piper amalago*'s leaves and stems make it easy to pick it out among other species of the *Piper* genus. This is helpful when conducting quality control process.

Introduction

According to Figueiredo and Sazima (2000) and Machado (2007), the Piperaceae family is made of approximately 3000 species covering tropical and subtropical areas. In Brazil alone, the Piperaceae consists of five genus and about 500 species mainly found in the Atlantic Forest (Souza, 2005; Souza and Lorenzi, 2005). It has several species that were popular for their medicinal use and have been studied for chemical composition and biological activities (Parmar et al., 1997; Jaramillo and Manos, 2001; Silva and Bastos, 2007; Facundo et al., 2008; Baldoqui et al., 2009).

Piper is one of the important genus of Piperaceae and comprises about 2000 species found all over the world (Joly, 2002; Silva et al., 2007). Ethno botanical studies reported the use of *Piper* in traditional medicine by several communities as an antioxidant, antimicrobial and antileishmanial (Regasini et al., 2009a,b; Carrara et al., 2012, 2013; Chander et al., 2014).

Piper amalago L. is popularly known in Brazil by the terms jaborandi-manso, jaborandi-falso and jaborandinhandi. It is a perennial plant which may attain 2–7 m height (Guimarães and Valente, 2001). Pharmacological studies have demonstrated anti-inflammatory (Carrara et al., 2012), antimicrobial, cicatrizing (Guimarães and Valente, 2001), and antileishmanial properties (Carrara et al., 2013). Additionally, it has been suggested that it has antioxidant properties since it contains vitexin and lupeol which have been isolated through phytochemical analysis (Rovani et al., 2013).

The serious problem of the herbal drugs replaced with alternative plants or tampered are common in Brazil. However, there is no requirement for the consumer market to publish official monographs for quality control (Kato et al., 2012). In that respect, recently several works have been devoted to study morpho-anatomy of the plants (Budel and Duarte, 2008, 2010; Oliveira et al., 2011; Squena et al., 2012; Youssef et al., 2013; Camilotti et al., 2014; Pereira et al., 2014a,b; Amorin et al., 2014; Folquitto et al., 2014; Jasinski et al., 2014).

There is a close structural similarity between the species of *Piper* (Gogosz et al., 2012) and there has not been any previous study of microscopic characters of *P. amalago*. Thus, this paper therefore seeks to study the microscopic characteristics of aerial vegetative organs of *P. amalago* for quality control purposes of phytotherapy industry.

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Fig. 1. *Piper amalago* L. (Piperaceae). A. Aspect of aerial parts and habit. B. Detail of leaves and inflorescence. C–E – Frontal view of leaves. C. Adaxial side of epidermis. D. Abaxial side of epidermis showing stomata. E. Abaxial surface, showing detail of the stomata. st: stomata. Bar = 5cm (A), 2 cm (B), 100 µm (E).

Materials and methods

Plant material

Aerial vegetative parts of *Piper amalago* L., Piperaceae, were collected at Curitiba, Paraná (24°18′ S and 49°37′ W) in July 2012. The vegetal material was identified by a taxonomist and compared with the voucher specimens deposited in the Municipal Botanical Museum of Curitiba, under register number 71947.

Anatomical analyses

Five specimens of *P. amalago* were used. Their leaves and stems were cut at about 5 cm from the apex. These were then put in containers containing FAA 70 solution (Johansen, 1940), and stored in 70% ethanol (Berlyn and Miksche, 1976).

The plants were segmented by use of hand while others were dehydrated, implanted in glycol-methacrylate (Leica historesin) then sectioned with the microtome Leica RM-2145. Toluidine blue was used to stain the transverse and longitudinal sections (O'Brien et al., 1964). Some sections were stained using a mixture of astra blue and basic fuchsine (Roeser, 1972).

