Anatomy and histochemistry of leaves and stems of *Sapium glandulosum*

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**Abstract**

*Sapium* belongs to Euphorbiaceae family and comprises 23 species. *Sapium glandulosum* (L.) Morong is popularly known in Brazil as “pau-leiteiro” and “leiteisinha” and it is used in traditional medicine to cicatrization. Its leaf extracts have shown analgesic, anti-inflammatory and antibacterial activities. The preliminary set of pharmacognostic tools used for quality assessment of medicinal plant parts is macro- and micro-anatomy and *S. glandulosum* has not anatomical and histochemical description. Thus the aim of this study was to investigate the anatomical and histochemical characteristics of the leaf and stem of *S. glandulosum* as a means of providing information for quality assessment of herbal industry. The leaves and stems were investigated by employing field emission scanning electron microscopy, light microscopy, and histochemistry techniques. The analysis showed that *S. glandulosum* had the following anatomical features: dorsiventral and amphistomatic leaves; paracytic stomata; tabular crystal druses; non-articulated and branched laticifers; midrib's biconvex shape with vascular systems in open arc with invaginated ends; petiole with a round shape and slight concavity on the adaxial side; six collateral vascular bundles in U-shaped organisation; a circular stem shape and a sclerenchymatous ring. In the histochemical tests lipopholic components were found in cuticle and in the leaf; phenolic compounds were met in the mesophyll and in the leaf; starch grains were found in the parenchymatous sheath; lignified elements were met in the sclerenchymatous ring in the cortex and in the perivascular sclerenchymatous caps, beyond in the vessel elements. These features are helpful when conducting a quality control process.

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**Introduction**

*Sapium* Jacq. is one of the most important genus of Euphorbiaceae. It consists of 23 accepted species (The Plant List, 2015) and deserves consideration because of the complexity involved in delimiting its species (Secco et al., 2012). It is formed mainly by neotropical species and is distributed in fields, savannas, seasonal forests, rainforests and woodlands (Sátiro and Roque, 2008; Pscheidt and Cordeiro, 2012).

This genus presents several species that are used in popular medicine, such as *S. chihsinianum* S.K. Lee, *S. discolor* (Champ. ex Benth) Muell. Arg., *S. rotundifolium* Hemsl., and *S. sebiferum* (L.) Roxb., which are used mainly to cicatrization (Al Muqarrabun et al., 2014). Some species of *Sapium* have been chemically and pharmacologically studied. Extracts and single components from this genus were reported to have promising biological activities such as antioxidant, antimicrobial, and cytotoxic effects (Hajdu and Hohmann, 2012; Al Muqarrabun et al., 2014).

*Sapium glandulosum* (L.) Morong, which is popularly known in Brazil as “pau-leiteiro” and “leiteiro”, is a tree that can reach 3–8 m in height and is among the most polymorphic species of *Sapium*. It is used in traditional medicine to treat hernias (Hajdu and Hohmann, 2012; Al Muqarrabun et al., 2014) and its use has been potentially recommended for the recovery of degraded areas (Ferreira et al., 2009).

The leaves of *S. glandulosum* contain anthracene derivatives, monoterpenes, tannins and flavonoids (da Silva et al., 2011, 2012). This species is latex-bearing and the latex has proteins with considerable proteolytic activity. This activity is notably inhibited by a serine protease inhibitor (Sobotka et al., 2014). The leaf extracts...
have shown analgesic, anti-inflammatory (Valle and Kaplan, 2000) and antibacterial activities (da Silva et al., 2012).

Classifying medicinal plants is a serious problem because of their common names. A single medicinal species frequently has a number of popular names and a popular name can occasionally be used for a range of plants (Upton et al., 2011). Some species of Sapium, such as S. glandulosum, S. arbutum (Müll.Arg.) Huber and S. sellowianum (Müll.Arg.) Klotzsch ex Baill (Agra et al., 2008), are popularly known in Brazil as “pau-leiteiro”, “leiteiro” or “burra-leiteira”. In this context the most important consequence in regard to the use of inappropriate folk names is the substitution of therapeutic and safe herbs by toxic vegetable species (Upton et al., 2011).

The preliminary set of pharmacognostic tools used for quality assessment of medicinal plant parts is macro- and micro-anatomy (Upton et al., 2011). Consequently, the aim of this study was to investigate the anatomical and histochemical characteristics of the leaf and stem of S. glandulosum as a means of providing information for quality control in the herbal industry. Furthermore, there are no previous papers in the literature about the pharmacobotanical characteristics of this taxon.

