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Morpho-anatomical study of the leaf and stem of pau-alecrim: *Holocalyx balansae*

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Abstract: *Holocalyx balansae* Micheli belongs to the Fabaceae family and is called pau-alecrim in Portuguese. In folk medicine, it is indicated to treat heart, hepatic and digestive upsets, as well as being considered anti-septic and diaphoretic. This work aimed to study the morpho-anatomy of the leaf and stem of this native medicinal species, in order to increase knowledge of the Brazilian flora and to contribute to pharmacognostic quality control. Samples of mature leaves and young stems were fixed and either sectioned free-hand or embedded in glycol-methacrylate and sectioned by microtome, then stained. Microchemical tests and scanning electron microscopy were also performed. The leaves are alternate, compound and paripinnate, and the leaflets have a lanceolate shape. Anomocytic and anisocytic stomata are found exclusively on the abaxial surface. The mesophyll is dorsiventral and the midrib, in cross-section, is flat on both sides, showing one collateral vascular bundle, encircled by a sclerenchymatic and crystalliferous sheath. The rachis and petiole present a collateral vascular bundle with a centric arrangement. The stem presents phellogen localized superficially. In the vascular cylinder, there is a sclerenchymatic sheath and continuous phloem and xylem cylinders, both traversed by narrow rays. Prismatic crystals of calcium oxalate are present in the leaf and stem.

Introduction

The family Fabaceae Lindley, also known as Leguminosae Jussieu, includes approximately 740 genera and 19400 species with a broad distribution and widely recognized importance (Mobot, 2009). Among the members of this taxon is the species *Holocalyx balansae* Micheli (Caesalpinioideae), known in Brazil as pau-alecrim, alecrim-do-campo and ibirapepê (Lorenzi, 2002; Carvalho, 2003), which is employed in heart failure, in hepatic and digestive disturbances and in the treatment of menstrual irregularity, being considered an antiseptic, diaphoretic and digestive in Brazilian popular medicine (Körbes, 1990).

This species is a leafy, semi-deciduous tree (Figure 1A), which reaches 15 to 25 m in height, with a winding stem 50 to 80 cm in diameter with dark gray, thin and almost smooth bark, and has compound leaves with numerous narrow leaflets (Burkart, 1979; Côrrea, 1984; Lorenzi, 2002).

Phytochemical studies have revealed the presence in the leaves of kaempferol-3,7-dirhamnoside (kaempferitrin), a flavonol glycoside with diuretic, anti-inflammatory and anti-capillary fragility activities (Muradian et al., 1975). Due to its ethnopharmacological importance and the lack of studies on this native medicinal species, the present investigation sought to obtain morpho-anatomical data regarding the leaf and stem of *H. balansae*, in order to increase the knowledge of Brazilian flora and to contribute to the pharmacognostic quality control of this potential plant drug, by comparing it with other medicinal species in the same family.

Material and Methods

Plant material

Samples of *Holocalyx balansae* Micheli, Fabaceae were collected in July 2008 from individuals cultivated in an open and sunny environment in the municipality of Curitiba, Paraná, approximately 25º25'S and 49º16'W, and at an altitude of 930 m. An exsiccate was identified at the herbarium of the Museu Botânico Municipal de Curitiba, and indexed under registration MBM 285665.

Methodology

Adult leaves and young stems, collected

between 5 and 20 cm from the stem apex, were fixed in FAA 70 (Johansen, 1940) and stored in 70% ethanol (Berlyn & Miksche, 1976). The description of the leaf external morphology, including the veining pattern, was based on Hickey (1974) and the sizes were determined from the simple means of the measurements taken from at least ten leaves and twenty leaflets.

For the anatomical description the material was sectioned free-hand, in transverse and longitudinal directions, including paradermal sections of the leaf. Sections were stained with astra blue and basic fuchsine (Roeser, 1972) and then mounted in 50% glycerin (Kraus & Arduin, 1997). Permanent slides were prepared from the fixed material, dehydrated in a graded ethanolic series and embedded in glycol methacrylate (Kraus & Arduin, 1997). The tissue was sectioned using a rotary microtome and then stained with toluidine blue (O'Brien et al., 1964). Finally, sections were mounted with Entellan®.