Micro chemical tests

The following standard solutions were used in the micro chemical tests: methylene blue to test for mucilage (Oliveira et al., 2005); hydrochloric phloroglucin to reveal traces of lignin (Sass, 1951); Sudan III for testing lipophilic compounds (Foster, 1949); ferric chloride to test for phenolic substances (Johansen, 1940); sulphuric acid to find out the chemical composition of the crystals (Oliveira et al., 2005) and iodine-iodide to test for starch (Berlyn and Miksche, 1976).

Some photomicrographs were captured by a light microscope Olympus CX 31equipped using a control unit of C 7070. The semi-permanent, permanent and microchemical test slides were then analyzed in the Laboratory of Pharmacognosy at the State University of Ponta Grossa for a detailed description of the leaf and stem tissues.

Scanning electron microscopy

During the scanning electron analysis (SEM), the material was desiccated in a graded ethanolic series and carbon (IV) oxide critical point apparatus called Balzers CPD-030. Finally they were coated with gold by Balzers Sputtering SCD-030. The scanning microscope Jeol JSM-6360LV was used to capture electron micrographs (Souza, 1998). The results were forwarded to the Federal University of Paraná's Electron Microscopy Centre.

Results and discussion

Piper amalago L., Piperaceae, (Fig. 1A and B), from a frontal view of the blade, shows straight epidermal cell anticlinal walls

