Revista Brasileira de Farmacognosia 26 (2016) 15–22

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.journals.elsevier.com/revista-brasileira-de-farmacognosia

Original Article

Ovary and fruit morphology and anatomy of *Amphilophium crucigerum*

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ARTICLE INFO

Article history:
Received 2 April 2015
Accepted 6 August 2015
Available online 28 September 2015

Keywords:
Crystals
Histochemical
Morphoanatomy
Ovary
Seed
Trichomes

ABSTRACT

Amphilophium crucigerum (L.) L.G. Lohmann, known as "pente-de-macaco" is a species of Bignoniaceae native to Brazil, and whose seeds are used in folk medicine. This study aimed to describe morphoanatomical features of this species of fruit to aid in its correct identification and pharmacognostic analysis. Samples of ovary, pericarp and seed were fixed with 3% glutaraldehyde, sectioned on a rotary microtome and analyzed by stereomicroscope. The results are shown in three parts: (1) The ovary presents peltate trichomes, long non-glandular trichomes and emergences in the epidermis; it is 2-carpellate and unilocular with two intruding parietal placenta; ovules are numerous on the placenta; it presents a large quantity of crystals. (2) The pericarp is woody, densely echinate and elliptic shape; it presents a 2-valved capsule and is septicidal; it presents emergences, stomata, lenticels, crystals and a large quantity of clustered stones cells. (3) Seeds are alate, exalbuminate and exotestal; there is a large amount of crystals in the exotestal region; it presents an endothelium and remnant endosperm. Histochemical tests showed the presence of lipophilic substances, polysaccharides, phenolic substances, alkaloids and a small quantity of starch. These pharmacobotanical features described for *A. crucigerum* are essential for the pharmacognostic analysis of the drug plant.

Introduction

The Bignoniaceae family is composed of approximately 406 species, predominantly neotropical (Lohmann, 2015). Only three tribes of this family occur in Brazil: Tecomeae, Crescentieae and Bignonieae (Sandwith and Hunt, 1974; Von Poser et al., 2000). *Amphilophium crucigerum* (L.) L.G. Lohmann belongs to the Bignonieae tribe, whose genus is composed of 28 liana species (Lohmann, 2015). *A. crucigerum* presents synonymy with *Pithecoctenium echinatum* and *Pithecoctenium crucigerum* among several other synonyms (Lohmann, 2015).

This species frequently grows on forest clearings and on the borders of highways. It blooms from October to December. In Brazil, *A. crucigerum* is popularly known as "pente-de-macaco" and is cultivated as ornamental (Sandwith and Hunt, 1974). Its fruits are used in folk medicine to treat neuralgia (Bye, 1979), inflammations, skin infections and headaches and as a calming agent (Franco and Fontana, 2005).

The Bignoniaceae species typically present iridoids, alkaloids, flavones, naphthaquinones, anthraquinones, tannins, and anthocyanins (Fischer et al., 2004). Iridoid glycosides were isolated from stems of *A. crucigerum*, showing an antioxidant potential against DPPH, by bioautography, and against acetylcholinesterase inhibitors (Martin et al., 2007).

Despite the pharmacological potential attributed to *A. crucigerum*, there are no reports to date describing diagnostic features for discriminating this species. Delimiting the generic level of the Bignonieae tribe has always been a problem according to Lohmann (2006), due to the lack of diagnostic features and because of overlapping patterns of morphological variation which make it difficult to identify. The present study aims to characterize the morphological and anatomical features of the ovary, pericarp and seed of *A. crucigerum*, describing useful structural features in order to improve its description and identification, as well to present a histochemical analysis. These features are essential for the pharmacognostic analysis of the drug plant.

Material and methods

Plant material

Amphilophium crucigerum (L.) L.G. Lohmann, Bignoniaceae, was obtained from the Southern region of Brazil, at 29°41′02″ S and 53°48′25″ W. The flowers and mature fruits, from seven individuals,

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http://dx.doi.org/10.1016/j.bjp.2015.08.006
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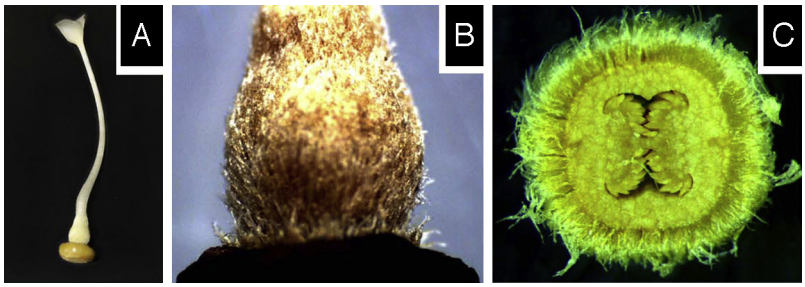

Fig. 1. Dissected flower of *Amphilophium crucigerum*. (A) General aspect of the gynoecium; (B) ovary showing trichomes (25×); and (C) cross-section of ovary (35×).

were collected from November to March. The collected material was identified by Gilberto Dolejal Zanetti and the voucher was registered under number 12872 SMDB at the herbarium of the Biology Department at the Federal University of Santa Maria.