Materials and methods

Plant material

The leaves and stems of Sapium glandulosum (L.) Morong, Euphorbiaceae, were collected from grown specimens in open and sunny areas in the Campos Gerais region of Paraná (24°18' S and 49°37' W), Brazil in October 2013. Mature leaves and stems (at least ten samples) obtained from the sixth node and below (median, intercostal and margin regions), as well as stem fragments from 5 to 15 cm from the shoot were prepared for the pharmacobotanical assays. The plant material containing inflorescences was used to prepare a voucher specimen, which was identified by Osmar dos Santos Ribas and stored at the Museu Botânico de Curitiba under the number 390589 MBM.

Pharmacobotanical assays

The leaves and stems of S. glandulosum were placed in a solution of FAA 70 (Johansen, 1940), and stored in 70% ethanol (Berlyn and Miksche, 1976). For the examination of leaf and stem material free-hand longitudinal and cross-sections were prepared. In the leaves it was included the midrib, internerual regions, and lateral veins. These materials were stained using Astran blue and basic fuchsin (Roer, 1972) and toluidine blue (O’Brien et al., 1964) to obtain semi-permanent slides. The diaphanisation of the leaves was performed by following the technique of Fuchs (1963); for the crystals descriptions He et al. (2012) were used.

Histochemical tests

The following standard solutions were employed in the histochemical 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); Hoeprner–Vorsatz test, modified by Reeve (1951) (aqueous 10% sodium nitrate, aqueous 10% acetic acid, aqueous 10% urea and, 2 N NaOH) and ferric chloride to test for phenolic substances (Johansen, 1940); Bouchardat reactive for nitrogen compounds (Borio, 1959); methylene blue to test mucilage (Oliveira et al., 2005) and iodine-iodide to reveal starch (Berlyn and Miksche, 1976).

Photomicrographs were captured using a Olympus CX 31 light microscope that was equipped with a C7070 digital camera. The semi-permanent and histochemical test slides were then analysed in the Laboratory of Pharmacognosy at the State University of Ponta Grossa (UEPG) for a detailed description of the leaf and stem tissues.

Field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray spectroscopy (EDS)

For the field emission scanning electron microscopy (Mira 3 Tescan) fresh leaves and stems were used. The samples were submitted in high vacuum with high accelerating voltage (15 kV). This method required the samples to be previously hydrated using increasing amounts of ethanol then dried in a critical point dryer. Afterwards, they were submitted to metallisation with gold (Quorum, model SC7620). Qualitative X-ray microanalyses were performed on certain crystals and in cells without crystals (control) using an EDS machine (Mira 3 Tescan) on the same variable-pressure microscope. This procedure was carried out at the multi-user laboratory (LABMU) of UEPG.

Results and discussion

The leaves of S. glandulosum (Fig. 1A, B), in frontal view, showed epidermal cells with straight to slight wavy anticlinal walls (Fig. 1C, F), which were relatively thin on both sides. The leaves were amphistomatic and the paraeystomatic stomata were observed predominantly on the abaxial side (Fig. 1C–E). On the adaxial side, they appeared only near the midrib as observed in Fig. 1F, G. They measured 35 μm in length on average and the striate cuticle was tangentially positioned in the subsidiary cells (Fig. 1C–E). Metcalfe and Chalk (1950) reported paraeystomatic stoma in the Euphorbiaceae tribe. Valle and Kaplan (2000) reported that S. glandulosum had amphistomatic leaves, while S. sellowianum (Müll.Arg.) Klotzsch ex Baill. had hypostomatic leaves. These authors affirmed that the distribution of stomata was a taxonomic feature that helps to separate these two species.

In cross-section, the epidermis was uniseriate and the cells were larger than the adaxial side. The cuticle was smooth and thin and reacted with Sudan III in the histochemical test (Fig. 1H). Druses were found in the epidermal cells (Fig. 1H). The mesophyll was dorsiventral and was formed by one layer of palisade parenchyma and about eight layers of spongy parenchyma. Small collateral vascular bundles were immersed in the mesophyll and they were surrounded by a parenchymatous sheath. Druses were also observed in the mesophyll (Fig. 1H).

Phenolic compounds are secondary metabolites responsible for adaptation and resistance to hostile environment factors. They are implicated not only in the defense mechanisms of plants against fungal pathogens but also against insect herbivores (Lattanzio et al., 2006). In the present study, phenolic compounds reacted positively with ferric chloride and Hoeprner–Vorsatz test and they are found in the mesophyll.

