

Comparative leaf anatomy and morphology of some neotropical Rutaceae: *Pilocarpus* Vahl and related genera

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Abstract Previous anatomical studies have been restricted to the foliar aspects of *Pilocarpus*. However, no anatomical studies analyzing the foliar aspects of *Pilocarpus* in relation to related genera have been carried out. Therefore, the aim of this study was to identify characters for future taxonomic and phylogenetic studies in Rutaceae, particularly in *Pilocarpus*, and to discuss the characteristics associated with the simple or compound leaf condition for the group. The petiole and the leaf blade of 14 neotropical Rutaceae species were analyzed, and the following characteristics were observed in all leaves studied: stomata on both surfaces; secretory cavities, including mesophyll type; camptodromous–brochidodromous venation pattern; and free vascular cylinder in the basal region of the petiole. Additional promising characters were identified for future taxonomic and phylogenetic studies in the Rutaceae family, especially for the *Pilocarpus* genera.

Keywords Leaf venation · Petiole · Simple and compound leaves

Introduction

The Rutaceae family includes about 160 genera and 1,900 species that are widely distributed in tropical and temperate regions around the world, but they are especially abundant in Australia and South Africa (Groppo et al. 2008; Cronquist 1988). In Brazil, there are 32 native genera with

154 species (Pirani 1999), of which 17 are *Pilocarpus* species (Skorupa and Pirani 2004). The phylogeny of Rutaceae is based on molecular (Chase et al. 1999; Scott et al. 2000; Samuel et al. 2001; Corazza-Nunes et al. 2006; Salvo et al. 2008; Groppo et al. 2008; Bayer et al. 2009; But et al. 2009) and morphological analyses (Oliveira 2007; Salvo et al. 2008).

The family is characterized by the presence of secretory cavities, particularly in the leaves and reproductive structures (Groppo et al. 2008). Observations on the morphology and anatomy of secretory cavities were made by Liu and Hu (1998) in the leaves of 22 genera, 40 species and two varieties of Rutaceae. According to these authors, there are three types of secretory cavities: a notch type (between the leaf teeth), a mesophyll type (subdivided into spongy parenchyma and palisade parenchyma) and a mixed type (with both notch and mesophyll cavities), suggesting that the mixed type of cavities developed from the notch and mesophyll types. Recently, Pan (2010) described the epidermal morphology of fossil leaf and modern leaf fragments of Rutaceae.

Anatomical studies on *Pilocarpus* have thus far been restricted to the foliar aspects. These studies include those of Marquete (1981) who analyzed the structures of the leaf blade and petiole of *P. organensis* Occhioni & Rizzini (= *P. pauciflorus* A. St.-Hil.), Gallardo and Jiménez (2002) who described xeromorphic leaf traits in *P. goudotianus* Tul. (= *P. racemosus* Vahl), and Spegazzini et al. (2002) who discussed leaf anatomy applied to the taxonomy of three species of *Pilocarpus* and correlated the anatomical characteristics of the leaf blade with the simple or compound condition of the leaf. However, no anatomical studies analyzing the foliar aspects of *Pilocarpus* in relation to related genera have been carried out. Therefore, the purpose of this comparative study was to assess anatomical

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and morphological variations which may be useful in species identification, as well as establish characteristics associated with leaf organization for the group, considering both simple and compound leaves.

Materials and methods

The samples of all Rutaceae species analyzed were kept in the herbarium at the University of São Paulo, São Paulo, Brazil, and included 14 species belonging to seven genera (Table 1): *Pilocarpus* (eight species), *Metrodorea* (one species), *Esenbeckia* (one species), *Raulinoa* (one species), *Balfourodendron* (one species), *Galipea* (one species) and *Helietta* (one species).

Fully expanded leaves situated from the third and fourth nodes of the plant from the tip were randomly sampled from 70 specimens. Samples of ten mature leaves of each species were collected, fixed in FAA (formalin/acetic acid/ alcohol 50%) and dehydrated in an ethanol series, and then stored in 70% ethanol (Johansen 1940). The leaf blade (including basal, middle, and distal parts) and petiole were embedded in Paraffin Plus following the methodology described by Johansen (1940) using a tertiary butyl ethanol

series (50–100%). Cross sections and longitudinal sections were stained with basic fuchsin and Astra blue according to the method of Kraus et al. (1998). To analyze the epidermis and stomata type, 100 mm² samples were diaphanized and stained with alcoholic safranin (Franklin 1945). Leaf blades were diaphanized according to the method of Strittmater (1973). All slides were then mounted with Entellan and studies by scanning electron microscopy (SEM) to visualize the cuticle using the methodology described by Silveira (1989).

All 14 Rutaceae species were compared according to their analyzed morphoanatomical characteristics using a presence/absence matrix (Table 2). The venation patterns of the leaf blades were classified according to methods of Hickey and Wolfe (1975) and Metcalfe and Chalk (1979).

Results and discussion

Leaf blade

The SEM analysis showed that the adaxial surface had a striated cuticle (Fig. 1a, b), except in *Pilocarpus* sp., *G. trifoliata* and *R. echinata* (Fig. 1c) which had a smooth cuticle. On the abaxial surface, a smooth cuticle occurred only in *P. pauciflorus*, *R. echinata* and *B. riedelianum*, while in the other species, the cuticle was striated (Fig. 1d–f). According to Spegazzini et al. (2002), a striated cuticle on both surfaces of the epidermis occurs in *P. jaborandi* and *P. microphyllus*, which corroborates the results obtained by Duval (1903) and Kaastra (1982) in relation only to *P. jaborandi*. Analysis of the morphoanatomy of the cuticle showed that *Pilocarpus* sp. and *P. pauciflorus* were the only species of the genus that presented a striated cuticle on the abaxial surface (Table 2).