Morphological and anatomical characterization

After dissection with stereomicroscope SZH10 (Olympus®) and M80 (Leica®), the samples were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (Gabriel, 1982) and submitted to vacuum for 6 h for improved infiltration. Subsequently, the samples were washed in 0.1 M sodium phosphate buffer, pH 7.2 (Gabriel, 1982), and then in distilled water. Tween 20 was utilized during 24 h for extraction of epicuticular waxes. Subsequently, the samples underwent dehydration in an ethyl alcohol series, followed by solutions of chloroform and pure ethanol (1:3, 1:1, 3:1, 1:1, 1:3), and finally of pure ethanol. The samples were pre-infiltrated in a 2-hydroxyethyl methacrylate (HEMA) and pure ethanol solution (1:1) during 12 h, followed in pure HEMA and embedding in the same resin, in a Teflon holder until reaching polymerization (Gerrits and Smid, 1983). Sections of 5 μm thickness were made using a RM2245 rotary microtome (Leica®). Toluidine blue O in 0.05% sodium benzoate buffer, pH 4.4 was used for staining (Feder and O'Brien, 1968). Permanent slides were deposited in the collection at the Structural Botany Laboratory of the Biology Department, UFSM. Observations and photomicrographs in bright field and polarized light were performed using a DM 2000 microscope (Leica®) with a DFC 295 image capture system (Leica®).

Histochemical analysis

Hand sections of seeds were prepared for histochemical tests for different purposes: Lugol's solution for starch (Jensen, 1962); Sudan III to detect lipophilic substances (Brundrett et al., 1991); Dragendorff to detect alkaloids (Furr and Mahlberg, 1981); PAS (periodic acid-Schiff) to detect total polysaccharides (Maia, 1979) and toluidine blue for lignins, pectins and phenolic compounds (O'Brien and McCully, 1981).

Results and discussion

Gynoecium morphology

The stigma is 2-lobed (Fig. 1A). The yellowish and densely hairy ovary is superior and cylindrical in the transversal section (Fig. 1B and C). The trichomes, situated in the ovary, reflected light when observed under the magnifying glass (Fig. 1B and C).

A relatively well-developed ring-shaped nectary is present at the base of the ovary (Fig. 1A), where there are stomata and trichomes similar to those found in the ovary. The type of nectary and the presence of trichomes in the nectary are considered invariable features, making them important in the determination of species of different genera, including *Amphilophium* (Rivera, 2000). Some of the features described above were also described for genus determination by Sandwith and Hunt (1974), Galetto (1995), Nogueira et al. (2013) and Lohmann and Taylor (2014).

Ovary anatomy

The ovary is 2-carpellate, unilocular and plurispermic, with two intruding parietal placenta (Fig. 2A and B), similar to that described by Fischer et al. (2004). The ovules are anatropous (Fig. 2C).

The outer epidermis is composed of one cell layer, and presents a large quantity of non-glandular trichomes (Fig. 3A and D). These trichomes are multicellular, long and non-branched. Studies carried out by Nogueira et al. (2013), in leaves of *A. crucigerum*, showed the presence of branched non-glandular trichomes. In the nectary was observed stomata (Fig. 3E).

In the ovary, emergences were also observed, besides a relatively fewer number of glandular trichomes at different stages of

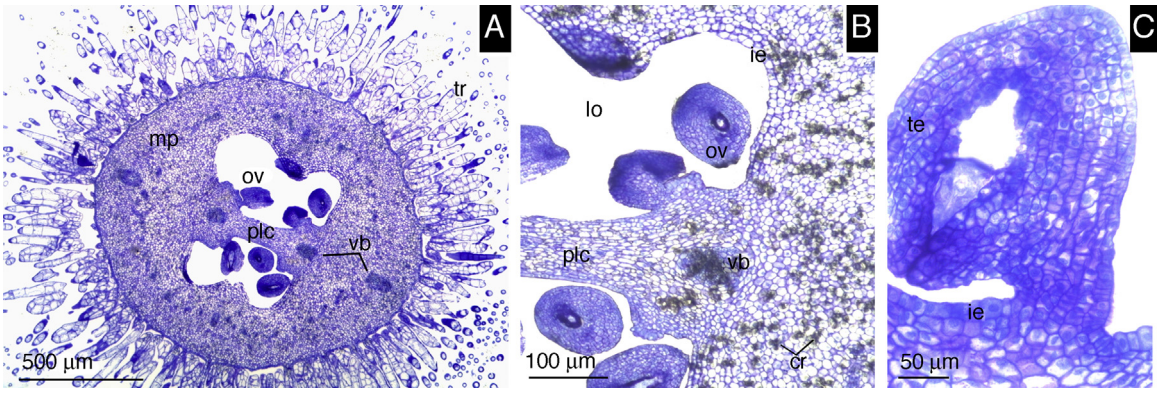

Fig. 2. Cross-section of ovary of *Amphilophium crucigerum*. (A) General aspect; (B) detail of intruding placentae; and (C) ovule. cr, crystals; *i.e.*, inner epidermis; lo, locule; mp, mesophyll; ov, ovule; plc, intruding placentae; te, tegument; tr, trichome; vb, vascular bundle.