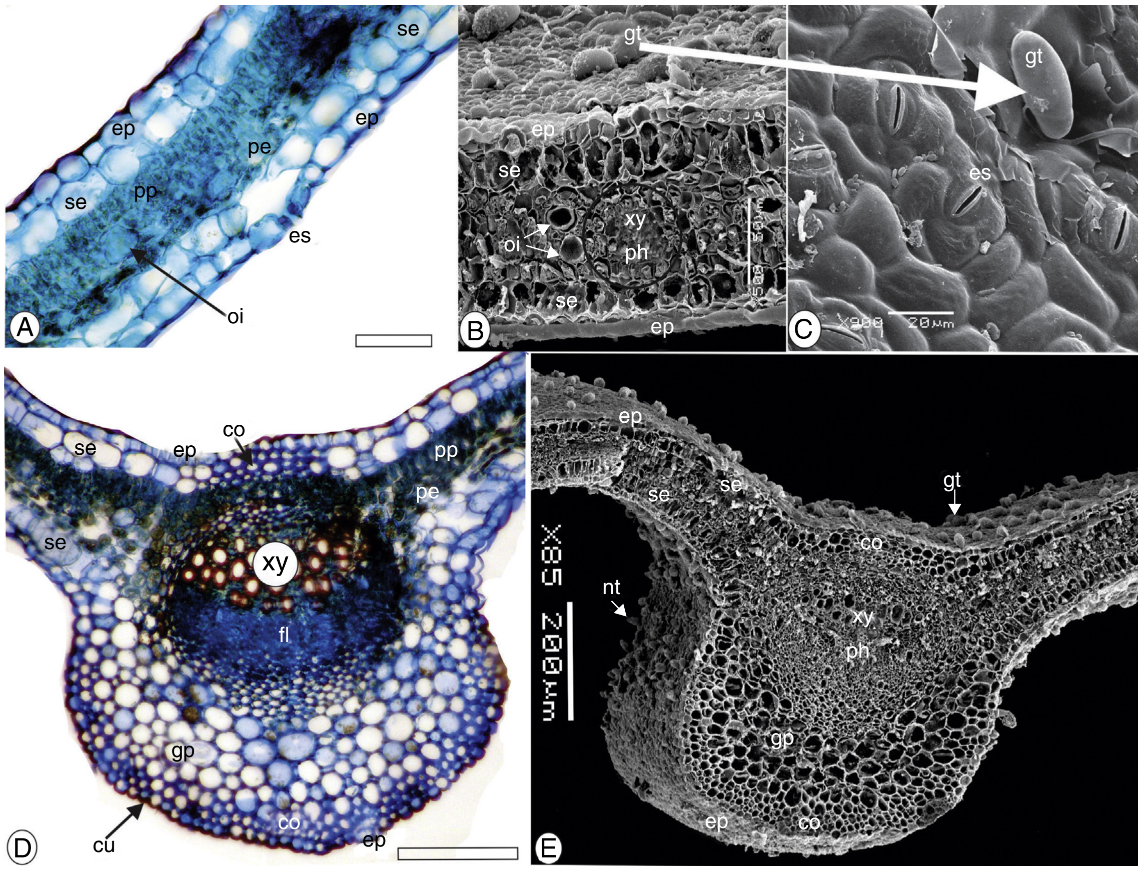

Fig. 2. *Piper amalago* L. (Piperaceae). Leaves. A. Blade organization showing dorsiventral mesophyll. B. Blade organization showing dorsiventral mesophyll. C. Adaxial side of epidermis, showing stomata and glandular trichomes. D. Midrib crossed by collateral vascular bundle. E. Midrib and blade organization. co: collenchyma, ep: epidermis, fp: fundamental parenchyma, gt: glandular trichome, oi: oily cell, nt: non-glandular trichome, ph: phloem, pp: palisade parenchyma, se: subepidermal layer, sp: spongy parenchyma, nt: non-glandular trichome, vb: vascular bundles, xy: xylem. Bar = 50 μm (A), 200 μm (D).

with relatively thin on both sides (Figs. 1C–E and 2C). On the epidermis' abaxial face (Figs. 1D, E and 2A) has tetracytic stomata (Figs. 1D, E and 2C). Hypostomatic leaves are common in Piperaceae (Metcalfe and Chalk, 1950), as described for *P. crassinervium* H.B.& K. (Albiero et al., 2005a), *P. hispidum* Sw. (Albiero et al., 2006), *P. aduncum* Vell., *P. cernuum* Vell., *P. dilatatum* Rich, *P. gaudichaudianum* Kunth, *P. betle* L. (Raman et al., 2012), *P. glabratum* Kunth, *P. lindbergii* C.DC., *P. solmsianum* C. DC. and, *P. umbellatum* Jacq. (Gogosz et al., 2012). Amphistomatic leaves were however found in *P. hispidinervum* C. DC. (Nascimento and Vilhena-Potiguara, 1999) and in *P. sarmentosum* Roxb. (Raman et al., 2012).

Several species of *Piper* have tetracytic stomata (Albiero et al., 2006; Gogosz et al., 2012). However, *P. arboreum* Aubl. has evidenced staurocytic (Souza et al., 2009), *P. crassinervium* has presented both the ciclocytic and tetracytic type (Albiero et al., 2005a), *P. betle* has showed anisocytic, anomocytic, tetracytic, polycytic, paracytic, amphicyclic and *P. sarmentosum* has presented anisocytic, anomocytic, actinocytic, cyclocytic and tetracytic type (Raman et al., 2012).

In transection, the epidermis of *P. amalago* is uniseriate and is covered by a thin cuticle. The stomata are located slightly above the other epidermal cells (Fig. 2A). A subepidermal layer was observed on both surfaces of the leaf (Fig. 2A and B). Multiple epidermis have been observed in species of *Piper* (Gogosz et al., 2012; Raman et al., 2012).

Variants are also present, such as, *P. aduncum* (Vianna and Akisue, 1997) has only one sub-epidermal layer on the adaxial side; *P. crassinervium* (Albiero et al., 2005a) has up to 3 sub-epidermal layers on the adaxial side; *P. gaudichaudianum* (Albiero et al., 2005b) has 1–2 layers on the abaxial and adaxial surfaces; *P. hispidum* (Albiero et al., 2006) has 3–4 layers on the abaxial side and, *P. arboreum* (Souza et al., 2009) has 2–4 layers on both sides of the blade.

A hypodermis was observed by Metcalfe and Chalk (1979) in species of Piperaceae, however, Takemori et al. (2003) argued that the multiple epidermis is constituted by the first layers leaf blade cells of several species of *Peperomia.* This is because the cells develop from periclinal division of protodermis. To investigate the exact origin of the sub-epidermal layer in *P. amalago* requires application of ontogenetic studies.

