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Original Article

Leaf and Stem Anatomy and Histochemistry of *Dalbergia ecastaphyllum*

Michelline V. Marques Das Neves[1], Nathalia Diniz Araújo[1], Eduardo De Jesus Oliveira[2] and Maria De Fátima Agra[1*]

ABSTRACT

Dalbergia ecastaphyllum (L.) Taub. is a shrubby of Tropical America and Africa. It is recognized as the main botanical source of red-propolis, and also by its uses in folk medicine. This work was performed by light and scanning electron microscopy in order to carry out an anatomical and histochemical study of leaves and stems of this species,to find distinctive characters to support the quality control of its ethnodrugs and derivatives. The leaf epidermis is hypostomatic with straight to curved anticlinal cell walls, papillose on the abaxial surface, with thickened cuticle and coated with epiculticular waxes as rosette. The mesophyll is dorsiventral, with palisade 2-3-layered and the spongy 4-6-layered. The petiole and midrib have vascular system collateral. The stem is cylindrical, with an uniseriate epidermis coated with thickened cuticle and angular collenchyma. The vascular system is a continuous ectofloic siphonostele. Resin idioblasts are concentrated in the inner area of the vascular tissue of the midrib, petiole and stem. The leaf and stem anatomy and histochemistry of *D. ecastaphyllum* provided distinctive characters for this species that can be used as an additional support for its taxonomy and for the quality control of their ethnodrugs.

Key words: Genus *Dalbergia*, Ethnobotany, Fabaceae, Leguminosae, Red propolis.

Michelline V. Marques Das Neves[1], Nathalia Diniz Araújo[1], Eduardo De Jesus Oliveira[2] and Maria De Fátima Agra[1*]

[1]Laboratório De Taxonomia E Farmacobotânica, Programa De Pós-Graduação Em Produtos Naturais E Sintéticos Bioativos, Centro De Biotecnologia, Universidade Federal Da Paraíba, João Pessoa, Paraíba, BRAZIL.
[2] Departamento De Ciências Farmacêuticas, Universidade Federal Dos Vales Do Jequitinhonha E Mucuri, Diamantina, Minas Gerais, BRAZIL.

Correspondence

Maria De Fátima Agra. Laboratório De Taxonomia E Farmacobotânica, Programa De Pós-Graduação Em Produtos Naturais E Sintéticos Bioativos, Centro De Biotecnologia, Universidade Federal Da Paraíba, João Pessoa, Paraíba, BRAZIL.

Email: agramf@ltf.ufpb.br

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INTRODUCTION

Dalbergia L. f., belonging to the Fabaceae family, has about 100 species with pantropical distribution and is considered the second largest genus of the tribe Dalbergieae Bronn ex DC.[1] In Brazil the genus is represented by about 40 species distributed in representative areas of different Brazilian ecosystems and in various types of vegetation, such as Caatinga, Cerrado, Atlantic Forest and Campos Rupestres.[2]

Dalbergia stands out for having species considered valuable for its decorative and fragrant woods.[3] Many species of the genus are used in traditional medicine for various purposes: treatment of pain, fever, inflammations, as sexual stimulant, and against worms and larvae.[4] Although some species of *Dalbergia* are referred to by their uses in folk medicine, only some of these uses have been studied biologically or pharmacologically in order to validate its traditional uses.[5] In addition, *Dalbergia* species also have been shown to be important sources of bioactive components, such as is of lavonoids (daidzein, formononetin and biochanin A), neoflavonoids, glycosides, cinnamylphenols, quinones and furans.[5-8]

Among the species of the genus stands out *Dalbergia ecastaphyllum* (L.) Taub popularly known in Brazil as "rabo-de-bugio", "marmelo-do-mangue" and "marmeleiro-da-praia", a shrubby species, distributed in tropical America and Africa[9]. In Brazil, it is found mainly in areas of restingas along the Brazilian coast.[1]

Propolis is a resinous mixture of substances collected by the bees, *Apis mellifera* mainly, from several plant sources including *Dalbergia ecastaphyllum*. Various biological activities have been reported for propolis, including: cytotoxicity,[10] antitumor,[11] antioxidant,[12] and antimicrobial.[13] Studies carried out in hives of *Apismellifera*, in northeastern Brazil, reported the presence of a new type of propolis in areas of the State of Alagoas, which was called "red-propolis", whose chemical composition and botanical origin were studied by Silva *et al.*,[8] Daugsch *et al.*[7] and Alencar *et al.*,[14] and the results indicate *Dalbergia ecastaphyllum* as the main species associated with the production of "red-própolis".