In parallel, microchemical tests were carried out with the following solutions: hydrochloric phloroglucin (Foster, 1949), for the detection of lignin; Sudan III (Sass, 1951) for lipophilic compounds; ferric chloride (Johansen, 1940) to visualize phenolic compounds; lugol (Berlyn & Miksche, 1976) for starch, and sulfuric acid (Oliveira & Akisue, 1997) to confirm the chemical nature of the crystals of calcium. The results were recorded by means of photomicrographs.

Ultrastructural analysis of the surface (scanning electron microscopy; SEM) was performed on the leaf blade of fixed samples, dehydrated in a graded ethanolic series and critical point dried in CO_2. These were adhered to a support, coated with gold and observed under a high vacuum in a scanning electron microscope, and the images obtained were subsequently digitalized (Souza, 1998).

Results

Leaf

The leaves (Figures 1B, 1C) are alternate, compound, paripinnate, short petiolate, with a chartaceous texture and approximately 12 cm in length. The leaflets are asymmetric, lanceolate, with an obtuso-mucronate apex, a rounded base, a slightly serrated margin and a reduced petiolule. The mean length was 1.6 cm, while the mean width was 0.3 cm. With regard to veining, the leaflets were classified as actinodromous, in which three or more primary veins diverge radially from a single point.

On the blade of the leaflet, in face view, the cells of the epidermis on both surfaces present anticlinal walls with a slightly wavy form (Figures 1D, 1E). The cuticle is smooth (Figures 1F, 1G) and the stomata are anisocytic and predominantly anomocytic, and restricted to the abaxial surface (Figures 1E, 1G). In transverse section, the epidermis is uniseriate, consisting of periclinally elongated cells and covered with a slightly thick cuticle (Figures 2A-2D). The stomata are found to be inserted on the same level in relation to the remaining epidermal cells and show evident external cuticular ledges (Figures 2C, 2D).

The mesophyll is dorsiventral, consisting of approximately two layers of palisade parenchyma and six strata of spongy parenchyma. The cells closest to the abaxial side tend to be elongated anticlinally. Collateral vascular bundles are distributed through the mesophyll, with those of medium size having sclerenchymatic caps (pericycle) adjoined to the xylem and the phloem (Figure 2C).

The midrib, in transverse section, is flat on both the adaxial and abaxial surfaces. Subjacent to the adaxial side palisade parenchyma is seen and, on the opposing side, spongy parenchyma. There is a major vascular bundle, ovate and collateral, surrounded in series by sclerenchymatic (pericycle) and crystalliferous sheaths (endoderm), the latter featuring prismatic crystals of calcium oxalate (Figure 2D).