Among the species studied, two types of glandular trichomes were observed: capitate (Fig. 2a–e) and peltate (Fig. 2f–h). The capitate type consisted of a basal cell, one or more peduncular cells with cutinized external walls, and the apical region that could be uni- or multicellular. The trichomes can be classified into four types:

1. Multicellular uniseriate stalk inserted into a depression in the epidermis, with a multicellular, circular apical region as in *E. leiocarpa* (Fig. 2a, b).
2. Multicellular uniseriate stalk, with a unicellular, pyriform apical region as in *G. trifoliata* (Fig. 2c).
3. Unicellular stalk, with unicellular globular apical region having thin walls as in *P. spicatus* (Fig. 2d), *P. giganteus*, *P. pauciflorus* (Fig. 2e) and *P. microphyllus*.
4. Unicellular stalk, with multicellular apical region as in *B. riedelianum* and *Helietta apiculata*.

Table 1 List of the voucher material species deposited in the SPF herbarium (Universidade de São Paulo). Classification according to Scholz (1964)

Taxonomic treatment and species	Voucher
Subfamily Rutoideae	
Tribe Galipeae	
Subtribe Galipeinae	
<i>Galipea trifoliata</i> Aubl.	Dias 230, 231
Subtribe Pilocarpinae	
<i>Esenbeckia leiocarpa</i> Engler	Dias 214
<i>Metrodorea flavida</i> K. Krause	Dias 229
<i>Pilocarpus alatus</i> Joseph ex Skorupa	Dias 247
<i>Pilocarpus giganteus</i> Engler	Dias 337
<i>Pilocarpus jaborandi</i> Holmes	Dias 252, 253, 254
<i>Pilocarpus microphyllus</i> Stapf ex Wardl.	Dias 235, 237, 238
<i>Pilocarpus pauciflorus</i> A. St.-Hil.	Dias 218
<i>Pilocarpus riedelianus</i> Engler	Pirani 6056
<i>Pilocarpus spicatus</i> A. St.-Hil.	Dias 206, 325
<i>Pilocarpus</i> sp.	Pirani 6055
<i>Raulinoa echinata</i> Cowan	Dias 257, 258, 259
Subfamily Toddalioidae	
Tribe Toddalieae	
Subtribe Pteleinae	
<i>Balfourodendron riedelianum</i> Engler	Dias 217, 345
<i>Helietta apiculata</i> Benth	Dias 216, 344

Table 2 Presence (1) and absence (0) matrix of the 14 neotropical Rutaceae species analyzed

Analyzed characteristics	<i>Pilocarpus</i> sp.	<i>P. microphyllus</i>	<i>P. pauciflorus</i>	<i>P. spicatus</i>	<i>P. giganteus</i>	<i>P. alatus</i>	<i>P. jaborandi</i>	<i>P. riedelianus</i>	<i>Galipea trifoliata</i>	<i>Esenbeckia letocarpa</i>	<i>Metrodorea flavida</i>	<i>Helietta apiculata</i>	<i>Balfouriodendron riedelianum</i>	<i>Raulinoa echinata</i>
Striated cuticle on the adaxial surface (1)	0	1	1	1	1	1	1	1	0	1	1	1	1	0
Smooth cuticle on the adaxial surface (2)	1	0	0	0	0	0	0	0	1	0	0	0	0	1
Striated cuticle on the abaxial surface (3)	1	1	0	1	1	1	1	1	1	1	0	1	1	0
Smooth cuticle on the abaxial surface (4)	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Nonglandular trichome (5)	0	1	1	1	1	1	1	0	1	1	1	1	1	0
Glandular trichome: multicellular uniseriate peduncle with a unicellular apical region (6)	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Glandular trichome: multicellular uniseriate peduncle with a multicellular apical region (7)	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Glandular trichome: unicellular peduncle with unicellular apical region (8)	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Glandular trichome: multicellular peduncle with multicellular apical region (9)	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Peltate trichomes (10)	0	0	0	0	0	1	1	0	0	0	1	1	0	1
Epidermal cells with straight anticlinal walls in the front view of the adaxial surface (11)	1	1	1	0	1	0	1	1	0	1	0	1	1	1
Epidermal cells with sinuous anticlinal walls in the front view of the adaxial surface (12)	0	0	0	1	0	1	0	0	1	0	1	0	0	0
Epidermal cells with straight anticlinal walls in the front view of the abaxial surface (13)	1	1	1	1	1	1	1	1	0	1	0	1	1	1
Epidermal cells with sinuous anticlinal walls in the front view of the abaxial surface (14)	0	0	0	0	0	0	0	0	1	0	1	0	0	0
Cyclocytic stomata (15)	0	1	0	0	0	0	0	0	1	1	1	1	1	0
Tetracytic stomata (16)	1	0	1	1	1	1	1	1	0	0	0	0	0	1
Epidermis in transverse section: adaxial cells > abaxial cells (17)	0	0	0	0	1	0	0	0	1	0	0	1	0	1
Epidermis in transverse section: adaxial cells = abaxial cells (18)	1	1	1	1	0	1	1	1	0	1	1	0	1	0
Palisade parenchyma arranged in one layer (19)	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Glandular cavities in the leaf blade (20)	1	1	1	1	1	1	1	1	1	0	1	1	1	1