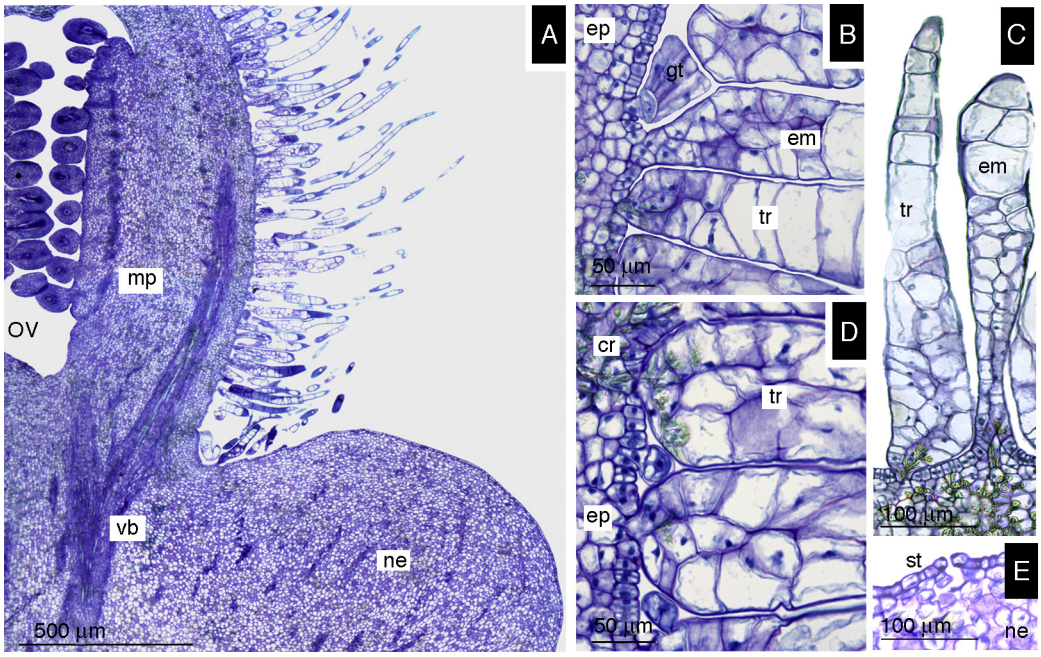

Fig. 3. Longitudinal section of the ovary of *Amphilophium crucigerum*. (A) General aspect of ovary; (B) outer epidermis; (C) trichome and emergence; (D) outer epidermis with crystals; and (E) stomata. cr, crystals; em, emergence; ep, epidermis cells; gt, glandular trichomes; mp, mesophyll; ne, nectary; ov, ovule; st, stomata; tr, trichomes.

development (Fig. 3B and D). The emergences possess wide bases and the subepidermal cells are only part of the base, possibly derived from one or two subepidermal cells (Fig. 3C). A large portion of this structure is made of cells derived from the epidermis. The arrangement and size of these cells are irregular. In addition, the emergences present a sharp apex defined by a single cell.

The glandular trichome is a peltate morphotype, composed of six cells located on the head of the trichome and a single cell on the stalk (Fig. 3B). According to Nogueira et al. (2013), the presence of peltate trichomes is very common in species of the Bignonieae tribe. The presence of trichomes is often associated with desiccation and/or protection against herbivores (Wagner et al., 2004).

The mesophyll is composed of parenchymatous tissue (Figs. 2A and 3A). The marginal vascular bundles bifurcate radially with half of the bundle facing toward a central position near the placentae. Thus, the ovary has two ventral large vascular bundles and two large dorsal vascular bundles on the carpel wall, besides many small lateral vascular bundles found on the ovary wall (Figs. 2A and 3A).

Numerous crystalliferous cells occur in the mesophyll (Fig. 2B). There is also a large accumulation of calcium oxalate crystals on the vascular tissue, which apparently obliterate some vessel elements, and therefore, without an identifiable form or type (Figs. 2B and 4A and B). The inner epidermis presents isodiametric cells that develop vacuoles. The epidermis, which covers the placenta, presents cells with dense cytoplasm that are smaller than the locular cells (Fig. 2B). The ovules are anatropous, unitegmic and tenuinucellate. They are located on the placenta and are numerous (Fig. 3A). Part of the results is in agreement with those usually described for Bignoniaceae and Bignonieae (Corner, 1976; Armstrong, 1985; Fischer et al., 2004, Lohmann and Taylor, 2014).