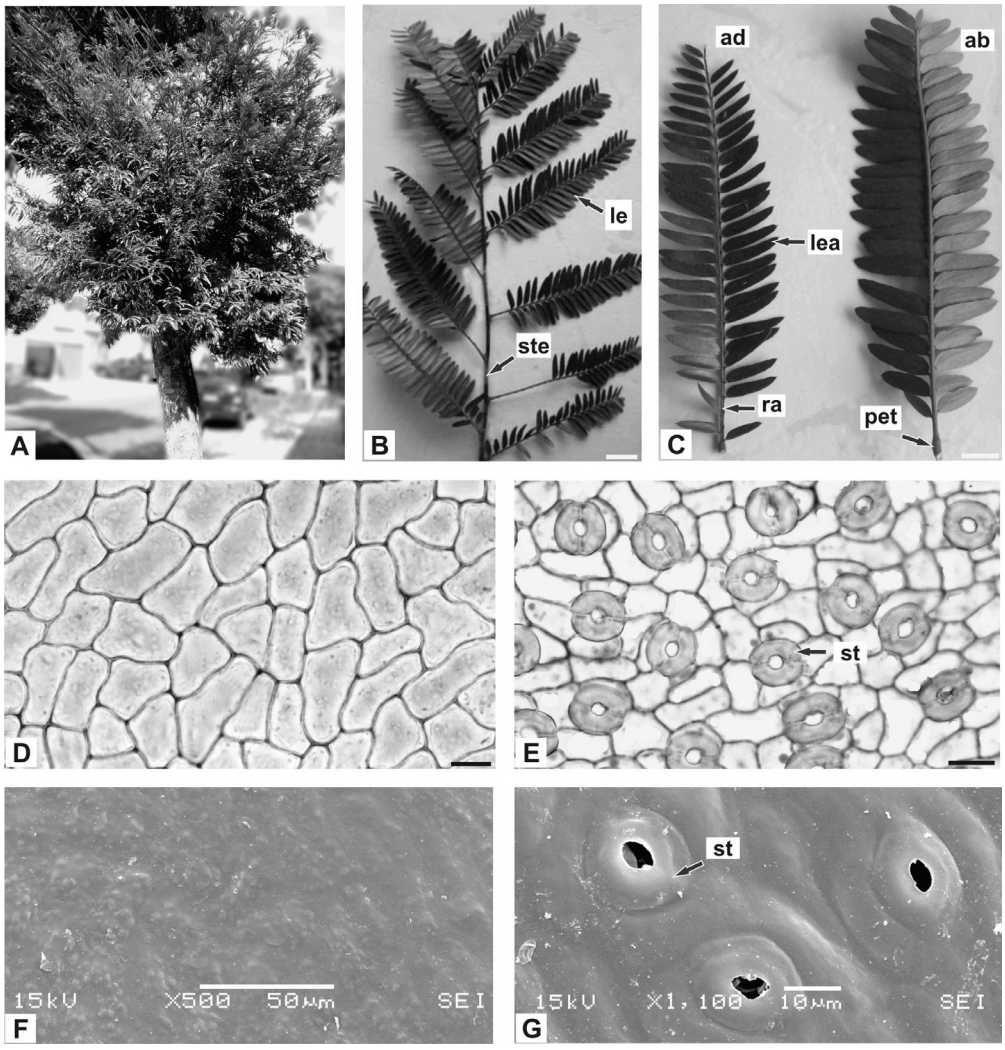
The rachis, in transverse section, presents a concave-convex shape, with small lateral projections from the adaxial side, each with an accessory bundle (Figure 2G). The cuticle is thick, forming cuticular flanges and covering the uniseriate epidermis (Figure 3A). A collateral vascular bundle with a cylindrical arrangement is noted, surrounded by a prominent sclerenchymatic sheath (Figures 2G, 3A).

The petiole (Figure 2E), in transverse section, has an approximately circular shape. There are cuticular flanges, a uniseriate epidermis and some simple non-glandular trichomes, which are uni- (Figure 2F) and multicellular. Next, there is a strand of annular collenchyma, accessory vascular bundles next to the adaxial surface and a larger vascular bundle of the collateral type, in a closed arc, with a cylindrical arrangement. Around this, a practically complete sclerenchymatic sheath (pericycle) is observed, as well as some stone cells in the ground parenchyma.

Cells containing amyloplasts, phenolic compounds and prismatic crystals of calcium oxalate are present in the leaflet (Figures 2C, 2D), in the rachis and in the petiole.

Stem

The stem (Figures 3B, 3C), in transverse section, presents a circular shape. The epidermis is uniseriate, with some non-glandular trichomes and, on the level analyzed, is in a detaching process. Phellogen is found in the superficial layers, forming the periderm



Figures 1A-F. *Holocalyx balansae* Micheli, Fabaceae: A. General aspect; B. Apical vegetative branch; C. Compound leaves, adaxial and abaxial sides, respectively; D, E. Leaf epidermis, in face view, adaxial and abaxial surfaces (LM); F, G. Leaf epidermis, in face view, adaxial and abaxial surfaces (SEM). Abbreviations: ab: abaxial side, ad: adaxial side, le: leaf, lea: leaflet, LM: light microscopy, pet: petiole, ra: rachis, SEM: scanning electron microscopy, st: stomatum, ste: stem. Bar = 2 cm (B), 1 cm (C), 20 µm (D, E)