The midrib, in transsection, was biconvex; however, the convexity was more conspicuous on the abaxial surface (Fig. 2A). The epidermis is uniseriate and it is covered by a striate and thick cuticle. The cuticle reacted with Sudan III (Fig. 2C). The cuticle is the most important barrier against uncontrolled water loss from leaves, stems, fruits and other parts of higher plants (Riederer and Schreiber, 2001). Cutin is the main component of the cuticle and is a lipophilic polymer that is deposited in and the top of the outer wall epidermal cells (Upton et al., 2011). Cuticle ornamentation is one of the most useful taxonomic characteristics of epidermis in leaves appearing as striations, ridges, or papillae (Barthlott et al., 1998; Upton et al., 2011).

Beneath the epidermis, on both sides, the chlorenchyma was interrupted and about six strata of angular collenchyma were apparent (Fig. 2A, C). The vascular system was represented by an
open arc with invaginated ends, which was surrounded by a sclerenchymatic sheath (Fig. 2A, B). The organisation of vascular system is relevant feature of species characterisation and differentiation (Almeida et al., 2017; Bobek et al., 2016). In the midrib of the leaf of Euphorbiaceae, the organisation of the vascular tissues was variable (Gaucher, 1902). The vascular system organisation can help S. glandulosum identification.

Several druses were evident in the ground parenchyma, mainly near the sclerenchymatic sheath (Fig. 2B, E, G). Druses are considered cluster crystals and are formed by aggregates having several sides and sharp points (Upton et al., 2011) as blockys, tabulars, styloids, and tetrahedral crystals (He et al., 2012). In the present work these druses were considered to be tabular crystal druses, as described by aforementioned authors for the Mimosoideae family.

Numerous laticifers were observed near the sclerenchymatic sheath and the vascular system (Fig. 2D–F); they were non-articulated and branched, as can be seen in the longitudinal section shown in Fig. 2F. Latex-secreting cells are named laticifers and occur in several families, including some Apocynaceae (Demarco et al., 2006), Campanulaceae (Folquitto et al., 2014), Papaveraceae (Upton et al., 2011) and Euphorbiaceae species (Demarco et al., 2013; Luz et al., 2015). The cell walls of the laticifers vary in shape, size, thickness, distribution and occurrence in the tissues and organs and in their occurrence in particular structures (Gales and Toma, 2007).
Pompert (1989) reported that the laticifers were smaller and less frequent in *S. haematospermum* than in*S. longifolium*.

According to Demarco et al. (2013), Euphorbiaceae presents non-articulated branching and articulated anastomosing laticifers. These authors reported that *S. haematospermum* presented two articulated laticifers systems in the leaf and stem, one that was formed of narrow laticifers and the other shaped by wide laticifers. Non-articulated laticifers are initialised from single cells at an early period in seedling growth; articulated laticifers are formed by chains of cells whose adjoining walls can occasionally break down, forming vessels (Rudall, 1987).

In the present study, the latex was a milky white fluid. In the histochemical tests it reacted positively in relation to lipophilic components with Sudan III (Fig. 3F); and to phenolic compounds in the Hoepfner–Vorsatz test (Fig. 2F) and with ferric chloride (Fig. 3E). The latex reacted negatively for mucilage and nitrogen components. Latex is a white fluid that is commonly exuded from the points of plant damage caused not only mechanically but also by...
insect herbivores (Konno, 2011). It is found in the vacuole of secretory cells known as laticifers, which include a complex combination of compounds such as phenolics, enzymes, terpenes, alkaloids, vitamins, mucilage, and lipids (Hagel et al., 2008; Folquitto et al., 2014; Luz et al., 2015). Latex has been attributed with cytotoxic (Luz et al., 2015), anti-tumour (Biscaro et al., 2013), anti-ulcer (Costa et al., 2012), and proteinase (Sobottka et al., 2014) activities, among others.

The petiole in the middle part and in cross-section had an almost round shape, however, with a slight concavity on the adaxial side (Fig. 3A). The epidermis had the same characteristics as the leaf blade and reacted with Sudan III in the microchemical test. Beneath the epidermis, there were about eight layers of angular collenchyma (Fig. 3B). Laticifers (Fig. 3E, F) as previously described for the midrib, could be observed near the vascular bundles. Druses were found in the ground parenchyma. The vascular system was formed by about six collateral vascular bundles (Fig. 3C) in a U-shaped organisation. Almeida et al. (2016) and Bobek et al. (2016) indicated the importance of the shape and vascular pattern of the petiole in cross-section and affirmed that these characteristics can be used as good markers in plants.

