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Original Article

Morpho-anatomical characters of *Limonium brasiliense* leaves

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ABSTRACT

Limonium brasiliense (Boiss.) Kuntze, Plumbaginaceae, is a coastal herb of the Southern Brazilian states and Rio de Janeiro State. In folk medicine, it is used in the treatment of female genitourinary tract infections and menstrual cycle irregularities. The aim of this study was to describe morpho-anatomical characters with details on venation pattern of *L. brasiliense* leaves, collected on the coast of Rio Grande do Sul State, in Southern Brazil. Leaf samples fixed in FAA50 (5% formaldehyde, 5% acetic acid and 90% ethanol: water mixture 50:50 v/v) and 1% glutaraldehyde and preserved in 70% ethanol, were histologically sectioned and analyzed by light and scanning electron microscopy. The *L. brasiliense* leaves were simple, entire, incomplete, oboval-lanceolate, venation penninervous and showed rosulated phyllotaxy, entire and revolute margin, with apex rounded to slightly retuse, base attenuate and venation pattern brochidodromous. On microscopic analysis, these leaves showed a thick and striated cuticle, salt glands, isobilateral mesophyll thicker in the apical region of the leaf blade, abundant aerenchyma in the petiole and main vein, collateral vascular bundles surrounded by fiber-sclereids, lipid substances in the cuticle and polyphenols in phloem cells.

Introduction

Plumbaginaceae Juss. is a cosmopolitan family comprising about 24 genera and 635 species (The Plant List, 2018), and only *Limonium* Mill. and *Plumbago* L. are found in Brazil (Flora do Brasil, 2018). *Limonium brasiliense* (Boiss.) Kuntze, popularly known as "baicuru, guaicuru or guaicurá" (Dias da Silva, 1920) is a native, non-endemic terrestrial plant, found in the mangrove and restinga vegetation of Southern Brazil, with confirmed occurrence also in Rio de Janeiro State (Funez et al., 2018). Simões et al. (1998) reported the presence of *L. brasiliense* in other countries, including Uruguay and Argentina.

Halophyte plants tolerate saline concentrations that kill 99% of other species (Flowers and Colmer, 2008). On average, more than 90% of the sodium in halophyte plants can be found in the aerial parts, mainly in the leaves (Flowers et al., 1977). One of the strategies used by this species for combating the ionic stress imposed by salinity and to avoid sodium toxicity in the cytosol is the compartmentalization of this ion, where it is used as an osmotic agent in the vacuole, with consequent ionic homeostasis (Ariza, 2012). Abiotic stress affects the biosynthesis, concentration, transport and storage of primary and secondary metabolites (Fraire-Velázquez and Balderas-Hernández, 2013). In fact, different salt concentrations induced a higher antioxidant capacity in *Lepidium latifolium* L., Brassicaceae (Boestfleisch et al., 2014). *Limonium* species are classified as crinohalophytes, due to the presence of glands or trichomes responsible for the excretion of salt (Ariza, 2012).

The chemical composition of *Limonium* species consist of amino acids, inorganic elements, vitamins, flavonoids, tannins, polysaccharides, alkaloids and organic acids (Lin and Chou, 2000; Eren and Özata, 2014). Flavonol glycosides, flavonol glycoside gallates, flavones, flavanones, flavan-3-ols and gallic acid have been isolated from the aerial part of *L. sinense* (Girard) Kuntze (Lin and Chou, 2000). Murray et al. (2004) reported the presence of hydrolysable and condensed tannins, leucoanthocyanins, flavonoids, β-sitosterol, saponins, and coumarin in *L. brasiliense*.

Regarding the biological activity of *Limonium* species, compounds isolated from *L. sinense* roots demonstrated a suppressive effect on replication of herpes simplex virus type-1 (Lin et al., 2000; Kuo et al., 2002) and the methanolic extract of *L. sinense* leaves displayed hepatoprotective activity in rats (Chaung et al., 2003).

The rhizome of *L. brasiliense*, sometimes erroneously referred to as roots (Antonelli-Ushirobira et al., 2015a), are used in popular medicine to regulate menstrual cycles (Lifchtz, 1981) and treat inflammation of the uterus and ovary, dysmenorrhea and vaginal

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discharge (Mentz and Schenkel, 1989), and as an antimicrobial (Rosito, 1975), anti-inflammatory (Moura et al., 1985) and antioxidant substance (Murray et al., 2004).