General information about the anatomy of *Dalbergia* was referred by Metcalfe and Chalk,[15] that reported the presence of secretory structures producing resins rich in bioactive compounds. Farooqui *et al.*[16] conducted studies of cuticular characters of three species of *Dalbergia*: *D. emarginata, D. latifolia, and D. sissoides*. Moreover, Khan *et al.*[17] recorded the

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same information on epidermal markers for *Dalbergia sisso*. Our work was motivated by the scarcity of anatomical studies for the genus and especially for *Dalbergia ecastaphyllum*, despite this species being recognized as the main botanical source of red propolis and many bioactive compounds[5] as well as by its traditional use in Folk medicine.[4]

This work was carried out in order to perform an anatomical and histochemical study of leaves and stems of *Dalbergia ecastaphyllum* to find distinctive characters that may constitute parameters to support its taxonomy as well as the quality control of its ethno drugs and derivatives.

MATERIAL AND METHODS

Plant Materials

Botanical expeditions and field observations were conducted on two populations of *Dalbergia ecastaphyllum*(L.) Taub., at May 2012, in two apiaries (7^{0}50'37.05"S e 34^{0}53'00.66"W Alt. 7m / 7^{0}49'57.90"S e 34^{0}56'02.38"W Alt. 64m), located at Igarassu, Pernambuco State, Brazil. Part of the samples was fixed in AFA (50%) for 48 hours, and then maintained in 70% ethanol[18]. The other part of plant material was pressed and dried, following the methods described by Mori *et al*.,[19] and the vouchers (*Neves 001, 002*) were deposited at the Herbarium Prof. LauroPires Xavier (JPB), of the Universidade Federal da Paraíba.

Anatomical Characterization

The anatomical analysis was performed on fresh, fixed and dried samples. Dried samples were properly rehydrated and then subjected to the same procedure cited for the fixed material.[20]

Cross sections were performed by free hand with the aid of blades in leaves (lamina and petiole) and stems of *Dalbergia ecastophyllum*. The cuts were clarified with sodium hypochlorite at 2%, washed in distilled water, neutralized with acetic acid to 1%, washed in distilled water and stained with safra blue, mounted with glycerinated gelatin 50%, and analyzed and photographed by optical microscopy.

For observation of the epidermis, paradermic sections were performed on both surfaces of the leaf, then clarified in a similar way to the transverse sections, and stained with safranin (1%), and mounted on glycerinated gelatin (50%). Observations and photomicrographs were performed on optical microscope (LeicaDM750, Switzerland) with Qwin system coupled to a video camera (Leica ICC50 HD) for image capture.

The classification of stomata and trichomes was based on Wilkinson[21] and Theobald *et al*.,[22] respectively.

Histochemical Characterization

Cross and longitudinal sections were made by free hand helped by cutting blades, in leaves (midrib and petiole) and stems under the following colorants and reagents: ferric chloride[18] and potassium dichromate[23] for phenolic compounds, 2,4-dinitrophenylhydrazine[24] for terpenoids, Wagner reagent[25] for alkaloids, sudan IV and sudan black B[26] for lipids, and acidified phloroglucinol[18] for lignin. The reaction was considered positive when specific, clearly visible and of a different color than the natural color of the substance being examined. Histological slides were also prepared without any processing (*in natura*), which were called "white".

Scanning Electron Microscopy

The scanning electron microscopy was used for micro morphological analysis of epidermis in dry material, to optimize the observation of the waxes and epidermal appendages, such as stomata, trichomes and papillas. The dried samples were placed on stubs with carbon ribbon, and subsequently coated with gold. The image capture was performed by a scanning electron microscope (Zeiss, model LEO 1430 VP, Cambridge, England). The terminology of epicuticular waxes morphology was done according to Barthlott [27] and Barthlott*et al*. [28]

RESULTS AND DISCUSSION

In front view, the epidermis of *Dalbergia ecastaphyllum* is hypostomatic, presenting a different pattern from that described by Metcalfe and Chalk[15] for *Dalbergia*, and by Kahn *et al*.[17] for *D. sisso* Roxb. ex DC., that have predominantly amphistomatic leaves (with stomata on both surface). Moreover, Farooqui *et al*.[16] recorded the amphyhypostomatic type for three additional species of the genus: *Dalbergia latifolia* Roxb., *D. sissoides* Graham, and *D. sisso*, which have predominantly hypostomatic leaves, with rare stomata on the adaxial surface.

In frontal view, the anticlinal cell walls of the epidermis are straight to curve on the adaxial surface (Figure. 1A), a feature also observed in other species of *Dalbergia* by Farooqui *et al*.[16] and Khan *et al*.,[17] and in species of some genera of Fabaceae, like *Bauhinia* L., according to Lusa and Bona.[29] On the other hand, the epidermis on the abaxial surface is predominantly papillose, except on subsidiaries cells of stomata (Figure. 1B, 2C-F). Similar pattern was referred for *Dalbergia latifolia* Roxb. by Farooqui *et al* [16]. The epidermal papillae on the abaxial surface is a common character in *Dalbergia,* and also present in other genera of Fabaceae like *Bauhinia*, according to Metcalfe and Chalk.[15]