The sac-like multicellular glandular trichomes found in *P. amalago* (Fig. 2B, C and E) are made of a short pedicel and an elongated apical cell. Similar trichome was witnessed in *P. regnellii* (Miq.) C. DC. var. *regnellii* (Silva and Machado, 1999), *P. regnelli* (Pessini et al., 2003), *P. mikanianum* (Kunth) Steud. (Duarte and Siebenrock, 2010), and *P. sarmentosum* Roxb. (Raman et al., 2012).

Glandular trichomes have the capability to secrete or store large quantities of specialized metabolites that defend the aerial parts of the plant against pathogens and herbivores (Tissier, 2012). The secretion has been showing biological activity and it has aroused the interest of pharmaceutical, pesticide, flavor and fragrance industries (Duke, 1994).

The conic trichomes are non-glandular. They are multicellular, uniseriate, consist five cells and a slightly acute apex (Figs. 2E and 3B). Trichomes that were found in several species of *Piper*, ranging from short to long all portrayed some similarity (Raman et al., 2012; Pessini et al., 2003; Albiero et al., 2006; Duarte and Siebenrock, 2010; Gogosz et al., 2012). These non-glandular

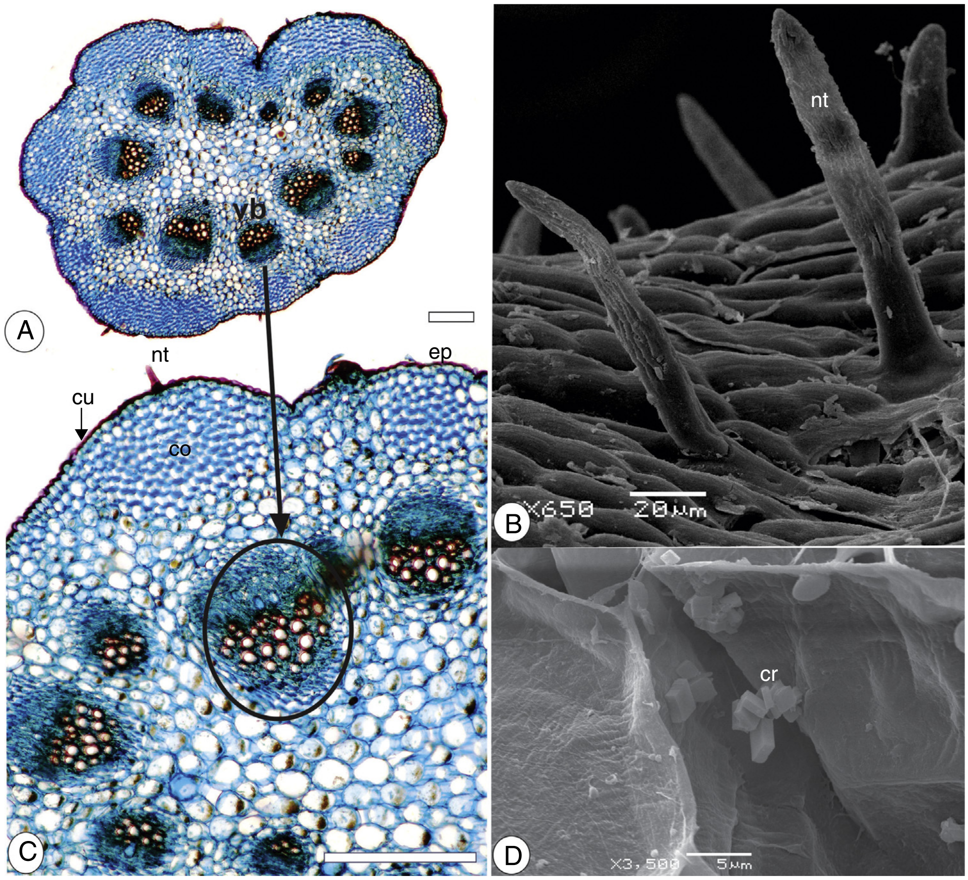

Fig. 3. *Piper amalago* L. (Piperaceae). Petiole. A. General aspect. B. Detail of the non-glandular trichomes. C. Petiole showing several collateral vascular bundles. D. Detail of prismatic crystals of calcium oxalate. co: collenchyma, cr: crystal of calcium oxalate, cu: cuticle, ep: epidermis, nt: non-glandular trichome, vb: vascular bundle. Bar = 200 μm (A, C).

trichomes have a variety of functions. They act as a means to prevent herbivores from feeding on the vegetable species, prevent excessive sunlight, controls leaf temperature as well as reduce water loss according to Wagner (1991) and Duke (1994).