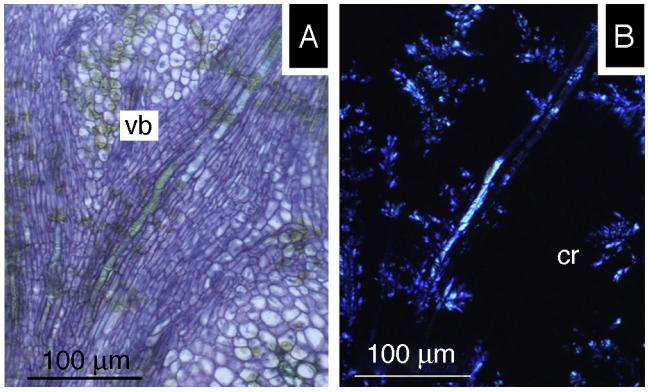

Fig. 4. Longitudinal section of the ovary of *Amphilophium crucigerum*. (A) Detail of parenchymatic and vascular tissues with crystal accumulation (bright field) and (B) crystals in the parenchymatic tissue and in vascular bundles after polarized light. cr, crystals; vb, vascular bundle.

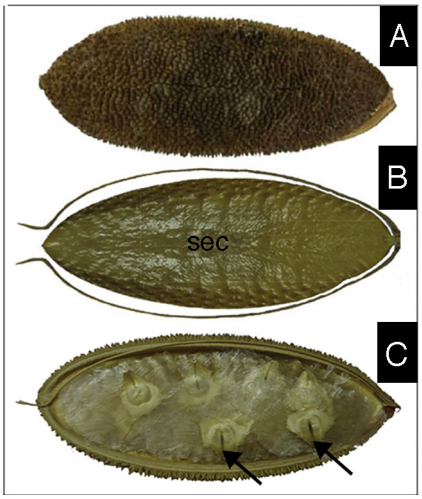

Fig. 5. Fruit of *Amphilophium crucigerum*. (A) pericarp: echinate projections; (B) seminiferous column and (C) seeds. sec, seminiferous column.

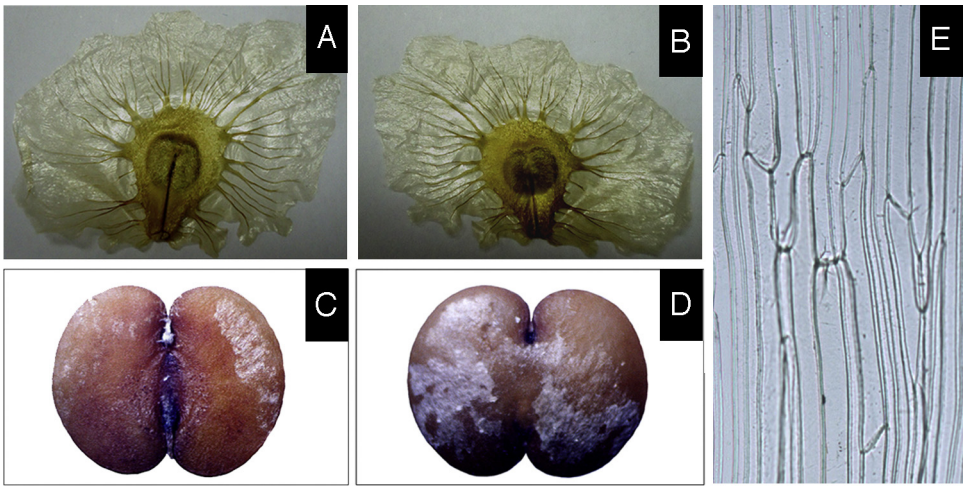

Fig. 6. Seeds of *Amphilophium crucigerum*. (A) General aspect of seed: dorsal face; (B) general aspect of seed: ventral face; (C) embryo covered coriaceous wrapper: raphe; (D) embryo covered coriaceous wrapper: anti-raphe; and (E) wing front view.

Fruit morphology

Pericarp

The fruit is elliptic, woody and densely echinate with approximately 12 cm in length, 6 cm in width and 2 cm in thickness, in the median region. It is composed of a 2-valved capsule slightly flattened with a thick pericarp and a seminiferous column (Fig. 5A and C).

The fruits are septicidal capsules, which is one of the key identifying features for the Bignonieae tribe (Gentry, 1980; Barroso et al., 1999; Fischer et al., 2004). The pericarps are biconvex with a dorsal vascular bundle. The fruit is greenish-brown to rust-colored, when dry.

Among the main features of *A. crucigerum* fruit, there are echinate (horn-like) projections of the exocarp, which is considered as morphological synapomorphies in this genus (Pool, 2007; Lohmann