Mota (1963) described the antimicrobial action of the dioxane extract of *L. brasiliense* leaves against *Escherichia coli*, *Salmonella typhi* and *Serratia marcescens*. Antonelli-Ushirobira et al. (2015b) reported that acute toxicity analysis and long-term safety assessment of a crude extract of *L. brasiliense* rhizomes had little or no toxicity in rats and mice, indicating a potential for medicinal use.

Detailed studies of the cells and tissues of medicinal plants with potential to become effective herbal medicines are essential for future pharmacognostic analysis, ensuring the detection of fraud or the use of erroneously identified plant samples. A morpho-anatomical characterization of *L. brasiliense* leaves was first carried out by Dias da Silva (1920) and should be extended for a better understanding of the tissues and cell types of this species. The aim of this study was to describe morpho-anatomical characters with details on venation pattern of *L. brasiliense* leaves, which would help in the recognition and establishment of quality control parameters of plant material from this species.

Materials and Methods

Plant material

Leaves of *Limonium brasiliense* (Boiss.) Kuntze, Plumbaginaceae, were collected in February 2013 on the Ilha dos Marinheiros (31°59′33″ S, 52°10′43″ W) in the city of Rio Grande, Rio Grande do Sul, Brazil. The collection of the plant material was registered with IBAMA-SISBIO under No. 11995-3, November 2, 2010, authentication code 46367613 and under the responsibility of João Carlos Palazzo de Mello. Access to the botanical material was authorized and licensed by CNPq, registration No. 010252/2015-0. The plant material containing inflorescences was used to prepare a voucher specimen, which was identified by Prof. Dr. Lilian Auler Mentz (Universidade Federal do Rio Grande do Sul) and stored at Universidade Estadual de Maringá Herbarium (HUEM) under registration No. 27725.

Morpho-anatomical analysis

The macroscopic characterization of the dried leaves of *L. brasiliense* was based on the notes of Rizzini (1977) and Oliveira et al. (2014). Mature leaves from the third to fifth node were fragmented and rehydrated in a boiling solution of 10% glycerin for 30 min. 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 and Arduin, 1997) for anatomical analysis under light microscopy (LM) and scanning electron microscopy (SEM), respectively, after storage in 70% ethanol (Johansen, 1940). For the examination of the leaf under LM, longitudinal, paradermal and transverse sections were prepared freehand with steel blades. These materials were bleached with sodium hypochlorite (30%), double-stained with astra blue (1%) and safranin (1%), and mounted on semi-permanent slides with glycerin gel (Kraus and Arduin, 1997).

For analysis under SEM, previously fixed leaves were sectioned. Afterwards, the samples 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 and 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 Shimadzu SS 550 SEM (at 15 kV) was used for ultrastructural analysis. The leaves were diaphanized according to Foster (1949). The descriptions by Ellis et al. (2009) were used for the analysis and description of the foliar venation pattern.

Histochemical tests

Histochemical tests were done with cross-sections, which were stained with the following: Lugol's iodine solution to reveal the presence of starch grains; iodinated zinc chloride, for lignin; Sudan IV glycerin, for lipophilic substances; ferric chloride, for polyphenols; and 60% chloral hydrate with 25% sulfuric acid, for calcium oxalate crystals (Johansen, 1940; Berlyn and Miksche, 1976; Kraus and Arduin, 1997; Farmacopeia Brasileira, 2010).

Powder analysis

The limb and petiole regions of *L. brasiliense* were fragmented with a knife mill (Marconi, TE048 series 890857). After rehydration of the plant material, slides were prepared for LM. The images of semi-permanent and histochemical test slides were obtained with an Olympus CX31 light microscope equipped with a Motic 3.0 digital camera, and analyzed using the program Motic Image Plus 2.0.