Tector trichomes, with short cells at the base, were observed on both surfaces of the by light and scanning electron microscopy (SEM). On the adaxial surface they are longer and sparse (Figure 2B). than on the abaxial surface, which are numerous and adnate to the surface (Figure. 2C-D). This type of trichome corroborate with Metcalfe and Chalk,[15] as well as common characteristic of *Dalbergia,* and also were reported by Farooqui *et al*.[16] for *Dalbergia sissoo.*

On the abaxial leaf surface of the studied species a large amount of epicuticular waxes was observed by SEM, as crystalloid of rosettes type, which were deposited on the epidermal papillae and paracitic stomata (Figure 2D-F). This pattern of epicuticular waxes was reported for *Bauhinia forficata,* another species of Fabaceae, by Lusa and Bona.[29] The adaxial surface showed very rough cuticle, however epicuticular waxes were not observed in this area (Figure 2A).

In transverse section, the epidermal cells of the adaxial surface were tabular and somewhat rounded (Figure 1C-D). followed by a layer of hypodermis that is formed by larger cells (Figure. 1C-D). The presence of a hypodermis in *Dalbergia* is a common character of this genus that was already reported by Metcalfe and Chalk.[15]

The pattern of mesophyll of *Dalbergia ecastaphyllum* is of the dorsiventral type (Figure 1C), with three-layered palisade parenchyma, and four to five-layered spongy parenchyma, and several collateral vascular bundles distributed throughout the mesophyll. The leaf margin, in cross section, showed to be slightly rounded (Figure 1F) toward the abaxial surface, with palisade and spongy parenchyma more homogeneous, with smaller and compact cells in this area (Figure 1F).

According to Metcalfe and Chalk,[15] due to the wide range of morphological types, leaves are considered a highly variable structure in Fabaceae that shows great plasticity, but it is usually dorsiventral, and less frequently isobilateral. Moreover, palisade and spongy parenchyma are tissues known to reveal responses related to light, soil and water variations, according to Esau,[30] Levitt[31] and Rozema *et al*.[32]

The midrib, in transverse section, has a plane-convex shape (Figure. 3A-B, D), with the vascular system constituted by a set of collateral vascular bundle. The distal portion of central vascular bundle exhibits a small semi-arch shape with the xylem and phloem polarized in the adaxial and abaxial surfaces, respectively (Figure 3A). The median and proximal portions are constituted by a set of vascular bundles, formed by a larger semi-arched shaped central one, and two lateral accessory bundles with circular shape (Figure B-D). Throughout the entire midrib, the perivascular region is marked by the presence of a continuous sclerenchymatic