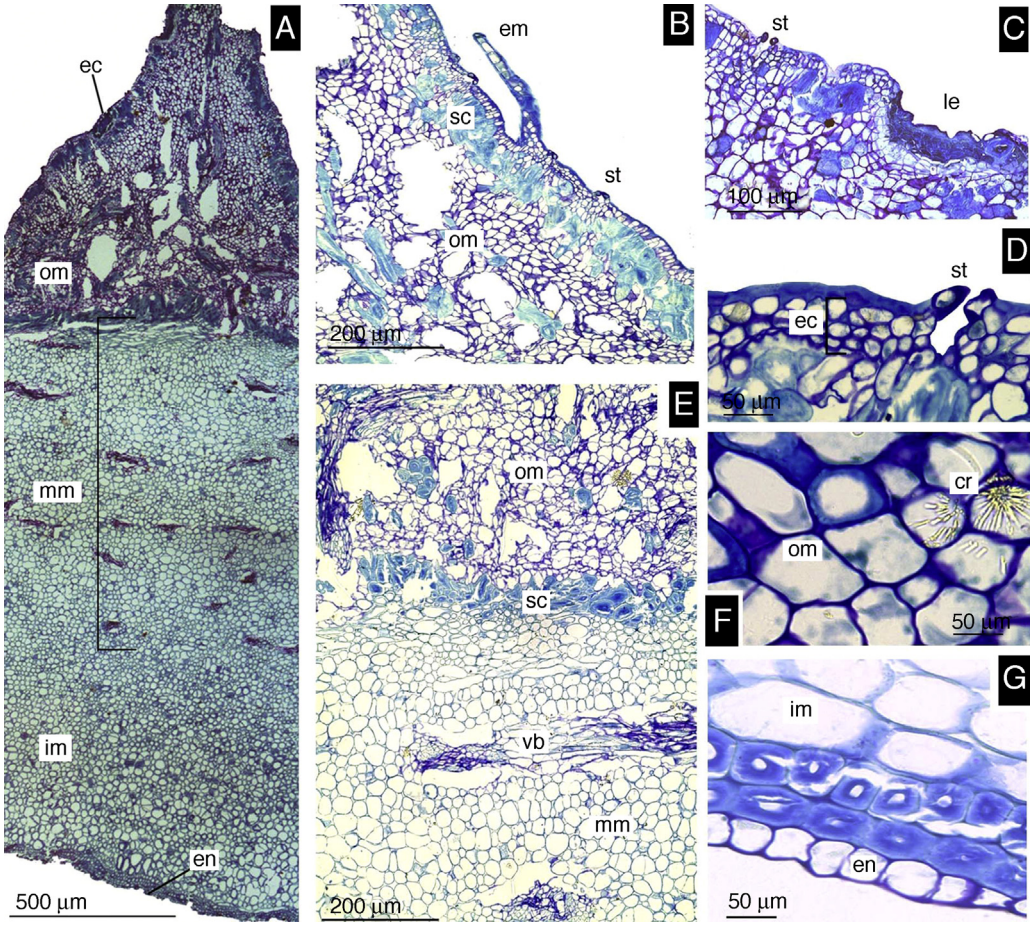

Fig. 7. Cross-section of the pericarp of *Amphilophium crucigerum*. (A) General aspect; (B) outer mesocarp; (C) lenticel; (D) exocarp with stomata; (E) mesocarp; (F) crystals; (G) endocarp. cr, crystals; ec, exocarp; em, emergence; en, endocarp; le, lenticel; im, inner mesocarp; om, outer mesocarp; mm, middle mesocarp; sc, stone cells; st, stomata; vb, vascular bundle.