Results and Discussion

The leaves of *L. brasiliense* (Fig. 1A, B) were simple, rosulated phyllotaxy, entire and incomplete, symmetrical, oboval-lanceolate, venation penninervous, with entire margin slightly sinuous and revolute, apex rounded to slightly retuse and attenuate base. The leaves were 12 cm long and 3 cm wide, glabrous, herbaceous in consistency, light green, odorless, astringent and rough to the touch. The petiole was concave–convex in cross-section with an invaginated base and length of 12 cm. The petiole and the midrib showed a convex salience on the abaxial side. The flowers of *L. brasiliense* were small and arranged in an open panicle (Fig. 1A).

The first morphological descriptions of *L. brasiliense* leaves were found in Flora Brasiliensis (Martius et al., 1840–1906) and Boletim da Associação Brasileira de Farmacêuticos (Dias da Silva, 1920). The latter publication reported the presence of a long invaginated petiole from 3 to 6 cm in length, smaller than that observed in the samples analyzed in the present study.

The primary venation pattern type of *L. brasiliense* leaves was pinnate, with a single primary vein (midrib). The secondary venation pattern was simple brochidodromous. The secondary veins did not end at the margin and were joined in prominent arches. The length of intersecondary veins was less than 50% of subjacent secondary veins and course were parallel to secondary vein and perpendicular to midrib, in all leaf blade portions (Fig. 1C). The organization pattern of tertiary veins was of the irregular reticulate type, in which the angles of anastomoses form irregular polygons (Fig. 1D). The areolas were irregular with polygonal shape, and freely ending in dendritic branched veinlets (Fig. 1D). At the margin of the leaf blade, the vein endings were incomplete, and there were terminal tracheids in this and other portions of the leaf blade (Fig. 1E).

The cuticle was thick and striated, whose details were best seen in SEM (Fig. 2B, E, F) but also observed in LM (Fig. 2A, C, D), with constant patterns in all leaf blade portions, and it reacted positively to Sudan IV in the histochemical test, revealing the presence of lipophilic substances. The thickened cuticle is an adaptation found in plants with water stress, such as halophytes, aimed at reducing transpiration (Ariza, 2012). In cross-section, the epidermis was uniseriate and the cells were slightly papillary on the adaxial side, with similar size on the two sides (Fig. 2C), different from that Dias da Silva (1920) said when described the epidermis of *L. brasiliense*