In *P. amalago*, the mesophyll is dorsiventral and formed of a layer of palisade and 2–3 layers of spongy parenchyma (Fig. 2A, B, D, and E). This description is consistent with that applied to the genus, however the number of layers of palisade and spongy parenchyma may vary (Albiero et al., 2005b, 2006; Santiago et al., 2001; Gogosz et al., 2012; Raman et al., 2012). A parenchymatous endoderm surrounds small collateral vascular bundles which are immersed in the spongy parenchyma (Fig. 2B). The mesophyll contains cells with essential oil (Fig. 2A) and phenolic compounds (Fig. 5A). Castro et al. (1997) says that these cells with essential oils are idioblasts with additional secretory functions. *Piper* has a variety of cells containing oil contents (Albiero et al., 2005a).

The cross section shows that the midrib shape is a plano-convex one and is particularly more convex on its abaxial face as seen in Fig. 2D and E. This feature was evident in *P. sarmentosum* (Raman et al., 2012). A concave-convex shape or almost concave-convex shape has been seen in several species of *Piper* (Albiero et al., 2005a, 2005b, 2006; Gogosz et al., 2012). In addition, a biconvex shape has also been found in *P. regnelli* (Pessini et al., 2003), *P. mikanianum* (Duarte and Siebenrock, 2010), *P. umbellatum* (Gogosz et al., 2012) and, *P. solmsianum* (Gogosz et al., 2012).

A slightly thickened and striated cuticle covers the single-layered epidermis. Close to three layers of angular collenchyma can be observed on both sides. The vascular system is represented by a single collateral vascular bundle in the ground parenchyma (Fig. 2D and E). A similar pattern is also evident in *P. glabratum* and *P. solmsianum* (Gogosz et al., 2012). However, *P. gaudichaudianum* (Albiero et al., 2005b), *P. diospyrifolium* (Souza et al., 2004), *P. crassinervium* (Albiero et al., 2005a), *P. hispidum* (Albiero et al., 2006), *P. regnelli* (Pessini et al., 2003) and, *P. mikanianum* (Duarte and Siebenrock, 2010) had a variable number of vascular bundles in the form of an open arc. A microchemical test using phloroglucin exposes lignin in fibers and in xylem as seen in Fig. 5B.

The petiole is short and is irregular in shape. It is also adaxially grooved (Fig. 3A and C). The epidermis has characteristics similar to those reported for the leaf blade (Fig. 3B and C). Multicellular uniseriate non-glandular trichomes are also present in the petiole (Fig. 3B). This has about eleven vascular bundles of varying sizes, arranged in a circle that is opposite the discontinuous angular collenchyma, which consists of 10–12 layers of cells (Fig. 3A and C). Although in some *Piper* species, the pattern of the petiole is quite similar to that found in this study. It was observed that the number of vascular bundles is not fixed but varies (Souza et al., 2004; Albiero et al., 2005a; Souza et al., 2009; Raman et al., 2012).

The petiole contains several prismatic crystals of calcium oxalate as in (Fig. 3D). Plants contain Calcium oxalate crystals in a variety of shapes and sizes. They are usually described as being prismatics, raphides, styloids, crystal sands and druses. They are responsible for some of the very essential roles such as removal of excess Ca^{2+}, detoxification of heavy metals and provide mechanical protection (Franceschi and Horner-Junior, 1980; Nakata, 2003).