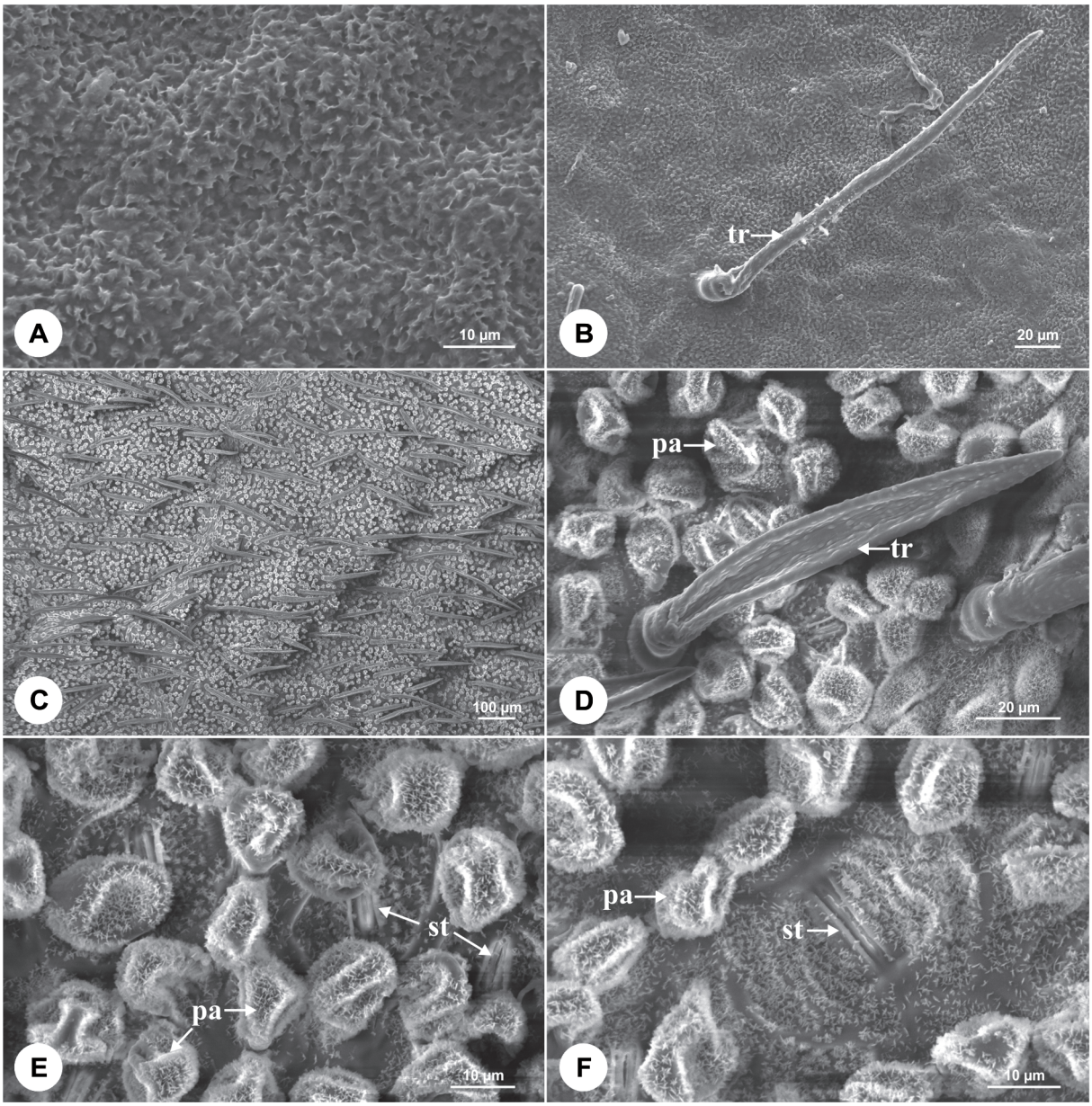

Figure 1: A-B. Leaf epidermis of *Dalbergia ecastophyllum*, in front view (*Neves 001*). A. Adaxial surface withcell walls anticlinal curved. B.Papillose epidermis at abaxial surface. C-F. Leaf blade in transverse section. C. Dorsiventral mesophyll with palisade parenchyma multisseriate. D. Detail of epidermis and sub-epidermal layer of the adaxial surface. E. Detail of cylindrical papillae on the abaxial surface. F. Detail of margin. Legends: (ep) epidermis, (hp) hypodermis, (pa) papillae, (pp) palisade parenchyma, (sp) spongy parenchyma, (st) stomata, (ts) and trichome scar.

sheath around the vascular system (Figure 3A-D). Adjacent to the epidermis, stands 1-3 layers of collenchyma of lignified cells of the angular type (Figure 3C). Resin idioblasts were observed in the medullar region, adjacent to the xylem (Figure 3A, E-F).

The petiole, in cross section, has a circular shape, having the cortex with 1-2 layers of collenchyma of the angular type, underlying the epidermis (Figure. 4A), followed by the fundamental parenchyma. In the distal and the median portions a sclerenchymatous sheath surrounding the collateral vascular system was observed, similar to that found on midrib, which is not evidenced in the proximal portion. The vascular system is formed by 2-7 vascular bundles, which are arranged in a somewhat irregular arch shape. The medullar portion is formed by the fundamental parenchyma in the central portion (Figure 4A-B), in which the presence of resin idioblasts underlying the xylem was observed.

According to Metcalfe and Chalk,[15] the morphology of petiole suffers little influence of the environment and represents a structure of taxonomic importance. The pattern of the vascular system of petiole in *D. ecastaphyllum* was also reported to be present in other Fabaceae, such as *Caragana arborescens* Lam., *Erythrina crista-galli* L., *Galega officinalis* L., amongst others.

The stem is cylindrical, with an uniseriate epidermis that is coated by a thick cuticle. In cross section, the collenchyma, adjacent to the epidermis, is the angular type with lignified walls. The perivascular region was marked by the presence of a discontinuous sclerenchymatic sheath around the vascular cylinder (Figure 4C-D) that is an ect of loic-siphonostele. The stem structure of *D. ecastaphyllum* showed isolated bundles of fibers surrounding the entire vascular system, and also resin idioblasts, similar to that reported for *Dalbergia* and other genera of