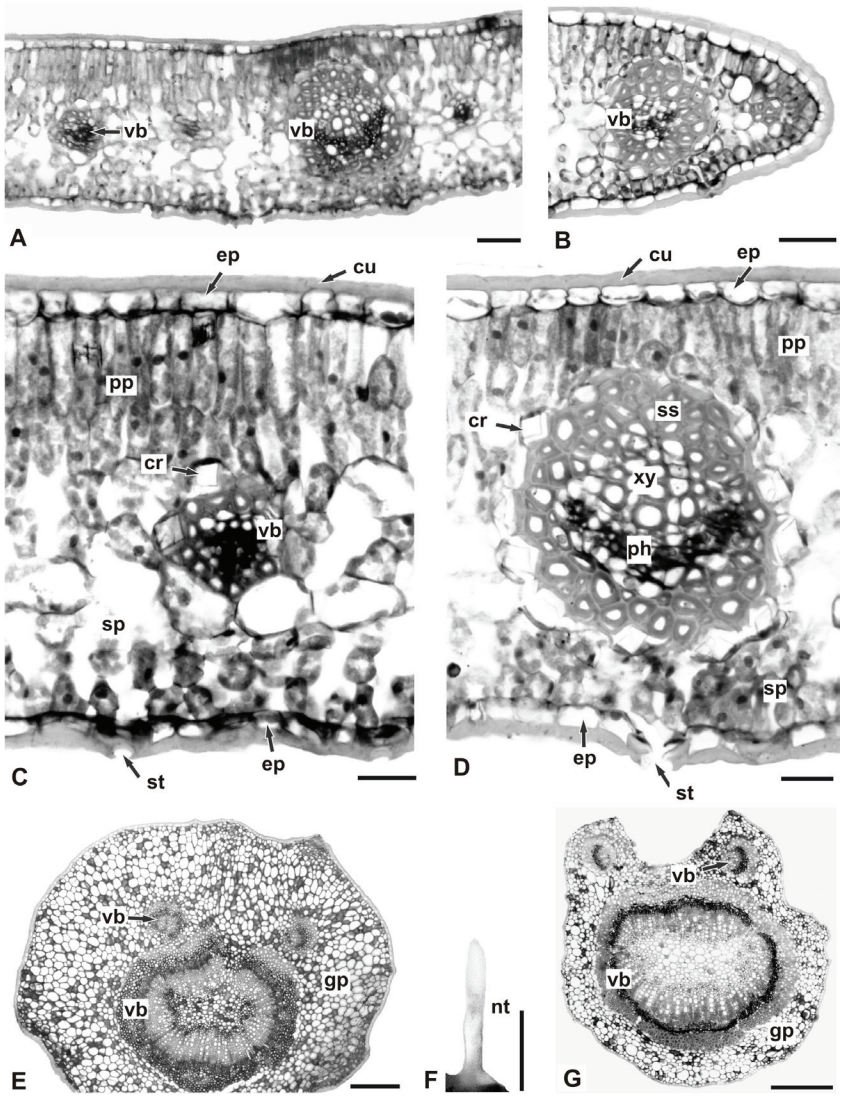
(Figure 3D). In the cortex, there is a continuous strand of collenchyma with annular thickening and cortical parenchyma. In the vascular cylinder there is a practically continuous sclerenchymatic sheath (pericycle) (Figures 3C, 3F), composed of fibers and some stone cells. Next are the continuous cylinders of phloem and xylem, both traversed by narrow parenchymatic rays. The phloem (Figure 3F) consists of sieve elements, parenchymatic cells and some fibers in distinct stages of lignification. The xylem is completely lignified, consisting of tracheary elements either isolated or in small groups (Figure 3F), parenchymatic cells and strands of fibers, which give a stratified aspect to this conducting system (Figure 3C). Amyloplasts and prismatic crystals of calcium oxalate are present in the cortex (Figure 3D), in the vascular system (Figure 3F) and in the pith (Figure 3E).

Discussion

The external leaf morphology of *H. balansae* observed in this work is in agreement with the general description by Barroso (1991) and Judd et al. (2007) for the family, and those by Corrêa (1984), Lorenzi (2002) and Carvalho (2003) for the species.

Taking into account anatomical characteristics, especially relevant in pharmacognosy, when the material to be analyzed is found broken or powdered, it can be seen that *H. balansae* corresponds to the Caesalpinioideae-Fabaceae since it presents anomocytic stomata on the abaxial side, dorsiventral mesophyll, collateral vascular bundles encircled by a sclerenchymatic sheath and crystals of calcium oxalate in the leaf (Metcalfe & Chalk, 1950).