Table 2 continued

Analyzed characteristics	<i>Pilocarpus</i> sp.	<i>P.</i> <i>microphyllus</i>	<i>P.</i> <i>pauciflorus</i>	<i>P.</i> <i>spicatus</i>	<i>P.</i> <i>giganteus</i>	<i>P.</i> <i>alatus</i>	<i>P.</i> <i>jaborandi</i>	<i>P.</i> <i>riedelianus</i>	<i>Galipea</i> <i>trifoliata</i>	<i>Esenbeckia</i> <i>letocarpa</i>	<i>Metrodorea</i> <i>flavida</i>	<i>Helietta</i> <i>apiculata</i>	<i>Balfouriodendron</i> <i>riedelianum</i>	<i>Raulinoa</i> <i>echinata</i>
Druses in palisade parenchyma (21)	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Vascular bundles arranged in an open arc in the midrib (22)	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Vascular bundles arranged in a closed arc in the midrib (23)	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Domatia (24)	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Uniform angle of the secondary veins (25)	1	1	1	1	1	1	0	1	1	1	0	0	0	1
Lower secondary veins more obtuse than the upper (26)	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Upper secondary veins more obtuse than the lower (27)	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Simple intersecondary veins (28)	1	1	1	1	1	1	1	1	1	0	1	1	1	1
Marginal ultimate venation incomplete (29)	0	1	0	0	0	1	0	1	0	1	1	1	0	0
Marginal ultimate venation looped (30)	1	0	1	1	1	0	1	0	1	0	0	0	1	1
Random reticulate pattern of the tertiary veins (31)	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Percurrent pattern of the tertiary veins (32)	1	0	0	1	1	0	1	0	0	1	1	1	1	0
Dichotomous branching of the tertiary veins (33)	0	1	1	0	0	1	0	1	1	0	0	0	0	0
Random reticulate pattern of the quaternary veins (34)	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Percurrent pattern of the quaternary veins (35)	1	0	0	1	1	0	1	0	0	1	1	0	1	0
Dichotomous branching of the quaternary veins (36)	0	1	0	0	0	1	0	1	0	0	0	1	0	1
Arcoles imperfect (37)	0	0	1	1	1	1	1	1	1	1	1	0	1	0
Arcoles incompletely closed meshes (38)	1	1	0	0	0	0	0	0	0	0	0	1	0	1
Simple veinlets (39)	0	1	1	1	0	0	0	0	1	1	1	0	0	1
Branched veinlets (40)	1	0	0	0	1	1	1	1	0	0	0	1	1	0
Papilla-like projections on the epidermis cells of the petiole (41)	0	0	0	0	1	0	0	0	0	1	0	1	1	0
Nonglandular trichomes in the petiole (42)	1	1	0	1	0	1	1	1	1	1	1	1	1	1
Peltate trichomes in the petiole (43)	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Capitate trichomes in the petiole (44)	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Glandular cavities in the petiole (45)	1	1	1	1	1	1	1	1	1	0	1	1	1	1

Table 2 continued

Analyzed characteristics	<i>Pilocarpus</i> sp.	<i>P. microphyllus</i>	<i>P. pauciflorus</i>	<i>P. spicatus</i>	<i>P. giganteus</i>	<i>P. alatus</i>	<i>P. jaborandi</i>	<i>P. riedelianus</i>	<i>Galipea trifoliata</i>	<i>Esenbeckia leiocarpa</i>	<i>Metrodorea flavida</i>	<i>Helietta apiculata</i>	<i>Balfouriodendron riedelianum</i>	<i>Raulinoa echinata</i>
Raphides in the petiole (46)	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Prismatic crystal in the petiole (47)	0	0	0	0	0	0	0	0	1	1	1	1	1	1
Druses in the petiole (48)	1	1	1	1	1	1	1	1	0	0	0	0	0	0
Pericyclic fibers on the petiole (49)	1	1	1	1	1	1	1	1	0	0	1	0	0	1

Studies on *Pilocarpus* (Duval 1903; Gallardo and Jiménez 2002; Spegazzini et al. 2002) have shown glandular trichomes on both leaf surfaces, but without indicating the type. Geiger (1897, apud Kaastra 1982) stated that glandular trichomes are present in all species of *Pilocarpus*, but are rare in *P. spicatus* and *P. microphyllus*. According to Kaastra (1982), the trichomes do not occur in the depression in these species. For *P. microphyllus*, the present study found that the trichomes are sparse and do not occur in the depression. It was further observed that the apical region of the trichome is unicellular, not multicellular, as mentioned by Spegazzini et al. (2002), who described trichomes as occurring in the depression of the epidermis in *P. microphyllus*. Among the *Pilocarpus* species studied, only *Pilocarpus* sp. showed multicellular uniseriate trichomes (see Table 2).