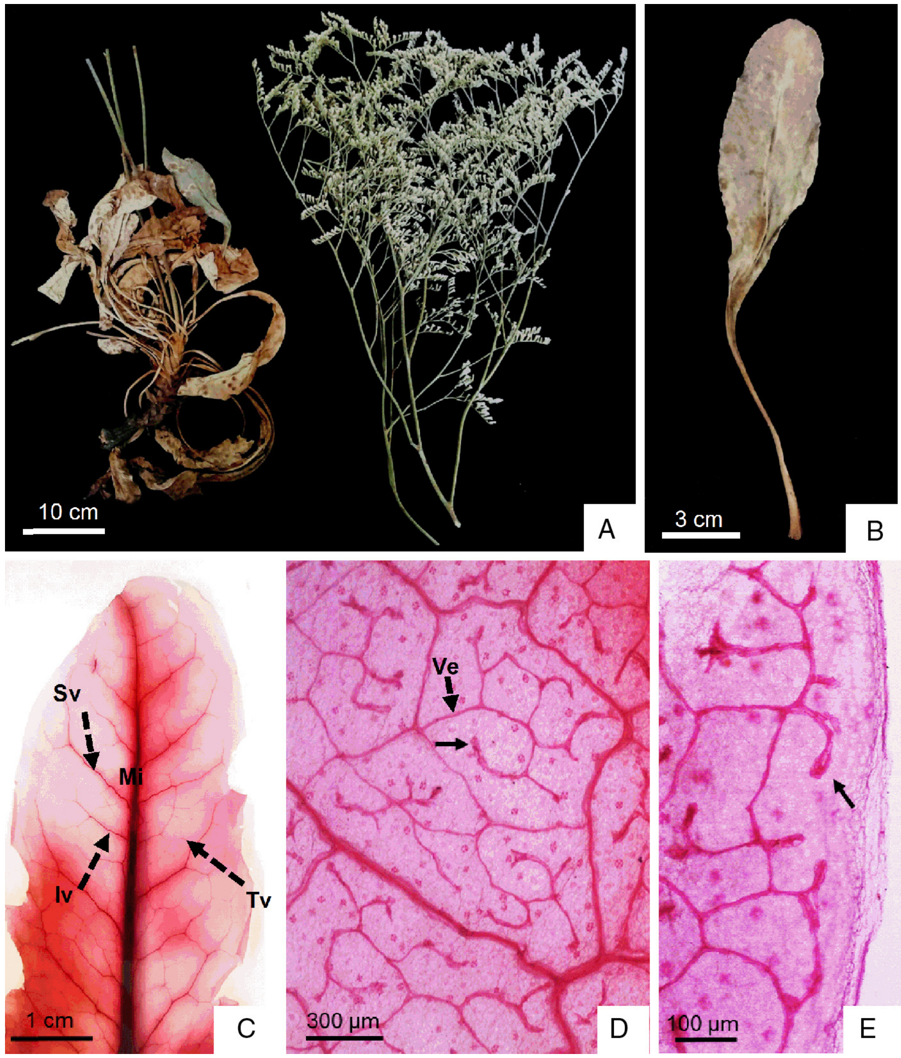

Figure 1. Macroscopic features and venation pattern of *Limonium brasiliense* leaves. (A) General aspect of the dry plant. (B) Morphological aspect of leaf. (C) Orders of venation. (D) Detail of irregular polygonal areolas and branched veinlets. (E) Margin of the leaf blade and terminal tracheids of veins (full arrows). Iv: intersecondary vein, Mi: midrib, Sv: secondary vein, Tv: tertiary vein, Ve: venule.

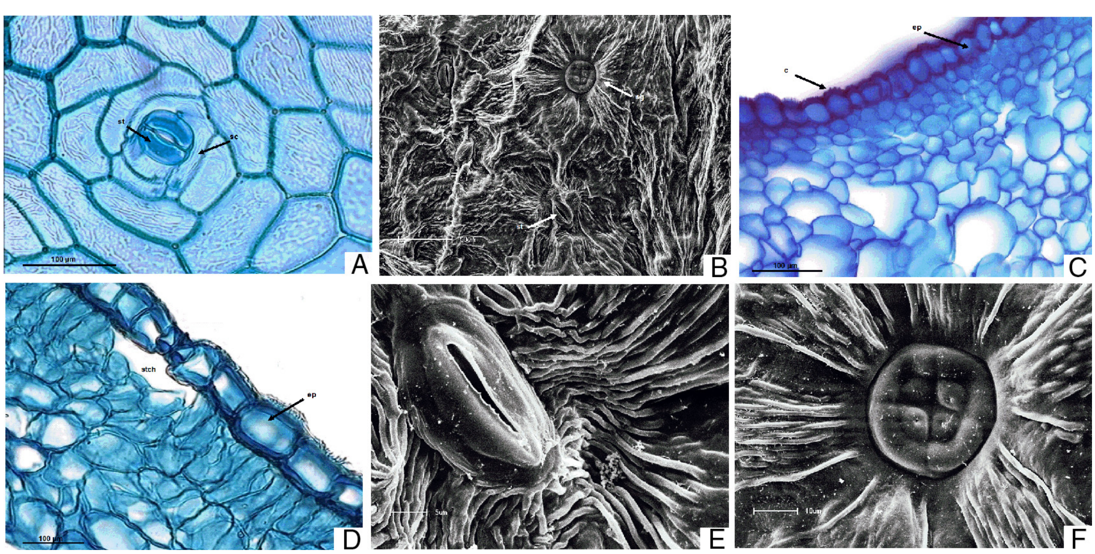

Figure 2. Epidermal cells of adaxial (B, C, E and F) and abaxial (A and D) sides of *Limonium brasiliense* leaves. Frontal view of epidermal cells and tetracytic stomata (A). General aspect of the striated cuticle (B). Cross-section of uniseriate epidermis with papillary cells (C). Detail of stomata in cross-section (D) and in frontal view (E). Salt gland in frontal view (F). c: cuticle, ep: epidermis, sc: subsidiary cells, st: stomata, stch: substomatal chamber, sg: salt gland. A, C and D: in LM; B, E and F: in SEM.

leaf. In frontal view, these cells had polygonal to irregular shapes and walls that were straight periclinal to slightly curved (Fig. 2A), characteristics previously described in the samples analyzed by Dias da Silva (1920).