In an incipient secondary structure, the stem is circular in shape (Fig. 4A). The epidermis is uniseriate and covered by a moderately thick cuticle (Fig. 4B) that reacted with Sudan III (Fig. 5D). As already mentioned for the leaf, non-glandular trichomes can be observed (Fig. 4D). There are some layers of angular collenchyma (Fig. 4A,

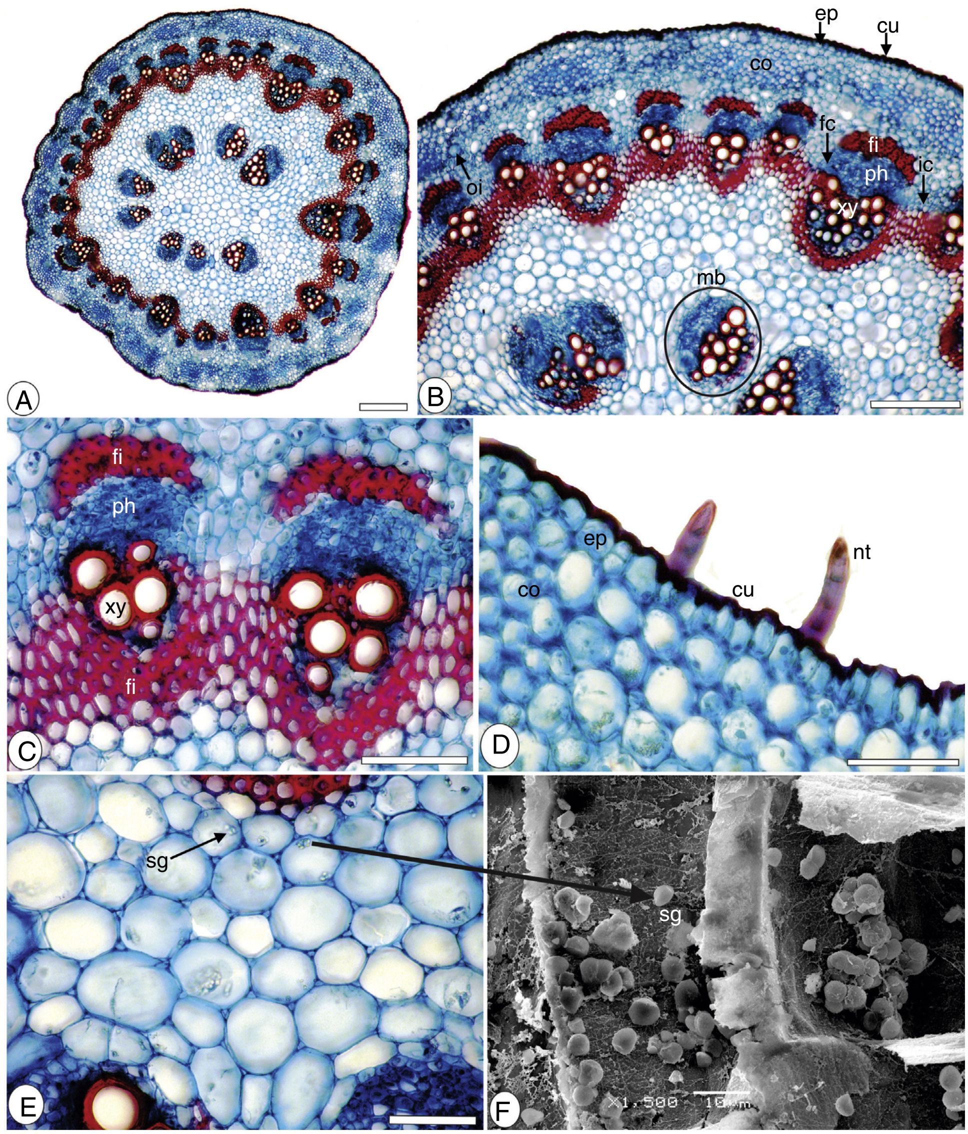

Fig. 4. *Piper amalago* L. (Piperaceae). Stem in cross section. A. General aspect. B. Detail of the previous figure. C. Detail of the sclerenchymatous ring, showing fibers. D. Epidermis and cortex area. E. Pith showing starch grains. F. Detail of the starch grains. cu: cuticle, ep: epidermis, co: collenchyma, fi: fibers, nt: non-glandular trichome, ph: phloem, pi: pith, sg: starch grains, xy: xylem. Bar = 200 μm (A, B), 50 μm (C, D, E).