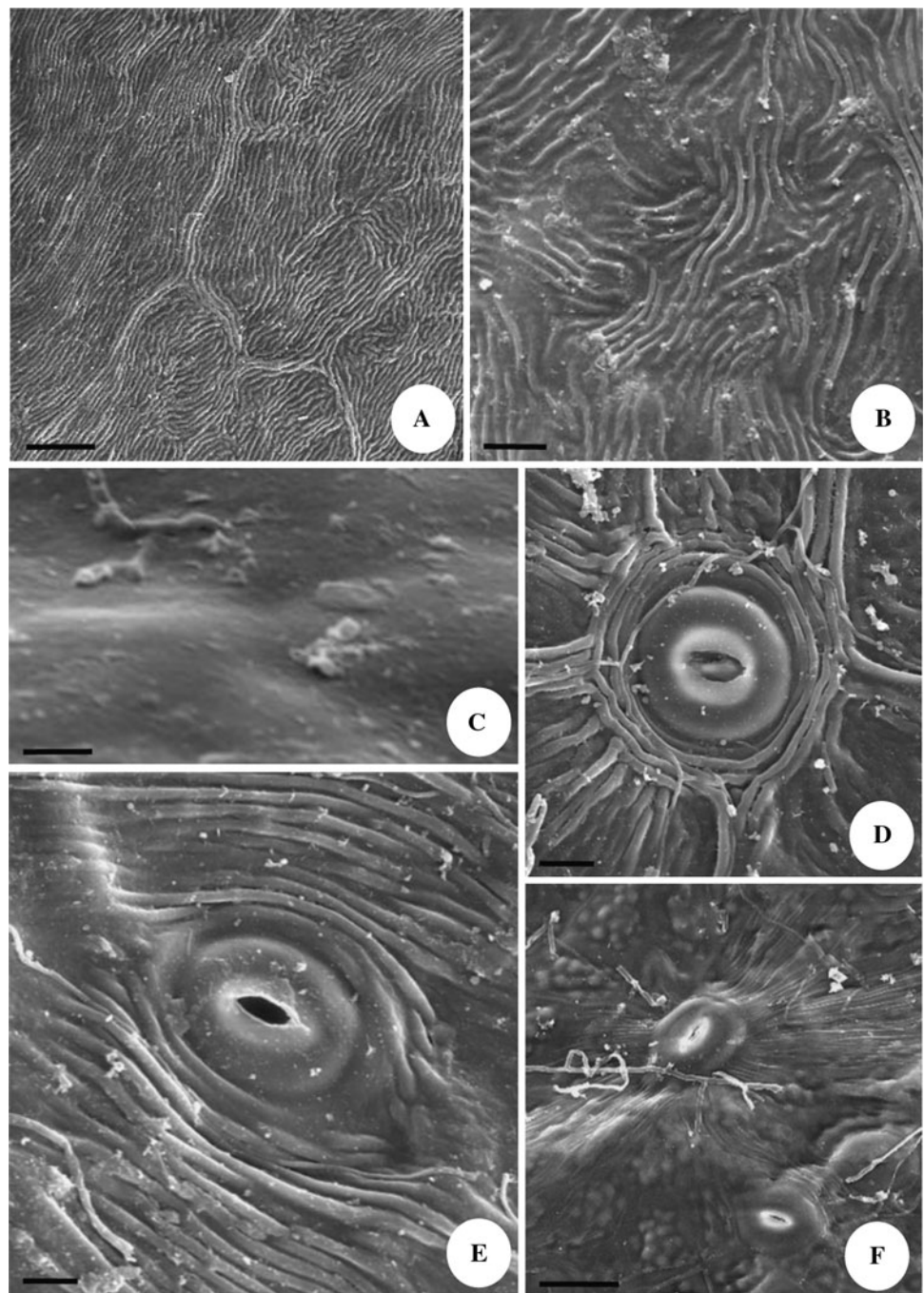
In frontal view (Fig. 3a–f) the epidermal cells on both sides have straight anticlinal walls in most species. Sinuous walls occur on both surfaces in *G. trifoliata* and *M. flavida*, but they occur only on the adaxial surface in *P. alatus* (Fig. 3c) and *P. spicatus*. In species in which the walls are straight, the epidermis on both surfaces has an outline ranging from square to pentagonal as in *E. leiocarpa*, pentagonal to hexagonal only on the adaxial surface as in *P. jaborandi* (Fig. 3a), and in other species it varies from rectangular to pentagonal.

According to Metcalfe and Chalk (1979), the anticlinal walls of epidermal cells may be straight or wavy. Duval (1903) observed that species of *Pilocarpus* with simple leaves usually have straight walls, while compound leaves have wavy walls. However, the author does not state what surface of the epidermis has this feature. Independent of the leaf pattern, all *Pilocarpus* species analyzed in this study have straight walls on the abaxial surface.

All species of *Pilocarpus* and related genera that we studied have hypostomatous leaves, with stomata at the same level of the epidermal cells, a characteristic of *Pilocarpus* cited by authors such as Duval (1903), Marquete (1981), Spegazzini et al. (2002) and Gallardo and Jiménez (2002).

Two types of stomata were found: cyclocytic stomata in *G. trifoliata*, *E. leiocarpa*, *M. flavida*, *P. microphyllus*, *H. apiculata* and *B. riedelianum*; and tetracytic stomata, with four subsidiary cells, two of them oblique in the upper portion and two oblique in the inferior part, in *Pilocarpus* sp., *P. alatus*, *P. jaborandi*, *P. pauciflorus*, *R. echinata*, *P. giganteus*, *P. riedelianus* and *P. spicatus*. Skorupa (1996) observed three types of stomata in *Pilocarpus*: anomocytic, tetracytic and stomata with four subsidiary cells, two oblique in the upper portion and two in the lower portion, similar to that found in our study. However, the author observed anomocytic stomata for *P. spicatus*, which differs from that found in our study. Other types of

Fig. 1 SEM micrographs of leaf epidermis. **a, b** Striated cuticle on the adaxial surface: **a** *Pilocarpus spicatus*; **b** *Metrodorea flavida*. **c** Smooth cuticle on the adaxial surface: *Raulinoa echinata*. **d–f** Striated cuticle on the abaxial surface (note stomata): **d** *Pilocarpus jaborandi*; **e** *Pilocarpus spicatus*; **f** *Pilocarpus microphyllus*. Scale bars **a**, **f** 20 μm ; **b, d, e** 10 μm ; **c** 5 μm