Limonium brasiliense leaves were amphistomatic, as observed in *Limonium sinuatum* (L.) Mill. by Oviedo de Blas (1992) and in other *Limonium* species analyzed by Colombo (2002). The stomata were predominantly anisocytic (Fig. 2A), a type also found in *Plumbago auriculata* Lam., *P. indica* L., and *P. zeylanica* L. and another genus of Plumbaginaceae (Galal et al., 2013), in *Limonium albidum* (Guss.) Pignatti, *Limonium lopadusanum* Brullo, and *Limonium intermedium* (Guss.) Brullo, native species of the Mediterranean Sea region (Colombo and Trapani, 1992), and in all *Limonium* species from the Iranian-Turanian region described by Akhani et al. (2013). However, in the present study, tetracytic stomata were also observed, a characteristic described by Dias da Silva (1920).

Akhani et al. (2013) observed the presence of three, four, and five subsidiary cells around the stomata, of the same size as other epidermal cells, and in some cases smaller ones in *L. gmelinii* (Willd.) Kuntze and *L. lobatum* (L. f.) Kuntze. The *L. brasiliense* stomata were located at the same level as the other epidermal cells (Fig. 2A, D). The presence of a relatively voluminous substomatal chamber in *L. brasiliense* (Fig. 2D) is another common characteristic of plants adapted to water stress, as observed in bromeliad species (Souza et al., 2005), Cactaceae (Dettke and Milaneze-Gutierre, 2008) and Eriocaulaceae (Scatena et al., 2004).

Another characteristic of *L. brasiliense* leaves was the presence of salt glands on both leaf blade sides (Fig. 2B, F). These structures prevent the damaging accumulation of ions in the tissues of halophyte plants, which can survive in flooded areas near the sea. The salt glands in *L. brasiliense* leaves were surrounded by 5–8 cells larger than that of the epidermis, arranged in a rosette, as described by Colombo and Trapani (1992) for *L. albidum*, *L. lopadusanum* and *L. intermedium*. These authors also described the internal structure of this gland as a cup with a circular ring, within which 12 cells are grouped as follows: four excretory, each with a characteristic pore, similar to the cell shown in Fig. 2F; four internal like a calyx; and four external as collector cells. Other species such as *L. bocconei* (Lojac.) Litard., *L. pignanttii* Brullo and De Martino, and *L. lojaconoi* Brullo also have salt glands formed by 12 cells (Colombo, 2002). These salt glands have two functions: the first is to reduce the elimination of water by the leaf, and the second is to eliminate salt, forming small scales on top of the glands, preventing excessive transpiration (Dias da Silva, 1920).

The mesophyll was isobilateral, and depending on the region of the leaf blade, there was a variation in the number of cell layers (Fig. 3). In the central and basal regions of the leaf blade, adjacent to the adaxial side, there were two layers of palisade parenchyma and one layer of this parenchyma on the abaxial side (Fig. 3B); while in the apical region (Fig. 3A, D), including its margin (Fig. 3C), there were 2–3 layers of palisade parenchyma on the adaxial side and 1–2 layers of this parenchyma on the abaxial side. The palisade parenchyma was absent only next to the biggest convexity on the basal region of the midrib (Fig. 3E). The spongy parenchyma, on the central region, consisted of 5–8 cell layers, with cells of different shapes and sizes, varying from isodiametric to irregular forms and with the presence of small brachiform expansions that allow the formation of intercellular spaces (Fig. 3B).

The midrib, in cross-section, was flat in the apical region and concave in the central and basal region (Fig. 3E) of the adaxial side and very convex in the abaxial side, as described by Dias da Silva (1920). The midrib epidermal cells were elongated, in