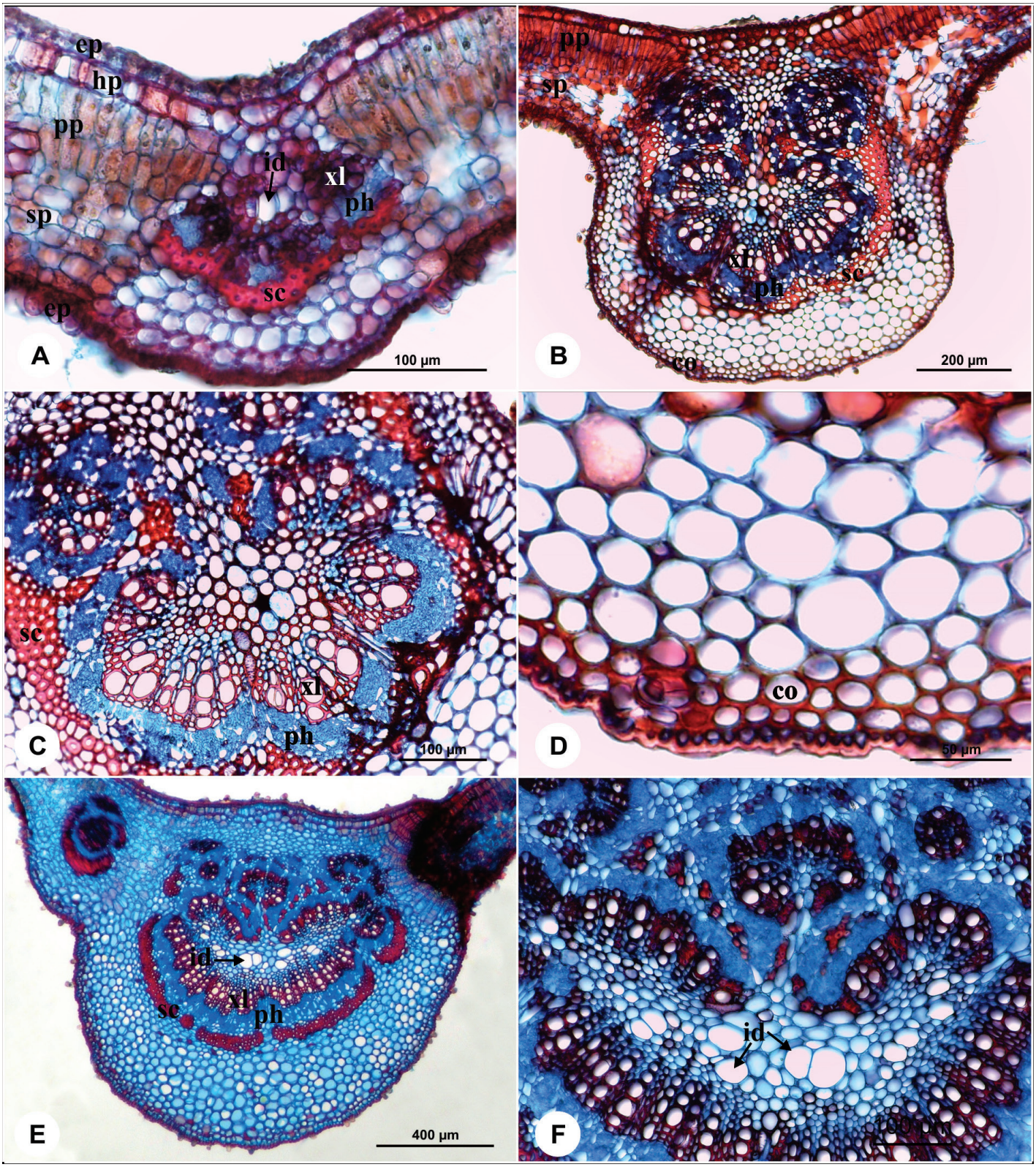

Figure 2: Scanning electron micrograph of the leaf epidermis of *Dalbergia ecastophyllum* (*Neves002*). A. General view of the adaxial surface. B. Detail of simple trichome on the adaxial surface. C-F. Indument and papillae of the abaxial surface: C. Detail of the papillae epidermis and trichomes. D. Detail of simple trichome on the abaxial surface. E. Detail of epidermal papillae with epicuticular waxes as rosettes. F. Detail of stomata and papillae covered by epicuticular waxes as rosettes. Legends: (pa) papillae, (st) stomata, (tr) trichome.