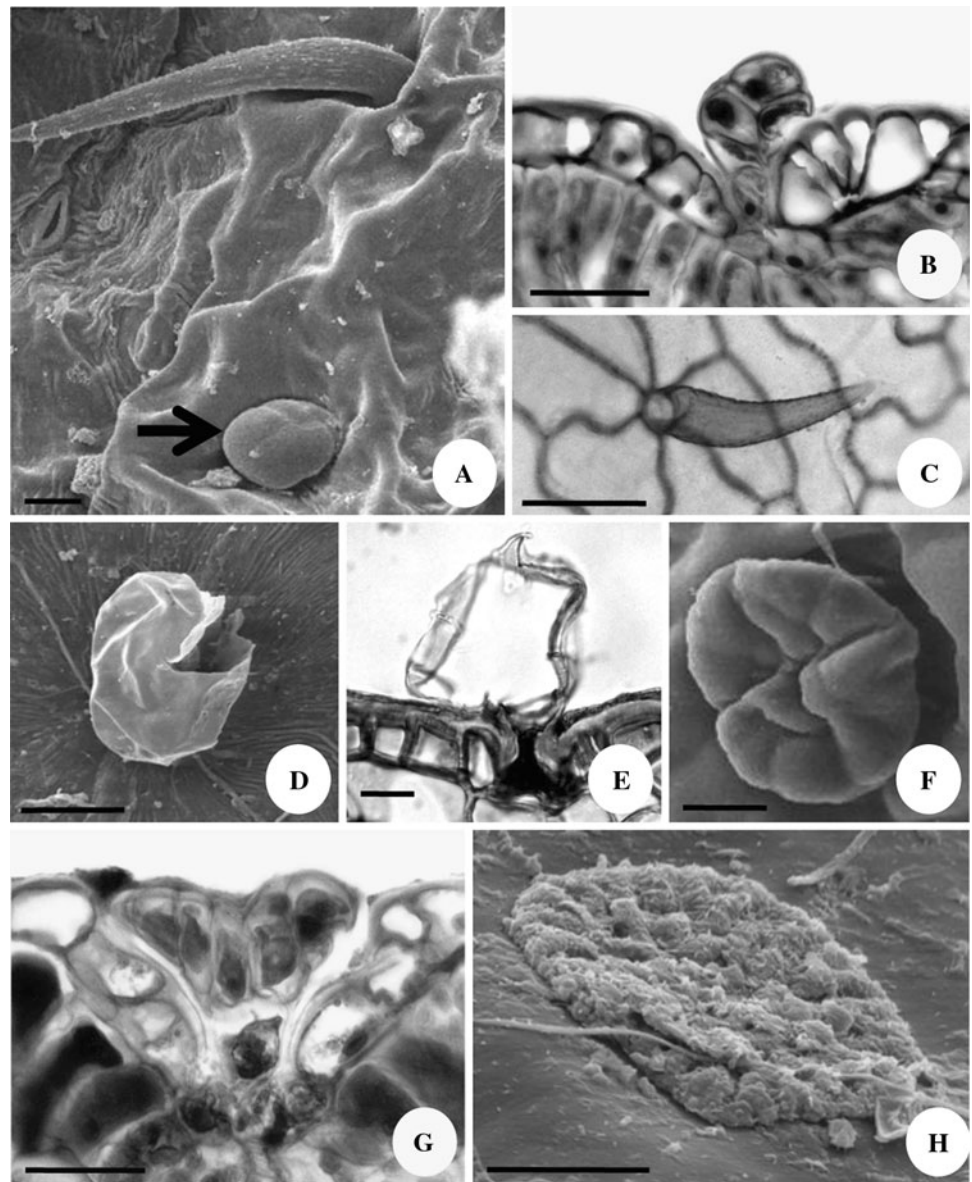


stomata, such as anisocytic and cyclocytic, were described by Marquete (1981) for *P. organensis* (= *P. pauciflorus*); Spegazzini et al. (2002) for *P. jaborandi*, *P. microphyllus* and *P. pennatifolius*; and Duval (1903) for *P. jaborandi*, *P. pennatifolius*, *P. spicatus* and *P. trachylophus*.

In transverse sections, the epidermis is uniseriate in all species studied (Fig. 4a–f), with square to rectangular cells on both surfaces. The adaxial cells are similar in size (lumen) to the cells on the abaxial surface in almost all

species, except in *G. trifoliata* (Fig. 4b), *R. echinata*, *H. apiculata* and *P. giganteus*, whose adaxial cells are larger. Two leaf anatomical characters, a papillose epidermis (Metcalf and Chalk 1979; Duval 1903) and a hypodermis with one or more layers (Metcalf and Chalk 1950; Kaastra 1982), have been described in some Rutaceae genera, including *Pilocarpus*. However, none of the species analyzed in the present study showed these features in the leaf blade. Finally, this study elucidated an

Fig. 2 Types of glandular trichomes. **a, b** *Esenbeckia leiocarpa* (adaxial surface): **a** SEM micrograph of leaf epidermis (arrow capitate trichome); **b** capitate trichome in transverse section. **c** Multicellular uniseriate trichomes on the adaxial surface in *Galipea trifoliata*. **d** SEM micrograph of leaf epidermis in *Pilocarpus spicatus* showing a capitate trichome on the abaxial surface. **e** Transverse section of the leaf of *Pilocarpus pauciflorus* showing a capitate trichome and the adaxial surface. **f–h** Peltate trichomes on the adaxial surface: **f** SEM micrograph of the leaf of *Metrodorea flavida*; **g** transverse section of the leaf of *Metrodorea flavida*; **h** SEM micrograph of the leaf of *Raulinoa echinata*. Scale bars **a, d, f** 20 μm ; **b, e, g** 40 μm ; **c** 80 μm ; **h** 5 μm