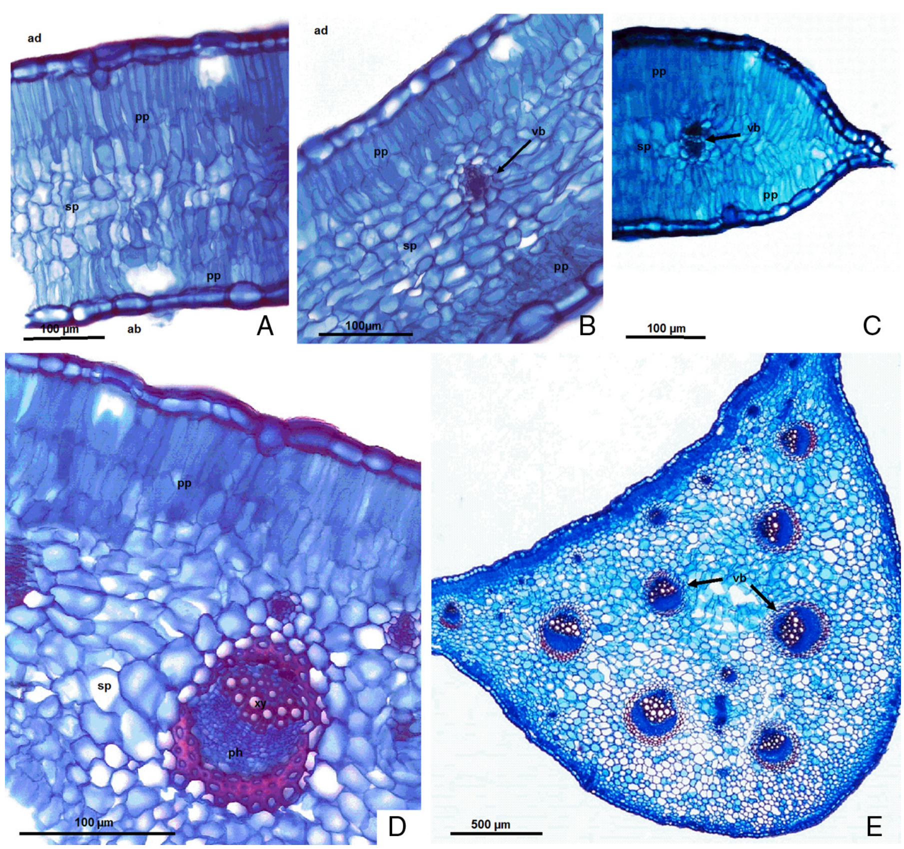

Figure 3. Mesophyll of *Limonium brasiliense* leaves. Cross-section of mesophyll in the apical (A), central (B) and (C) margin regions. Detail of the vascular bundle in the apical region, in cross-section (D). Cross-section of midrib in the basal region (E). ab: abaxial side, ad: adaxial side, ph: phloem, pp: palisade parenchyma, sp: spongy parenchyma, st: stomata, vb: vascular bundles, xy: xylem. A, B, C, D and E: in LM.

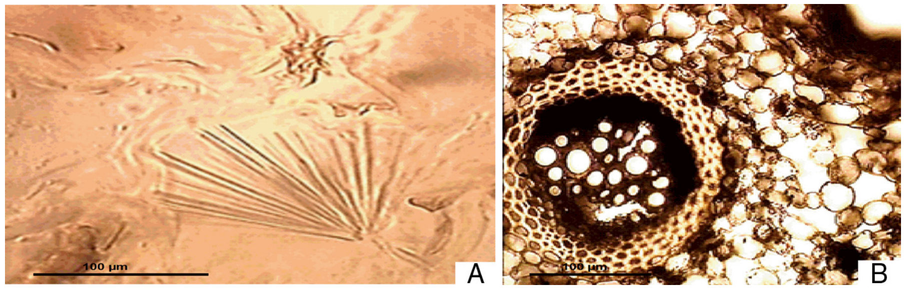

Figure 4. Histochemical tests of *Limonium brasiliense* leaves. Reorganization of calcium oxalate into acicular crystals after reacting with the chloral hydrate with sulfuric acid solution (A). Phloem reaction with ferric chloride (B). A and B: in LM.