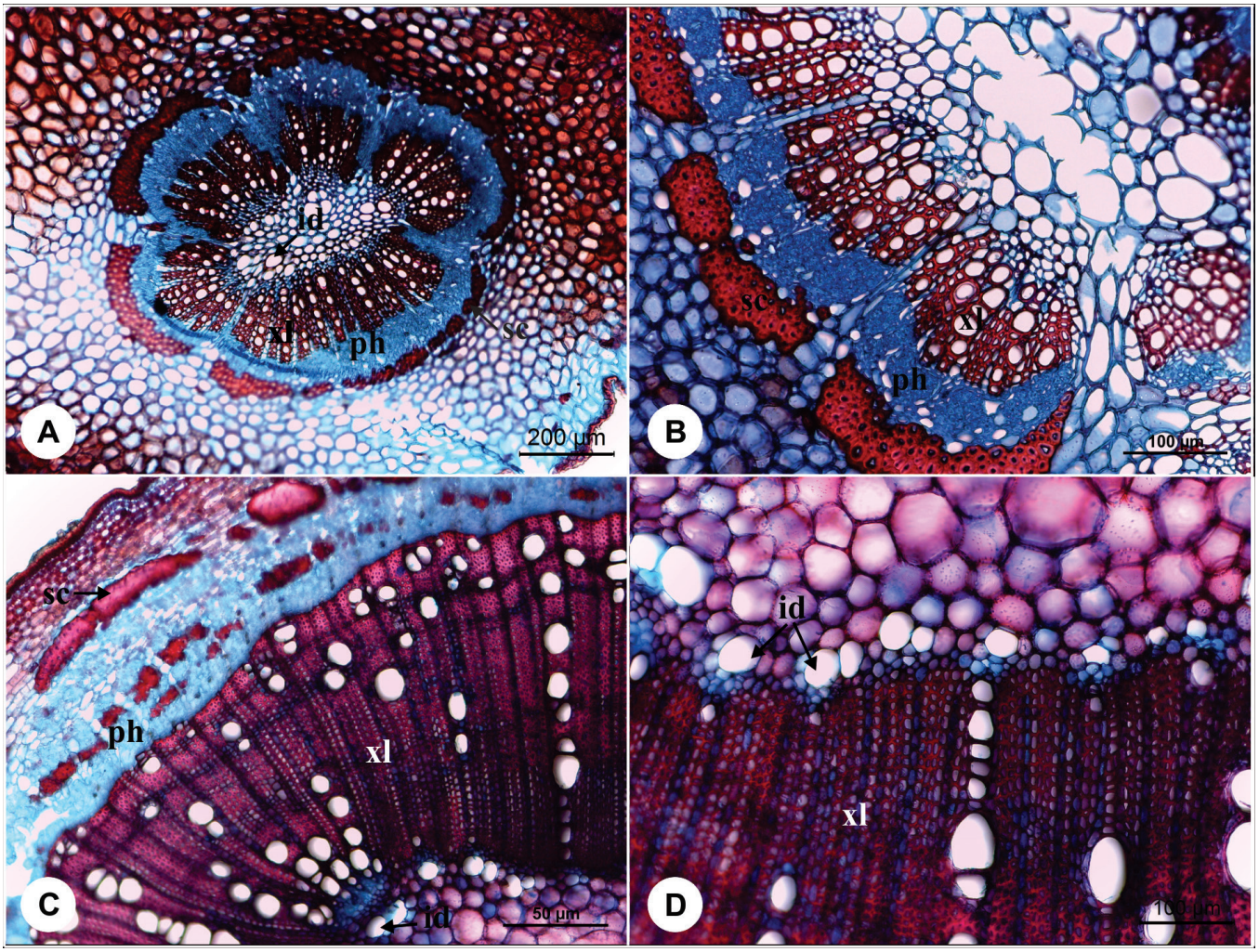

Figure 3: Leaf midrib in transverse section of *Dalbergia ecastophyllum* (Neves*001*).A. General appearance of the midrib at the distal portion. B. General appearance of the midrib at the median portion. C. Detail of vascular system of the midrib at the median portion. D. Detail of angular collenchyma of the midrib at the median portion. E. General appearance plane-convex of the midrib at the proximal portion. F. Detail of vascular system and resin idioblasts of the midrib at the proximal portion. Legends: (co) collenchyma, (ep) epidermis, (hp) hypodermis, (ph) phloem, (pp) palisade parenchyma, (sc) sclerenchyma, (id) idioblast, (sp) spongy parenchyma, (xy) xylem

Fabaceae by Metcalfe and Chalk.[15] In *D. ecastaphyllum* resin idioblasts were observed in the internal region of the vascular tissue of leaf and stem. They occur mainly concentrated in areas adjacent to the xylem of the midrib and petiole, and also in the stem. In transverse section they appear as cells with primary walls that contain hydrophilic substances, such as phenolic compounds (Figure 5C-F, L-O), terpenoids (Figure 5G, P). and alkaloid salts (Figure 5H, Q). Lipidic substances were present only in the cuticle (Figure 5I, R-S) and were absent in resin idio blasts.

According to Fahn,[33] secretory tissues occur in most vascular plants, but differing in structure, topographic position and in the materials secreted. The presence of resin idio blasts observed in the midrib and petiole leaf, and in stem (vascular system) of *D. ecastophyllum* could be the area of formation of the resin used by bees to produce red propolis. Secretory structures are resin producers, which are rich in bioactive compounds, already mentioned for *Dalbergia* by Metcalfe and Chalk[15] and Farooqui *et al.*[16] Secretory elements containing tannins, resins and proteins were previously reported by Metcalfe and Chalk[15] for many species of different genera of Fabaceae, including *Dalbergia.* Secretory elements of *D. ecastophyllum* are found into the vascular system of the midrib, petiole and stem, which differ from the secretory elements of *Myrocarpus*, in which the secretory structures are located in the cortex of midrib.[34]

Phytochemical studies have reported the presence of phenolic compounds such as flavonoids, isoflavonoids, as well as other substances in species of *Dalbergia* referred by Liu *et al.*[35], Zhao *et al.*[36] andSaha *et al.*,[5] amongstothers, as well as by Matos *et al.*[37]for*Dalbergia ecastophyllum.*The presence of phenolic compounds was evidenced by histochemical tests performed in leaf and stems of *D. ecastaphyllum*.