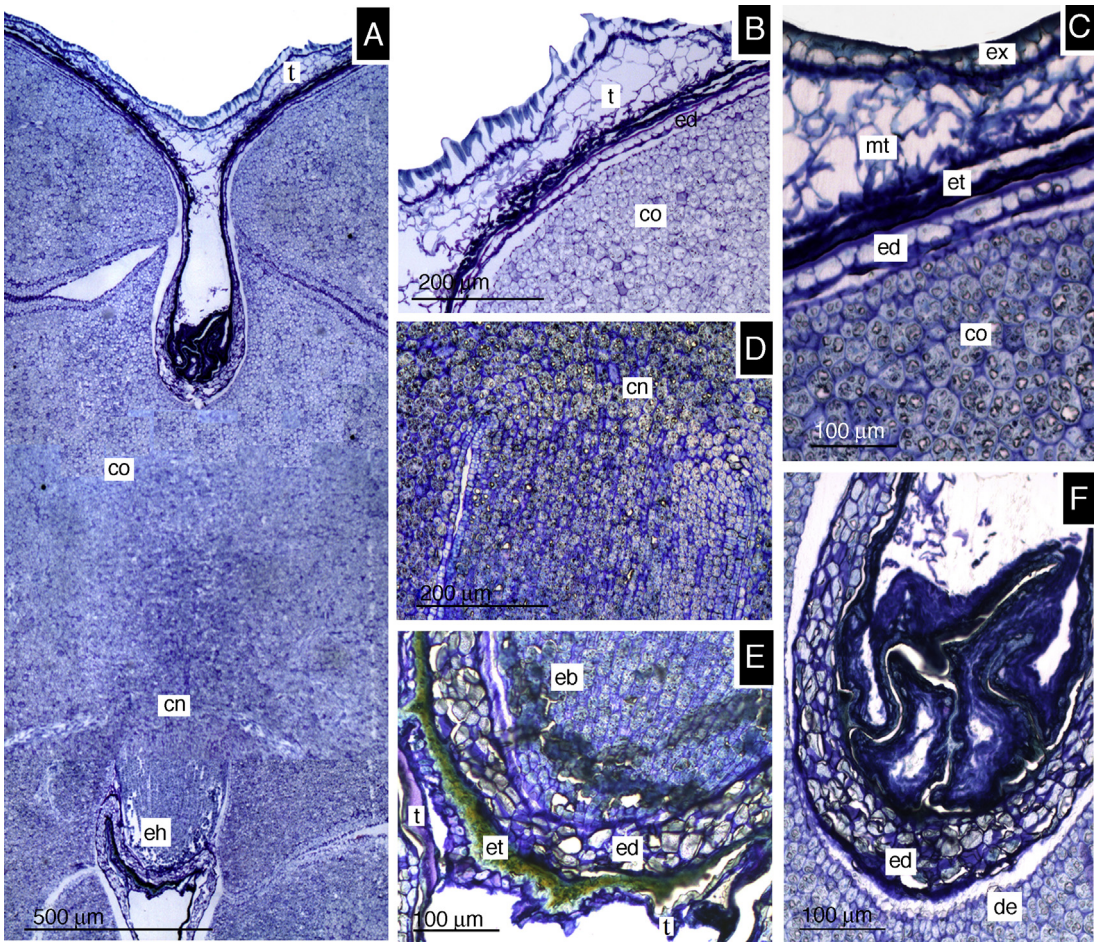

Fig. 8. Longitudinal sections through the lateral plane of the seeds of *Amphilophium crucigerum*. (A) General aspect; (B) testa; (C) endosperm and endothelium; (D) detail of the cotyledon node; (E) hypocotyl radicle axis; and (F) calazal region. cn, cotyledon node; co, cotyledon; de, cotyledon dorsal epidermis; eb, embryo; ed, endosperm; eh, hypocotyl radicle axis; et, endothelium; ex, exotesta; mt, mesotesta; t, testa.

and Taylor, 2014). According to Gentry (1973), these echinate projections in the valves may serve as protection against predators to allow the ripening of fruits and seeds.

Seed morphology and classification of the mature embryo

A. crucigerum presents winged seeds along the length of the fruit and occupying the entire locule (Fig. 5C). The seeds present a brownish-gold color and an evident seminal nucleus (Fig. 6A–D). They have bright translucent wings and papyraceous consistency with approximately 5 cm in width and 2.5 cm in height. The papillate seed coat is another synapomorphy of the genus (Lohmann and Taylor, 2014). The wings consist of lateral expansion of the tegument and chalazal region. The number of cell layers reduces toward the edge of the seminal nucleus and of the wing, where only one cell layer occurs (Fig. 6E).

According to Fischer et al. (2004), in *Pithecoctenium*, *Anemopaegma* and *Jacaranda*, the seminal body is enveloped by a large wing. The same can be observed for other taxa from this family, such as *Tabebuia ochracea* (Sampaio et al., 2007), *T. chrysotrica* (Souza et al., 2005) and *Macfadyena unguis-cati* (Souza et al., 2008), where the chalazal portion of the seed expands and becomes part of the wing, except in *T. caraiba* (Ferreira and Cunha, 2000). However, in some Bignoniaceae there is an almost vestigial chalazal expansion of the wing, as in *Tecoma stans* (Renò et al., 2007).

Fruit anatomy and histology

Pericarp

The pericarp is composed of three different portions (Fig. 7A). The outer portion comprises the exocarp. Below exocarp occurs the mesocarp, divided into three regions. The inner portion comprises the endocarp.

The exocarp is composed of one to three cell layers. Lenticels and emergences are present (Fig. 7B–D). The outer periclinal wall of the cells is thick and lignified (Fig. 7D). Stomatal guard cells occur slightly above the surface of the exocarp (Fig. 7D).

The outer mesocarp is formed during fruit differentiation through the proliferation of outer tissue, resulting in horn-like projections (Fig. 7A). The outer mesocarp presents parenchymatous cells and clustered stones cells (Fig. 7B) that form an almost continuous layer. At the base of the horn-like projections, there are layers of sclerified cells, demarcating the outer mesocarp from the middle mesocarp (Fig. 7A and E). Needle-like crystals are present on the outer mesocarp in a radial organization (Fig. 7F).

The middle mesocarp is composed of vascular bundles, and parenchymatous tissue with thin sclerified walls (Fig. 7E). The inner mesocarp is predominantly composed of tissue similar to that found in the middle mesocarp, but there are no vascular bundles (Fig. 7A). In addition, the inner mesocarp presents two isodiametric cell layers with thick walls. The endocarp is formed by a single layer, with thin-walled cells (Fig. 7G).