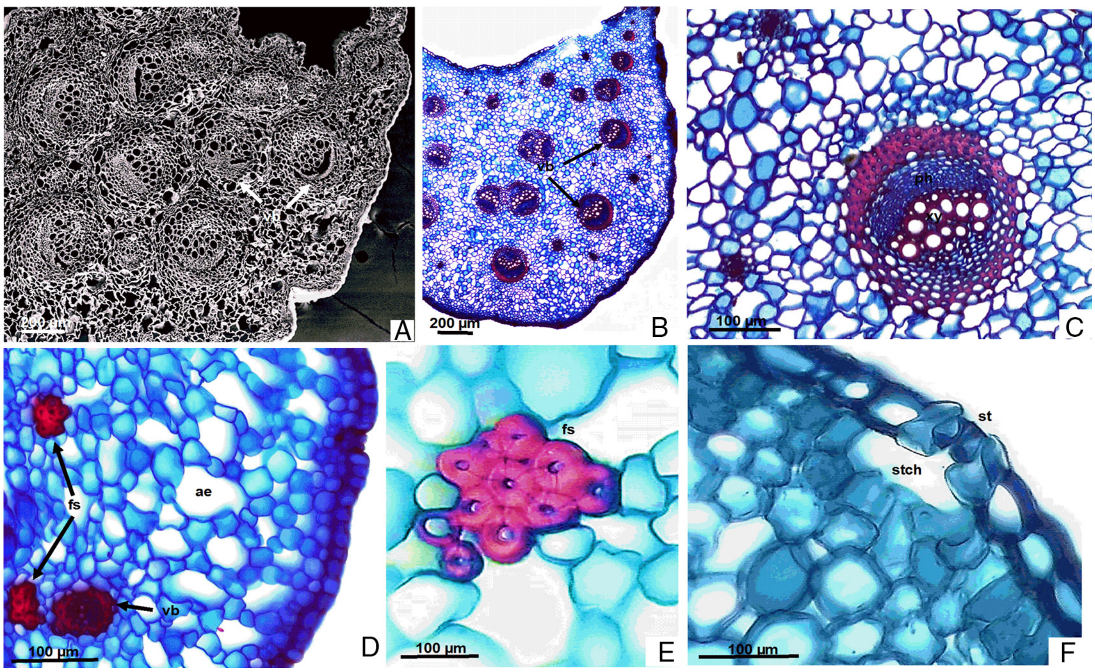

Figure 5. Petiole of *Limonium brasiliense*. Cross-section of petiole in the central region (A and B). Detail of vascular bundles of petiole in the central region, in cross-section (C). Typical aerenchyma and clusters of fiber-sclereids (D). Detail of sclerenchyma, in cross-section (E). Cross-section of stomata in the central region (F). ae: aerenchyma, fs: fiber-sclereid, ph: phloem, st: stomata, stch: substomatal chamber, sg: salt gland, vb: vascular bundles, xy: xylem. A: in SEM; B, C, D, E, F, G, H and I: in LM.

cross-section, and there were stomata and salt glands, similarly on the two sides of the leaf blade. The midrib vascular bundles were collateral (Fig. 3E), and there were fiber-sclereids in the phloem that completely or partially enveloped each bundle of conductive tissues, as found in *L. axillare* (Forssk.) Kuntze, *L. iranicum* (Bornm.) Lincz., *L. otolepis* (Schrenk.) Kuntze, and *L. reniforme* (Girard) Lincz. (Akhani et al., 2013). There were fewer vascular bundles toward the apical region of the leaf blade and wide variations between the specimens analyzed. Anastomoses between two or more sets of vascular bundles were also observed. The presence of smaller vascular bundles between the other bundles was common, as observed by Akhani et al. (2013) in different species of *Limonium*. In the vessel elements, perforated plaques of a simple type were observed. Rare idioblasts with prismatic calcium oxalate crystals close to the vascular bundles and in the mesophyll parenchyma were observed after positive reaction with 60% chloral hydrate and 25% sulfuric acid (Fig. 4A), give rise of acicular crystals of calcium sulphate (Oliveira et al., 2014). Histochemical tests also showed that fiber-sclereids in vascular bundles contained lignin, reacting positively with iodinated zinc chloride, and that the elements making up the phloem reacted positively with ferric chloride (Fig. 4B), indicating the presence of polyphenols. No starch grains were detected in the specimens analyzed.