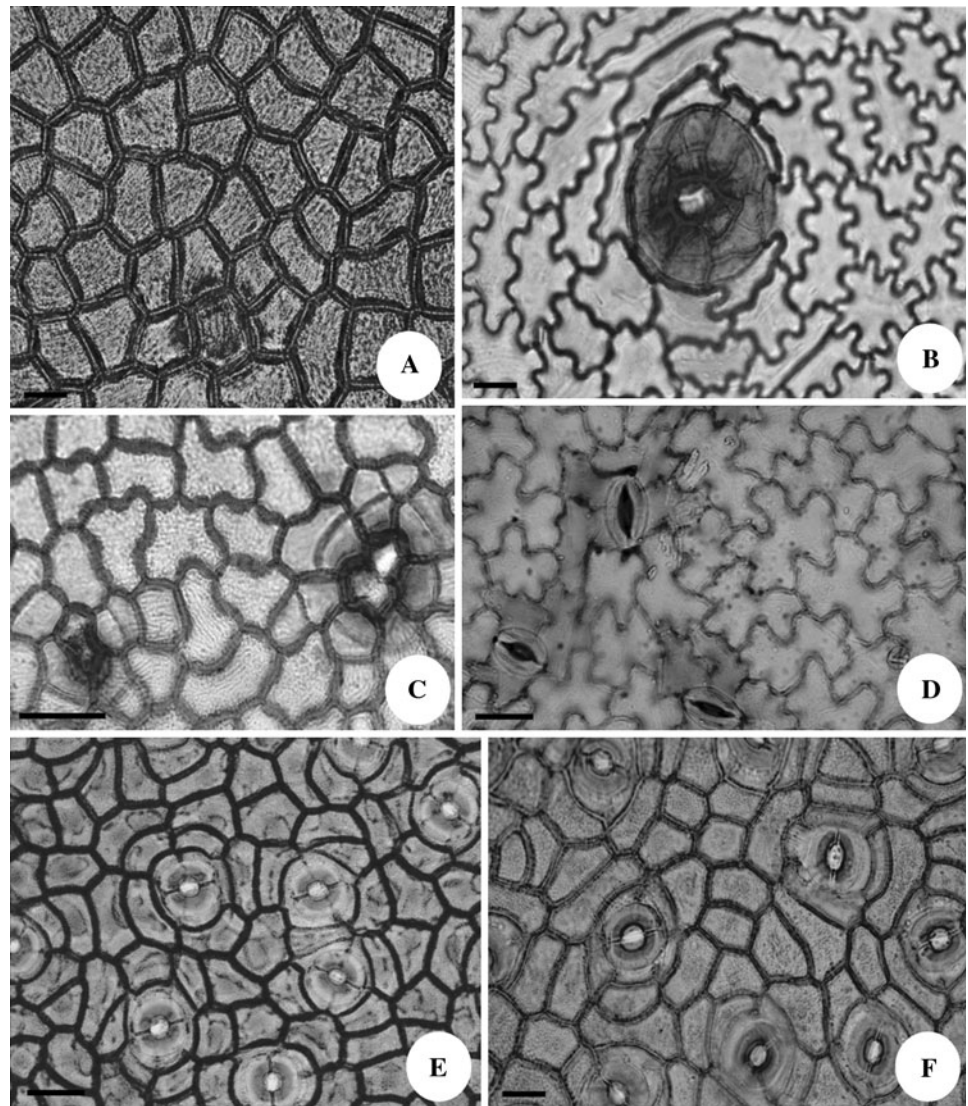
important characteristic not previously reported in the Rutaceae literature: a periclinal division in some epidermal cells in the transverse section of the mesophyll of *H. apiculata* (Fig. 4c).

A dorsiventral mesophyll was observed in all *Pilocarpus* species and related genera, which corroborates the data of Duval (1903), Marquete (1981), Liu and Hu (1998), Gallardo and Jiménez (2002) and Spegazzini et al. (2002). Duval (1903) attempted to associate the number of layers with the leaf pattern (simple or compound leaf), i.e., the palisade parenchyma has two layers in simple leaf species and one or two layers in compound leaf species. Marquete (1981) and Spegazzini et al. (2002) analyzed only compound leaves, describing a single layer of palisade parenchyma, which corroborates Duval's proposition. In our study, we found the ratio between the number of palisade

parenchyma cells and epidermal cells to be 1:1 in *P. alatus*, *P. giganteus* and *P. spicatus* (Fig. 4d), 3:1–5:1 in *P. jaborandi* (Fig. 4a), *B. riedelianum* and *H. apiculata* (Fig. 4c), and 2:1 in the other species.

Calcium oxalate druses were observed in palisade parenchyma cells in all species of *Pilocarpus* (Fig. 4e), and rod-shaped crystals were seen in the other genera (Fig. 4f). Raphides were described only in *G. trifoliata*, and only prismatic crystals could be observed in *E. leiocarpa*, *R. echinata*, *M. flavida*, *H. apiculata* and *B. riedelianum*. These idioblasts are present in the cortex, vascular system and the medullar region of the petiole. The crystals can also be distributed in the spongy parenchyma and in the midrib region. In addition, we observed septal cells transversally with calcium oxalate druses in each compartment in all species of *Pilocarpus* analyzed in the present study. This

Fig. 3 Epidermal cells in the frontal view. **a–c** Adaxial surface: **a** epidermal cells with straight anticlinal walls in *Pilocarpus jaborandi*; **b** epidermal cells with sinuous anticlinal walls in *Metrodorea flavida*; **c** epidermal cells with sinuous anticlinal walls in *Pilocarpus alatus*. **d–f** Abaxial surface: **d** epidermal cells with sinuous anticlinal walls in *Galipea trifoliata*; epidermal cells with straight anticlinal walls in *Pilocarpus pauciflorus* (**e**) and *Pilocarpus spicatus* (**f**). Scale bars 40 μ m



feature was also mentioned by Duval (1905 apud Kaastra 1982), Metcalfe and Chalk (1950), Marquete (1981) and Spegazzini et al. (2002). The terminology suggested in our study was “compartment” of the palisade parenchyma instead of “chambered cells” of the palisade parenchyma, as used by Duval (1905 apud Kaastra 1982).

The shape of the vascular system of the midrib is a closed cylinder in almost all species (Fig. 5a–e), except *P. microphyllus*, which is open (Fig. 5f). Collateral vascular bundles and pericyclic fibers surrounding the bundles were observed in all species, corroborating the results obtained by Geiger (1897 apud Kaastra 1982) for *Pilocarpus* species.