CONCLUSION

The leaf and stem anatomy and histochemistry of *D. ecastaphyllum,* performed by light and electron microscopy, provided a set of distinctive characters for this species, which can serve as a parameter to be used as an additional support for its taxonomy and the quality control of its drugs.

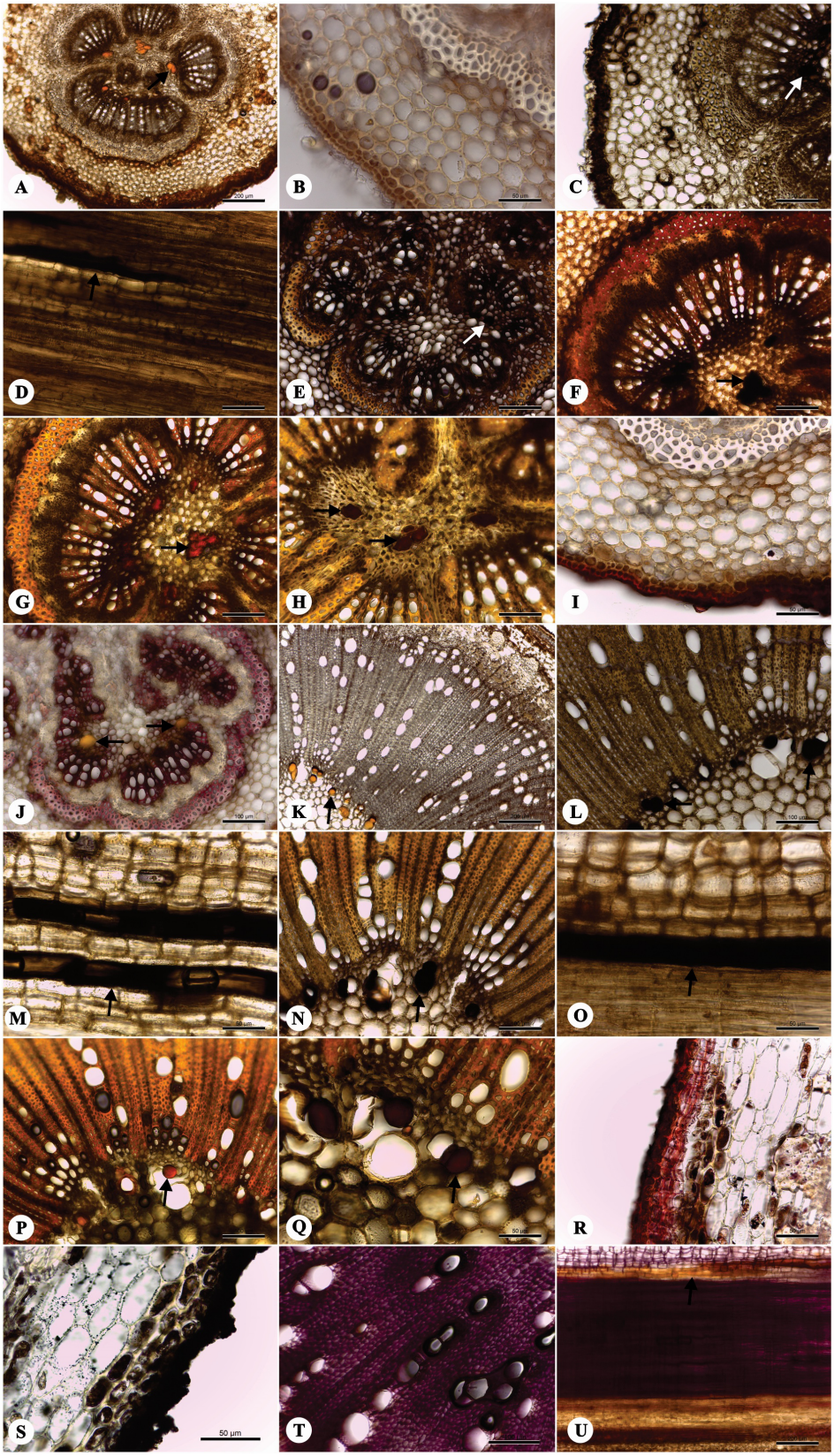

Figure 4. A-B: Leaf petiole of *Dalbergia ecastophyllum* in transverse section (*Neves 001*). A.Detail inside the petiole. B. Detail of the vascular system of the petiole. C-D. Stem in cross section. C. Detailof a portion stem. D. Detail of stem showing the location of idioblasts. Legends: (co) collenchyma, (ph) phloem, (sc) sclerenchyma, (id) idioblast, (xy) and xylem.