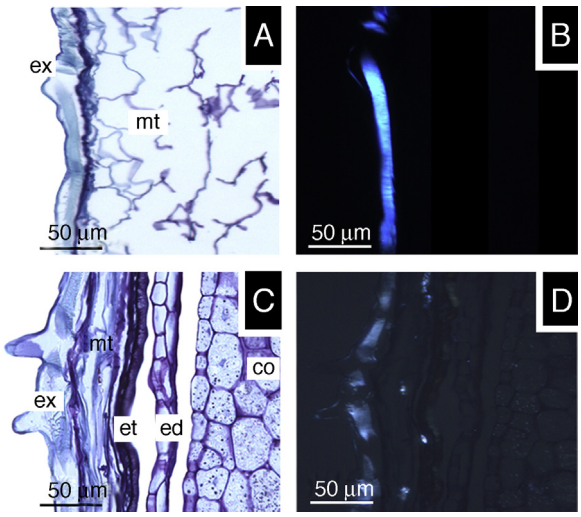

Fig. 9. Longitudinal section of the seed of *Amphilophium crucigerum*. (A) testa; (B) wall thickening, after polarized light; (C) details of the raphe region (testa); and (D) crystals on testa and on cotyledon, after polarized light. cn, cotyledon node; co, cotyledon; ed, endosperm; eh, hypocotyl radicle axis; ex, exotesta; de, cotyledon dorsal epidermis; et, endothelium; mt, mesotesta.

Seed

The seeds are exalbuminous in *A. crucigerum* (Fig. 8A), as they also are in *Tabebuia ochracea* (Sampaio et al., 2007). This remnant endosperm is formed by a layer of parenchymatous cells without any type of nutritive reserve (Fig. 8B and C). However, in the region near to the raphe, the endosperm presents three or four cell layers, which gradually lessen until forming a single cell layer (Fig. 8E and F). This feature is typical in Bignoniaceae, as described by Corner (1976), Esau (1985) and Johri et al. (1992).

These authors report Bignoniaceae seeds as exalbuminous with absent or scarce endosperm, except in some species, such as *Pyrostegia venusta*, which presents an evident endosperm (Gabrielli and Castro, 1995).

The embryo presents juxtaposed, 2-lobed, foliaceous cotyledons from the apex to the base, and a yellowish coriaceous wrapper. The embryos are on average 0.8 cm in length, 0.6 cm in width and 0.1 cm in thickness (Fig. 6C and D). The embryo presents a continuous axial and straight hypocotyl-radicle axis, which is very small in comparison to the cotyledon lobules (Figs. 6D and 8A). In contrast, *T. stans* presents a more evident hypocotyl-radicle axis (Renò et al., 2007). In this section, embryos present an oblong-transverse and lenticular shape (Fig. 8A), which are features of Bignoniaceae (Barroso et al., 1999; Ferreira and Cunha, 2000; Sampaio et al., 2007).

The seed is exotestal (Fig. 8A–C), possessing elongated cells with secondary radial walls in the form of bands with the presence of crystals (Fig. 9A–D). The parietal thickening of the exotestal cells are more developed in the seminal nucleus, which has never been reported for this genus.

In the mesotestal tissue composed of sclerenchymatous parenchyma, mature cells undergo compression from the expanding embryo (Figs. 8C, 9A, C, and 10A, B). The mesotesta in the chalazal region presents a greater number of cells and cell remnants, in contrast to the micropylar region, which presents only cell remnants (Fig. 8A and D–F).

The histological analysis revealed phenolic compounds in the micropylar region very close to the mesotestal cells cited above, together with cell remnants from the vascular tissue in the chalazal region (Fig. 8A, E, and F). The endotesta is absent because in this family the inner epidermis is destroyed during the development of the gynophyte.

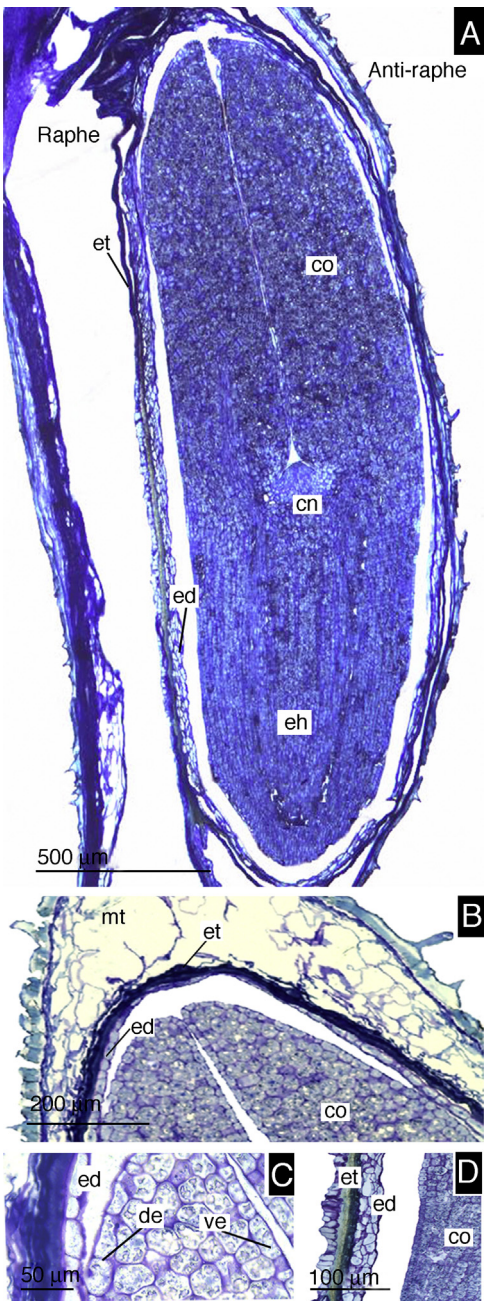

Fig. 10. Longitudinal section through the anteroposterior plane of the seed of *Amphilophium crucigerum*. (A) General aspect; (B) distal region (seed wing); (C) cotyledon; (D) endosperm and endothelium. cn, cotyledon node; co, cotyledon; ed, endosperm; eh, hypocotyl radicle axis; ex, exotesta; de, cotyledon dorsal epidermis; et, endothelium; mt, mesotesta; ve, cotyledon ventral epidermis.