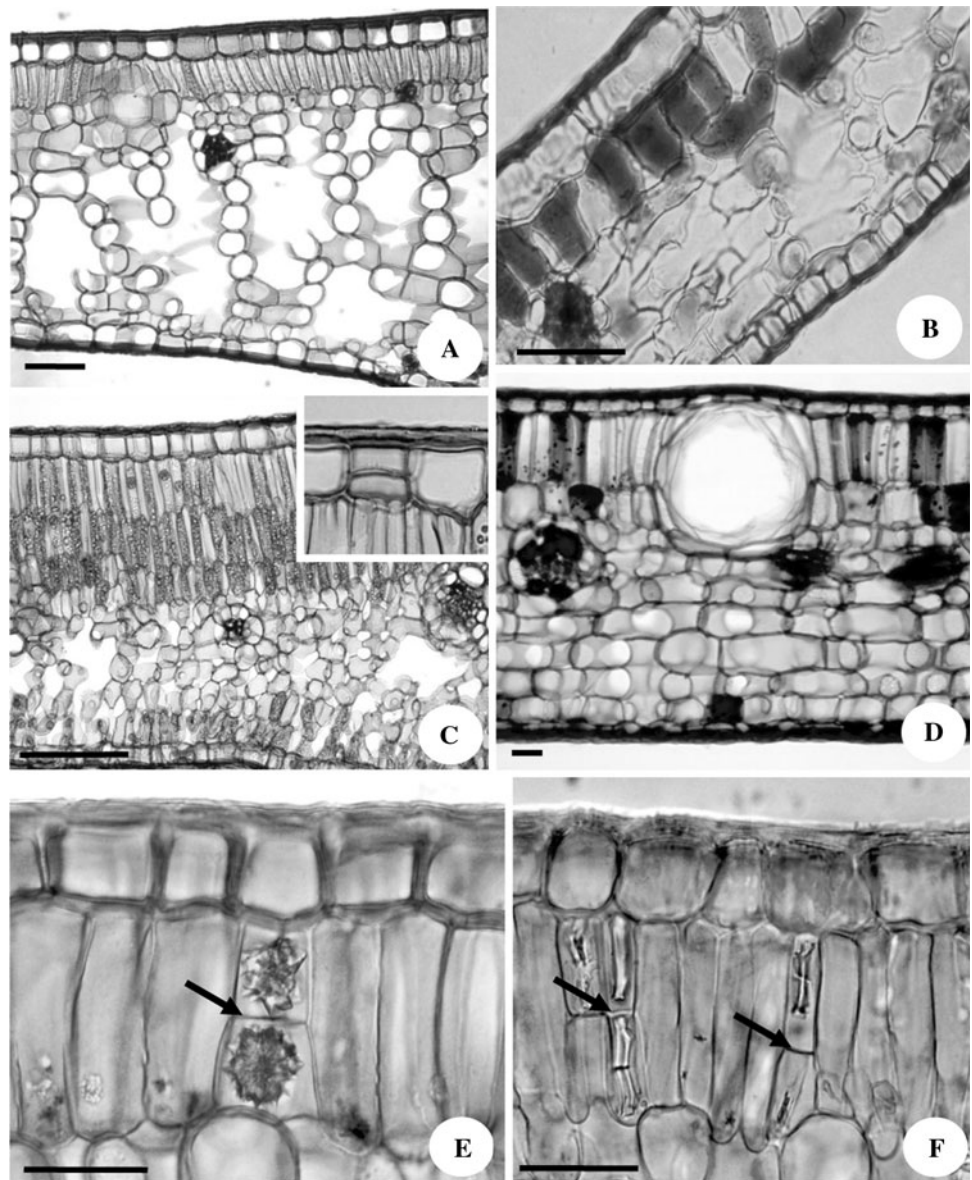
Venation

In all the leaves studied, the type of venation was pinnate with a camptodromous–brochidodromous foliar venation

pattern where second-order veins are joined (Fig. 6a). The brochidodromous pattern was described by Marquete (1981) for *Pilocarpus* species and Pan (2010) for Rutaceae leaf fossils (*Vepris* sp. and *Clausena* sp.). According to Skorupa (1996), *P. giganteus* and *P. spicatus* have a eucamptodromous leaf venation where second-order veins diminish and terminate within the leaf blade.

The spacing between the secondary veins is irregular in all species. However, the angle formed by these veins increases towards the leaf base, as in *P. jaborandi* and *B. riedelianum*, but decreases toward the base, as in *M. flavida* and *H. apiculata*. Otherwise, the spacing between secondary veins remains uniform throughout the leaf, as observed in other species. Most species have intersecondary veins, which have a width and route similar to secondary veins, but are thinner and do not reach the margin. The intersecondary veins may be weak, as in *G. trifoliata*, *M. flavida*, *H. apiculata*, *B. riedelianum*, and *R. echinata*,

Fig. 4 Transverse sections of the leaf blade. **a** *Pilocarpus jaborandi*; **b** *Galipea trifoliata*; **c** *Helietta apiculata* (inset detail of the periclinal division in an epidermal cell); **d** *Pilocarpus spicatus*; **e** *Pilocarpus pauciflorus* (arrow druses in palisade parenchyma); **f** *Esenbeckia leiocarpa* (arrows rod-shaped crystal in palisade parenchyma). Scale bars **a** 80 μm ; **b**, **e**, **f** 40 μm ; **c** 200 μm ; **d** 320 μm