In comparison with other medicinal species in

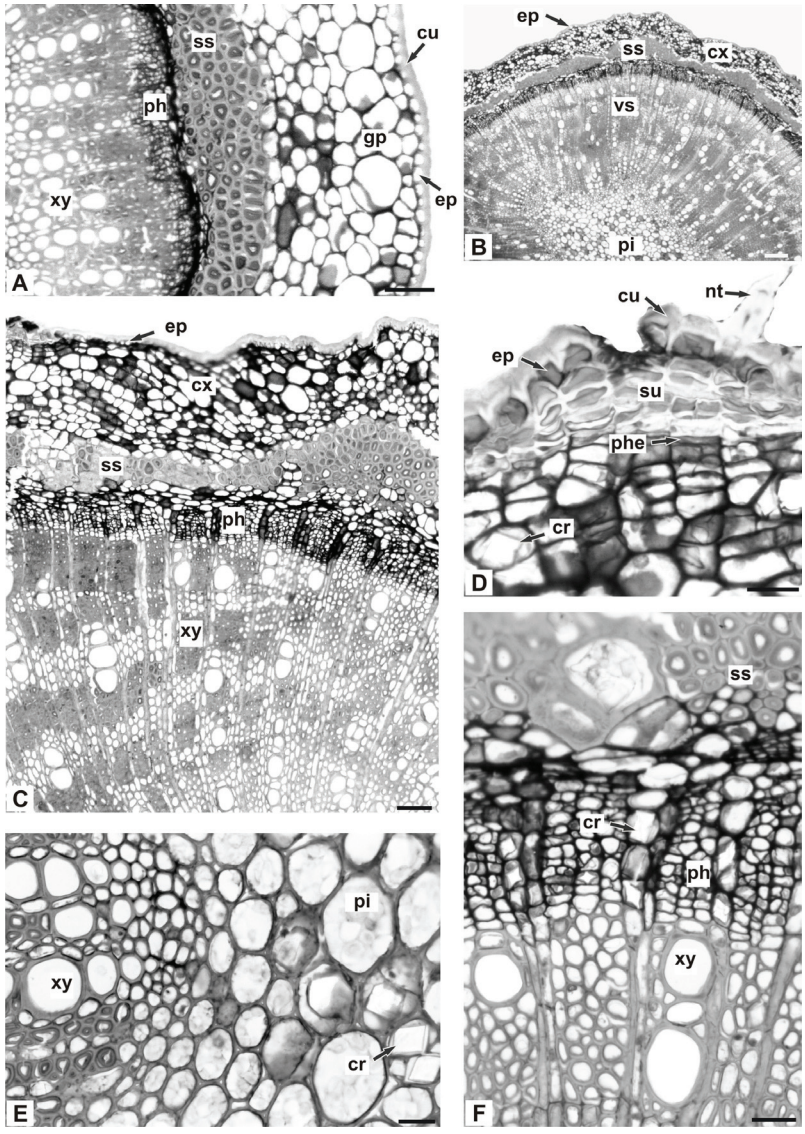


Figures 2A-G. *Holocalyx balansae* Micheli, Fabaceae. Leaf, in transverse section: A. Overall aspect of the leaf blade; B. Leaf margin; C. Intervein region, showing minor vascular bundle; D. Midrib; E. Petiole; F. Non-glandular trichome on the petiole; G. Rachis. Abbreviations: cr: crystal of calcium oxalate, cu: cuticle, ep: epidermis, gp: ground parenchyma, nt: non-glandular trichome, ph: phloem, pp: palisade parenchyma, sp: spongy parenchyma, ss: sclerenchymatic sheath (pericycle), st: stomatum, vb: vascular bundle, xy: xylem. Bar = 100 µm (E, G), 50 µm (A, B, F), 20 µm (C, D).

the family, *H. balansae* differs from *Senna alexandrina* Miller (F. Bras. IV, 1996), on account of it possessing leaflets with unicellular non-glandular trichomes covered with a papillose cuticle, paracytic stomata on both epidermal surfaces, isobilateral mesophyll and druses of calcium oxalate. While the species investigated here had various features in common with *Chamaecrista nictitans* subsp. *patellaria* (DC. ex Collad.) H.S. Irwin & Barneby (Osorio & Akisue, 1996) and *C. trichopoda* (Benth.) Britton & Rose ex Britton & Killip (Francino et al., 2006), these present paracytic stomata on an amphistomatic leaf as distinguishing elements.