The endothelium presents membranous layer of cells that envelop the entire embryo (Fig. 10A). This structure presents phenolic compounds that acquire a yellowish color, even in the presence of toluidine blue (Fig. 8E).

The presence of an endothelium and scarce endosperm (ab initio) is common in species of Bignoniaceae, which are unitegmic and tenuinucellate (Souza et al., 2005; Lohmann and Taylor, 2014). *Pyrostegia venusta* presents endosperm with two to six layers (Gabrielli and Castro, 1995).

In the longitudinal section, the epidermal cells of the cotyledons are slightly tangentially elongated, in both the dorsal and ventral region (Fig. 10C). The mesophyll is parenchymatous with accumulation of alkaloids, lipophilic compounds such as fixed and volatile

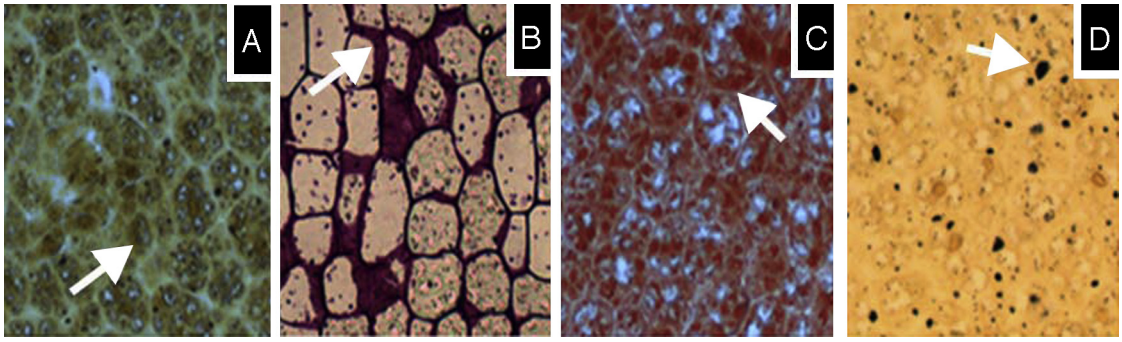

Fig. 11. Histochemical analysis of embryo of *Amphilophium crucigerum*. (A) Dragendorff (alkaloids); (B) PAS (polysaccharides); (C) Sudan III (oils); and (D) Lugol (starch).

oils, as well as a scarce presence of starch. PAS-positive polysaccharides were identified on the cell walls and in the cytoplasm (Fig. 11A–D).

In *A. crucigerum*, the membranous wrapper located between the embryo and the testa is similar to the structure found in Bignoniaceae, a common feature in the family (Gabrielli and Castro, 1995; Costa, 2003; Souza et al., 2005; Renò et al., 2007). This membranous wrapper is composed of cells containing phenolic compounds, which play a role in protecting against predators and microorganisms, and increase the hardness of the tegument and give color to the seed (Beltrati and Paoli, 2006).

Understanding the main groups of secondary metabolites in medicinal plants is important as a starting point for studies on biological activity or for the isolation and identification of bioactive compounds, especially those used in folk medicine. Phenolic compounds are linked to antioxidant, anti-inflammatory and analgesic activities, among many other biological effects (De Oliveira et al., 2012; Figueiredo-Rinhel et al., 2013). Alkaloids are associated to a greater number of activities, including antimicrobial and analgesic activities (Hu et al., 2013). Thus, the presence of these types of compounds corroborates with the use of this species in folk medicine.

Structural details of cells are relevant when observing torn or ground material, both for pharmacognostic control and for quick identification of species, as reported by Oliveira et al. (2003), Verdam et al. (2012), Jasinski et al. (2014), and Santos et al. (2015). Seeds of *A. crucigerum* present morphological and anatomical features common to the family. In addition, specific features allow the identification and reliable analysis of the species, including: presence of trichomes in the ovary and part of the nectary; large quantity of crystals on floral tissue; exotestal cells with secondary radial walls with the presence of crystals on seed wings and the presence of a remnant endosperm in the seed; and a positive test for alkaloids in the embryo.

Authors' contributions

RC contributed to the collection of plant samples, preparation of herbarium, running the laboratory work, analysis of the data and drafting the paper. MPM and JMSO designed the study, supervised the laboratory work and 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.

Acknowledgments

The authors thank the Federal University of Santa Maria and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support.

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