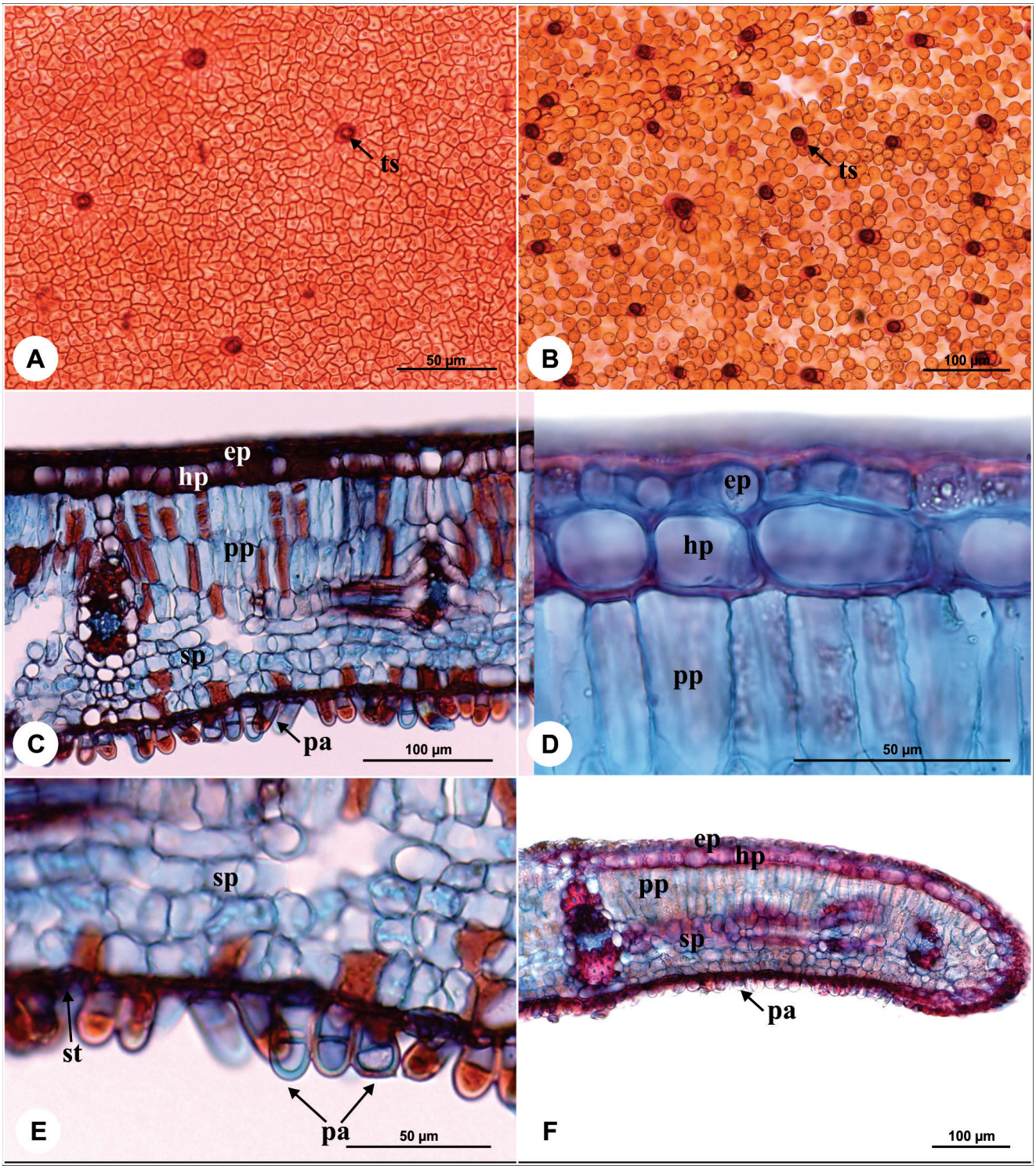

Figure 5: Histochemical tests in leaves and stems of *Dalbergia ecastophyllum* under different colorants and reagents (*Neves 001, 002*). A-J. Longitudinal and transverse sections of leaves: A-B. White proof. C-D. Secretory elements colored black by iron chloride III indicating the presence of phenolic compounds. E-F. Secretory elements colored black by potassium dichromate as a positive reaction for phenolic compounds. G. Secretory elements colored red-orange by 2,4-dinitrophenylhydrazine for the presence of terpenoids. H. Secretory elements colored reddish-brown by Wagner reagent as positive reaction for alkaloids. I. Cuticle colored with Sudan IV as a positive reaction for lipids. J. Lignified elements of xylem and sclerenchyma colored by acidified phloroglucinol. K-U. Stems in longitudinal and transverse sections. K. White proof. L-M. Secretory elements colored black by iron chloride III, indicating phenolic compounds. N-O. Secretory elements colored black by potassium dichromate as a positive reaction for phenolic compounds. P. Secretory elements colored red-orange by 2,4-dinitrophenylhydrazine indicating the presence of terpenoids. Q. Secretory elements colored reddish-brown by Wagner reagent, as a positive reaction for alkaloids. R. Cuticle colored with Sudan IV as a positive reaction for lipids. S. Cuticle colored with Sudan black B as a positive reaction for lipids.T-U. Lignified elements of xylem and sclerenchyma colored by acidified phloroglucinol. Legend: (arrow) idioblast.

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