B, and D). The endoderm containing some starch grains observed in Fig. 5E. *Piper mikanianum* (Duarte and Siebenrock, 2010) and *P. diospyrifolium* Kunth (Souza et al., 2004) displayed identical characteristics.

Vascular bundles are arranged in two circles within the vascular cylinder. The outer circle shows several vascular bundles and there may be perivascular fiber caps adjoining the phloem (Fig. 4A–C). About nive vascular bundles called medullary bundles are located in the inner circle (Fig. 4A and B). The vascular bundles show evidence of intrafascicular cambia (Fig. 4B). In the perimedular region, there is a sinuous sclerenchymatic sheath formed by several layers of lignified cells (Fig. 4A–C). These cells were seen to react with the phloroglucin (Fig. 5C) during the microchemical test. The pith occupies much of the stem's volume and is composed of parenchyma cells. Here starch grains can be found (Fig. 4E and F). Oily cells and prismatic crystals of calcium oxalate are also found in the stem.

The arrangement of vascular bundles of *P. amalago* is in agreement with the description by Cronquist (1981). Cronquist (1981), in his research found that, as a result of the growth exchange-rate, the outer circle of bundles gives rise to solid cylinders of phloem and xylem, while in the outer circle, the bundles remain individualized.

The arrangement of two concentric circles bundles separated by a sclerenchymatous ring is also typical of *Piper*, and was observed in *P. nigrum* (L.), *P. colubrinum* Link (Ravindran and Remarshree, 1998), *P. hispidum* (Albiero et al., 2006), *P. gaudichaudianum* (Albiero et al., 2005b), *P. crassinervium* (Albiero et al., 2005a), *P. diospyrifolium* (Souza et al., 2004), *P. regnelli* (Pessini et al., 2003), *P. arboreum* (Souza et al., 2009), *P. mikanianum* (Duarte and Siebenrock, 2010) and *P. sarmentosum* (Raman et al., 2012).

In most species of *Piper*, the crystals and oily cells, starch grains and phenolic compounds are located in the mesophyll, fundamental parenchyma of the midrib, petiole and, stem (Gogosz et al., 2012). According to Fahn (1988), these elements are common in Piperaceae.

Smith (2010) suggests that most plants accumulate carbon in form of starch and there is a huge difference in granule size and shape between the different organs in a plant and between species. On the other hand, the phenolic compounds found in these plants are particularly meant for protection and as antioxidants. They are responsible for adaptation and resistance to adverse environmental factors. They give plants a high resistance to microorganisms (Rocha et al., 2011).