With regard to the species *Bauhinia* L., which presents bilobate leaves that are apparently simple due to the partial fusion of two leaflets, some discordant features should be noted. These include the predominantly paracytic stomata on both the leaf surfaces, druses of calcium oxalate and a midrib containing a single collateral vascular bundle in an open arc in *B. variegata* L. (Albuquerque et al., 2000; Duarte et al., 2007). Combining these features, the presence of capitate glandular trichomes enables the distinction from *B. forficata* Link (Oliveira et al., 2001) and the species originally published as *B. blakeana* Dunn by Ferreira et al. (2003).

In relation to the leaf anatomy of *Bauhinia microstachya* (Raddi) J.F. Macbr., a creeping shrub that



Figures 3A-F. Holocalyx balansae Micheli, Fabaceae: A. Detail of the rachis, in transverse section; B, C. Aspect of the stem organization; D. Peripheral position of the phellogen; E. Prismatic crystals of calcium oxalate in the stem pith; F. Detail of the sclerenchymatic sheath and the vascular system. Abbreviations: cr: crystal of calcium oxalate, cu: cuticle, cx: cortex, ep: epidermis, gp: ground parenchyma, nt: non-glandular trichome, ph: phloem, phe: phellogen, pi: pith, ss: sclerenchymatic sheath, su: suber, vs: vascular system, xy: xylem. Bar = 100 µm (B), 50 µm (C), 20 µm (A, D, E, F).

presents bilobate leaves, a ribbon-like flattened stem and tendrils (Duarte & Debur, 2003), the microscopic distinction of *H. balansae* is made difficult because the two species share a number of characteristics. The divergent observations represented by a leaf epidermis with sinuous anticlinal walls in face view, slightly verrucose cuticle covering non-glandular trichomes and the simultaneous occurrence of paracytic and anomocytic stomata in *B. microstachya*, which could possibly be useful in the distinction of these species, may be attributed to environmental influences (Esau, 1977; Mauseth, 1988).

Based on the findings presented here, the aspect of the epidermis, in general, and in particular the epidermal appendages, contributes to the characterization of these species. Even so, this should be analyzed carefully, since genera not closely related in the family may present similar elements. According to a study by Zou et al. (2008), on the micromorphology of the leaf epidermis of *Cercis* L., a taxon considered to have a phylogenetic position of recognized antiquity in Fabaceae, there is agreement on characteristics among the representatives investigated and *H. balansae*, such as slightly wavy anticlinal walls in face view, anomocytic stomata and a hypostomatic leaf, besides the occasional occurrence of non-glandular trichomes.

On the other hand, clearly diverse leaf organization can be seen from a comparison with *Acacia podalyriifolia* A. Cunn. ex G. Don (Duarte & Wolf, 2005), whose modified leaf, known as a phyllodium, consists of a reduced blade and an expanded petiole with a photosynthetic function. In this exotic species, a striated cuticle with wax filaments is found, concentrically arranged mesophyll and a midrib containing two collateral vascular bundles facing each other, in a manner contrary to the description of *H. balansae*.

With regard to the stem, the characteristics observed for the species under study are in line with the general stem organization in the family, for example: peripheral phellogen, collenchyma in the external cortical region, pericycle formed by different sclerenchymatic cells arranged in a continuous ring, the occurrence of crystals of calcium oxalate, as well as fibers in the phloem and narrow rays in the xylem (Metcalfe & Chalk, 1950). According to these same authors, in Caesalpinioideae-Fabaceae there is usually abundant and paratracheary parenchyma in the xylem, which would explain the stratified aspect of this vascular system in *H. balansae*.

The morpho-anatomical description of the leaf and stem of *H. balansae* provides complementary information on native Brazilian flora, as well as contributing to the characterization of this medicinal species, through the establishment of comparisons with other Fabaceae. However, the taxonomic relevance of these features is debatable, because although they enable a distinction between some representatives of the family, there is structural similarity with other species.

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