Starch is widely distributed throughout plant tissues, but is commonly found in highest concentrations in roots, rhizomes, and fruits (Upton et al., 2011). In the present study starch grains were met in the ground parenchyma of the midrib (Fig. 2F) and in the vascular bundle sheath of petiole and reacted with iodine-iodide in the histochemical test (Fig. 3D). Not only the presence but also the structure of starch granules can be important for taxon identification (Upton et al., 2011). In S. glandulosum they were very small and rounded and/or ovate and appeared compound aggregates of two or more granules.

In transection, the stem presented a circular shape (Fig. 4A). The epidermis appeared in a single series with thickened cuticle. There were several layers of cells in the cortex (Fig. 4B, C). The sclerenchyma was formed by thickened cells containing lignin, leading to a sclerenchymatous ring in the cortex (Fig. 4C) and in the perivascular sclerenchymatous caps (Fig. 4B). They reacted with hydrochloric phloroglucin reagent (Fig. 4C) and with methylene blue (Fig. 4B). Lignin is polymer high phenolics which confers water resistance, strength, and elasticity. It is deposited among the cellulose microfibrils of primary and/or secondary cell walls (Upton et al., 2011). The presence of perivascular sclerenchymatous caps helped the S. glandulosum identification.

The endodermis was formed by a layer of cells. The vascular cylinder presented cambia, forming phloem outward and xylem inward. As expected, lignin was also found in vessel elements (Figs. 2B, 3C, 4B). Laticifers, as previously reported for the midrib and petiole, appeared close to the vascular system and to the sclerenchyma (Fig. 4D). The pith was composed of relatively small parenchymatous cells with thin walls (Fig. 4A).
Crystal idioblasts were gathered in the stem (Fig. 4E, F), as observed in the mesophyll, midrib and petiole. In the present study, the crystals were analysed for their elemental composition and the spectra showed prominent peaks for calcium (27.74%), carbon (16.7%) and oxygen (55.56%), as can be seen in Fig. 5, indicating that these crystals were formed of calcium oxalate. Crystals have been identified in some studies as calcium oxalate by using EDS (He et al., 2012; Almeida et al., 2016; Swiech et al., 2016).

The functions of crystals in plants are to act as an internal reservoir for calcium, to provide tissue rigidity, ionic balance, to remove calcium, magnesium, oxalic acid, aluminium and other heavy metals, and also to act as a protective device against for-
aging animals (Franceschi and Nakata, 2005; He et al., 2012; Silva et al., 2014). The presence or absence of crystals, their type and their chemical composition, can be characterised as taxonomic features (Meric, 2009). With reference to the chemical composition, excess calcium is habitually precipitated in calcium salts such as carbonate, citrate, malate, oxalate, phosphate, silicate and sulphate (Weiner and Dove, 2003).

Crystals of calcium oxalate are most commonly reported in higher families and they occur in most organs and tissues in the vegetable species. However, their size and number are responsive to changes in the concentration of calcium in the environment (Nakata, 2003; Franceschi and Nakata, 2005). Crystals of calcium oxalate are formed from endogenously synthesised oxalic acid and Ca taken from the environment, and they are formed and accumulated in species-specific morphologies (Meric, 2009).

Anatomical characterisation is an inherent part of practically all pharmacopoeias and is one of the primary identification tests necessary for pharmacopeial compliance. The individual structural elements are comparatively frequent within the same plant organs, but the ways in which the tissues, elements and cells are set within a plant organ permits diagnosis to be performed and lend support to the assessment of herbal drugs (Upton et al., 2011).

The main anatomical characters were highlighted in this pharmacobotanical study, which was performed to provide more information about the standardisation of the S. glandulosus species in order to support the quality control of this vegetable material. The following characteristics are helpful when conducting the quality control process: dorsivenular and amphistomatic leaves; paracytic stomata; calcium oxalate tabular crystal druses; non-articulated and branched laticifers; biconvex with vascular systems in open arc with invaginated ends; petiole with round shape and slight concavity on the adaxial side; six collateral vascular bundles in U-shaped organisation; circular stem shape, sclerenchymatous ring in the cortex and perivascular sclerenchymatous caps.

The histochemical test showed the presence of the phenolic and phenolic compounds in the cortex; phenolic compounds in the mesophyll; stalk grains small and rounded and compound aggregates of two or more granules; lignified elements were met in the sclerenchymatous tissue in the cortex and in the perivascular sclerenchymatous caps, beyond in the vessel elements.

Authors' contributions

EAA, DGF, LECL and KSP assisted in carrying out the laboratory work. EAA contributed in collecting the plant material and its identification. PVF performed the scanning electron microscopy (FESEM) analysis. JMB created the project, supervised the laboratory work, and wrote the paper. 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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References