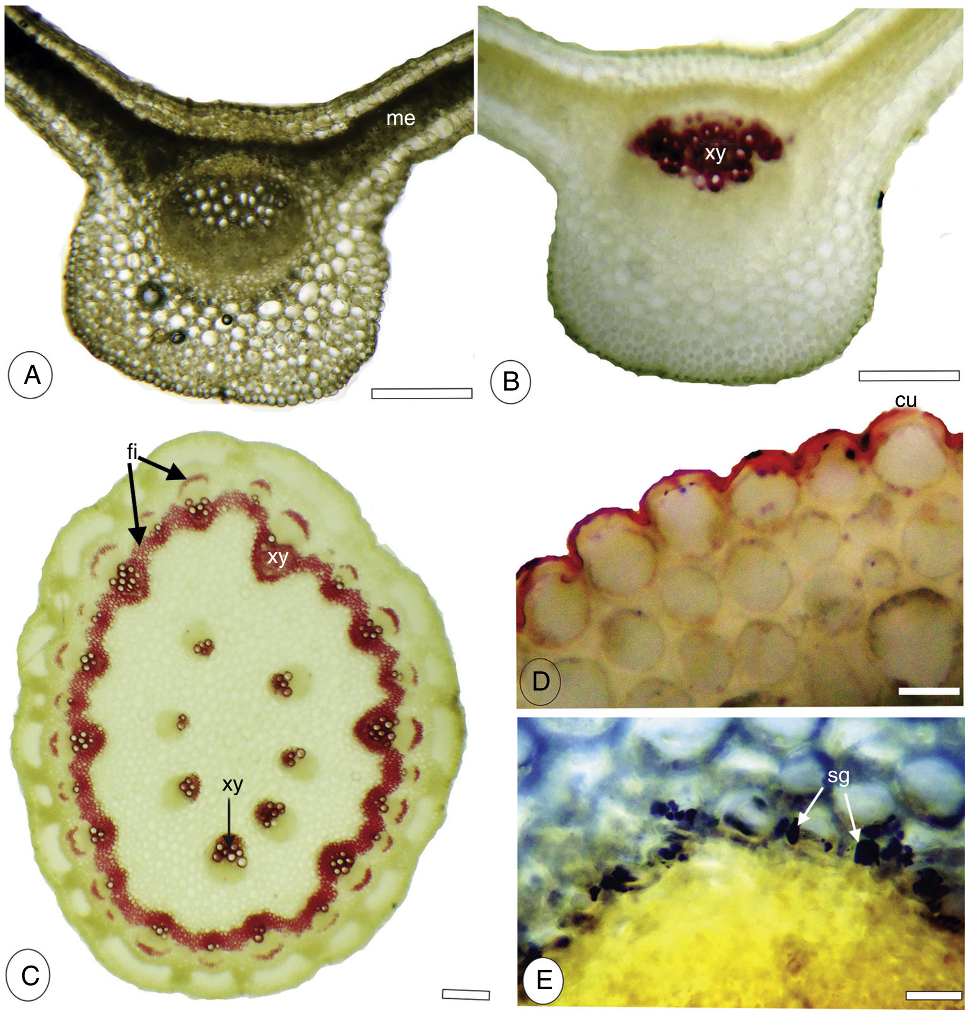

Fig. 5. *Piper amalago* L. (Piperaceae). Leaf and stem in cross section. Microchemical analysis. A. Mesophyll in reaction using ferric chloride. B. Midrib showing xylem in reaction using hydrochloric phloroglucin. C. Stem showing xylem and fibers in reaction using hydrochloric phloroglucin. D. Stem demonstrating cuticle in reaction using Sudan III. E. Stem showing endoderm with starch grains using iodine-iodide. cu: cuticle, fi: fibers, me: mesophyll, sg: starch grain, xy: xylem. Bar = 200 μm (A–C), 20 μm (D), 10 μm (E).

Conclusion

As expected, the leaf and stem anatomy of *Piper amalago* were similar to other *Piper* species. The features observed should be evaluated as a representative of the entire species, even though several structures can be highlighted as distinguishable among the species of the genus. The main characteristics include a hypostomatic leaf, a sub-epidermal layer on the surfaces, sac-like glandular trichomes, conical non-glandular trichomes, dorsiventral mesophyll, a plano-convex midrib with a single collateral vascular bundle, short petiole with irregularly shaped and adaxially grooved. At the caulinar level that was analyzed, the shape is circular and there are collateral vascular bundles arranged in two rings (the outer one in the vascular cylinder and the inner one in the pith). In the perimedular region, a sinuous sclerenchymatic sheath is apparent.

Authors' contributions

VLPS assisted in collecting and identifying plant samples, making a herbarium, conducting laboratory experiments, data analysis and drafting the paper. JMB supervised the laboratory work. IJMR undertook a critical reading of the manuscript. CRCF assisted in the scanning electron microscopy (SEM) analysis. EA was responsible for preparing the permanent slides. All the authors have read the final manuscript and agreed to its submission for appraisal.

Conflicts of interest

The authors declare no conflicts of interest.

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