Typical aerenchyma was seen in the most peripheral portions of the base of the midrib and throughout the petiole, especially in the middle region toward the enlarged base (Fig. 5D). This type of parenchyma is common in plants that inhabit soils subject to flooding, such as *L. brasiliense*, where there is an accumulation of gases in the intercellular spaces, preventing tissue anoxia. In cross-section, the parenchyma cells were oval to polygonal and of different sizes, but always with a thin cell wall (Fig. 5F). In longitudinal section, these cells were rectangular with a straight and sinuous cell wall. The vascular bundles were collateral (Fig. 5A, B, C) and fiber-sclereids were observed alone or in small clusters near the concavity of the abaxial side, in the basal region of the petiole (Fig. 5D, E).

The main anatomical characteristics found in the analysis of *L. brasiliense* leaf powder were the presence of the following: epidermal fragments with striated cuticle (Fig. 6C), salt glands and stomata (Fig. 6A, B, C); fragments with voluminous epidermal cells; mesophyll with a variable number of palisade parenchyma strata (Fig. 6D); rectangular fragments of parenchyma cells that might

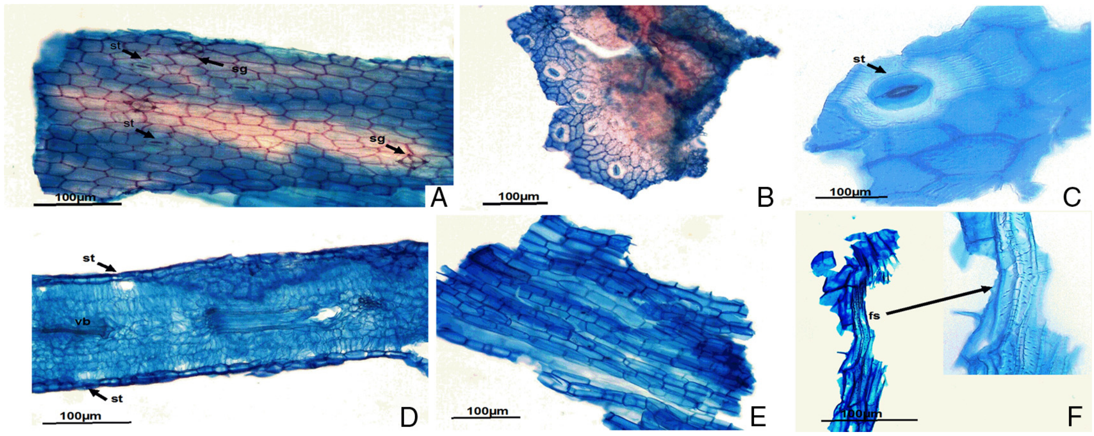

Figure 6. Fragments observed in rehydrated powder of *Limonium brasiliense* leaves. Epidermal cells (A, B and C). Mesophyll (D). Detail of fundamental parenchyma of the midrib (E). Fiber-sclereid (F). fs: fiber-sclereid, st: stomata, sg: salt gland, vb: vascular bundles. A, B, C, D, E, F: in LM.

have originated from the petiole and midrib (Fig. 6E), with fiber-sclereids present as well (Fig. 6F).

Conclusions

The analyses allowed a detailed morphological and anatomical description of *L. brasiliense* leaves, providing the necessary help to carry out the quality control of this species. The main morpho-anatomical characters of *L. brasiliense* leaves were as follows: leaf blade simple, oboval-lanceolate and venation penninervous, with whole margin slightly sinuous and revolute, apex rounded to slightly retuse and attenuate base; very thick and striated cuticle; salt glands on both leaf blade sides; isobilateral parenchyma; aerenchyma abundant in the petiole, being present also in the midrib; and collateral vascular bundles surrounded by fiber-sclereids. Variations in the number of vascular bundles and strata of palisade parenchyma were observed in the midrib and mesophyll, respectively, and should be considered in pharmacognostic analysis, to avoid an incorrect diagnosis of *L. brasiliense* leaves.

Authors' contributions

NCG conducted the laboratory work, prepared the plant material for microscopic analysis, and wrote and formatted the manuscript. DCM assisted with the review, analysis, discussion and formatting of the manuscript. MAMG supervised the laboratory work, performed the microscopic analyses, and supervised the writing. JCPM was responsible for conceiving the project and assisted with the writing, review, and supervision of the study.

Conflicts of Interest

The authors declare no conflicts of interest.

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