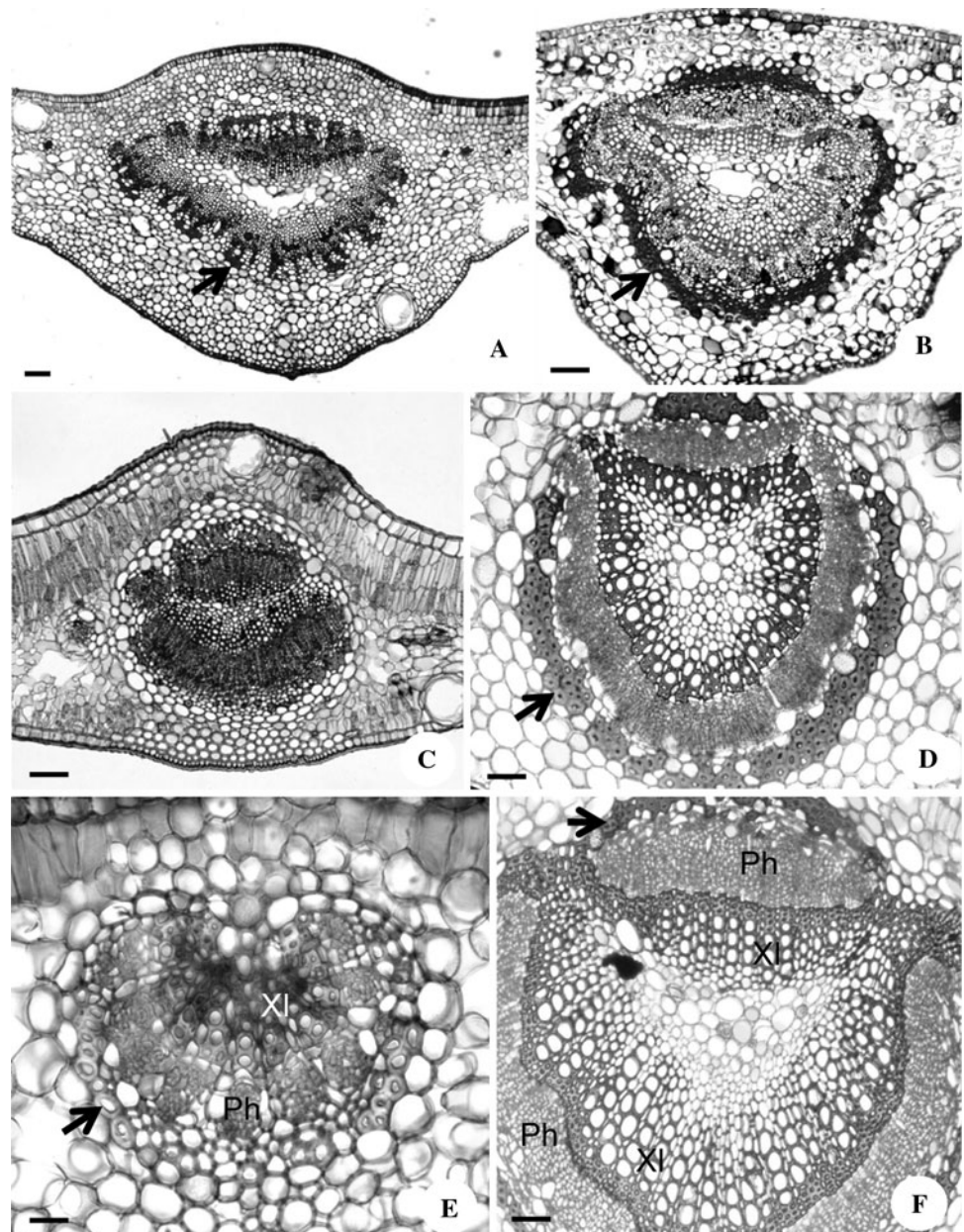
or strong, as in other species of *Pilocarpus*. Only *E. leiocarpa* has no intersecondary veins. The ultimate marginal venation is incomplete, consisting of free vascular endings in the leaves of *P. microphyllus* (Fig. 6b), *P. alatus*, *H. apiculata*, *E. leiocarpa*, *M. flavida* and *B. riedelianum*. In other species, the ultimate marginal venation is complete, in the form of an arc (Fig. 6c).

The tertiary and quaternary veins are also classified according to the leaf arrangement. In the species studied, the tertiary venations are alternate percurrent; dichotomizing, where the tertiary veins are freely branching, as in *P. microphyllus* (Fig. 6d), *P. pauciflorus*, *P. alatus*, *P. riedelianus* and *G. trifoliata*; or random reticulate, as in *H. apiculata* (Fig. 6e) and *R. echinata*. The leaves studied also have three types of quaternary venations: regular

polygonal reticulate as in *G. trifoliata* and *P. pauciflorus*; dichotomizing as in *P. riedelianus*, *P. microphyllus*, *P. alatus*, *H. apiculata* (Fig. 6e) and *R. echinata*; and alternate percurrent in the other species.

Leaf areolas are classified according to their development, and four types have been identified: well-developed, imperfect (Fig. 6f), incomplete (Fig. 6g), and absent. The majority of studied leaves have an imperfect areola. However, in *R. echinata*, *H. apiculata*, *Pilocarpus* sp. and *P. microphyllus*, the areola is incomplete, with one or more sides of the network of veins not limited. Similar to *Pilocarpus* species analyzed by Skorupa (1996), veinlets, either simple or branched, were observed in *Pilocarpus* species, as well as the genera *Helietta*, *Balfourodendron* and *Esenbeckia*, examined in this study.

Fig. 5 Transverse sections of the midrib region. **a–e** Closed cylinder in *Pilocarpus giganteus* (**a**), *Pilocarpus pauciflorus* (**b**), *Helietta apiculata* (**c**), *Pilocarpus jaborandi* (**d**), and *Metrodorea flavida* (**e**). **f** Open cylinder in *Pilocarpus microphyllus*. Scale bars **a** 320 μm , **b**, **c** 160 μm , **d–f** 80 μm ; arrows pericyclic fibers, **XI** xylem, **Ph** phloem



Anatomy of the petiole

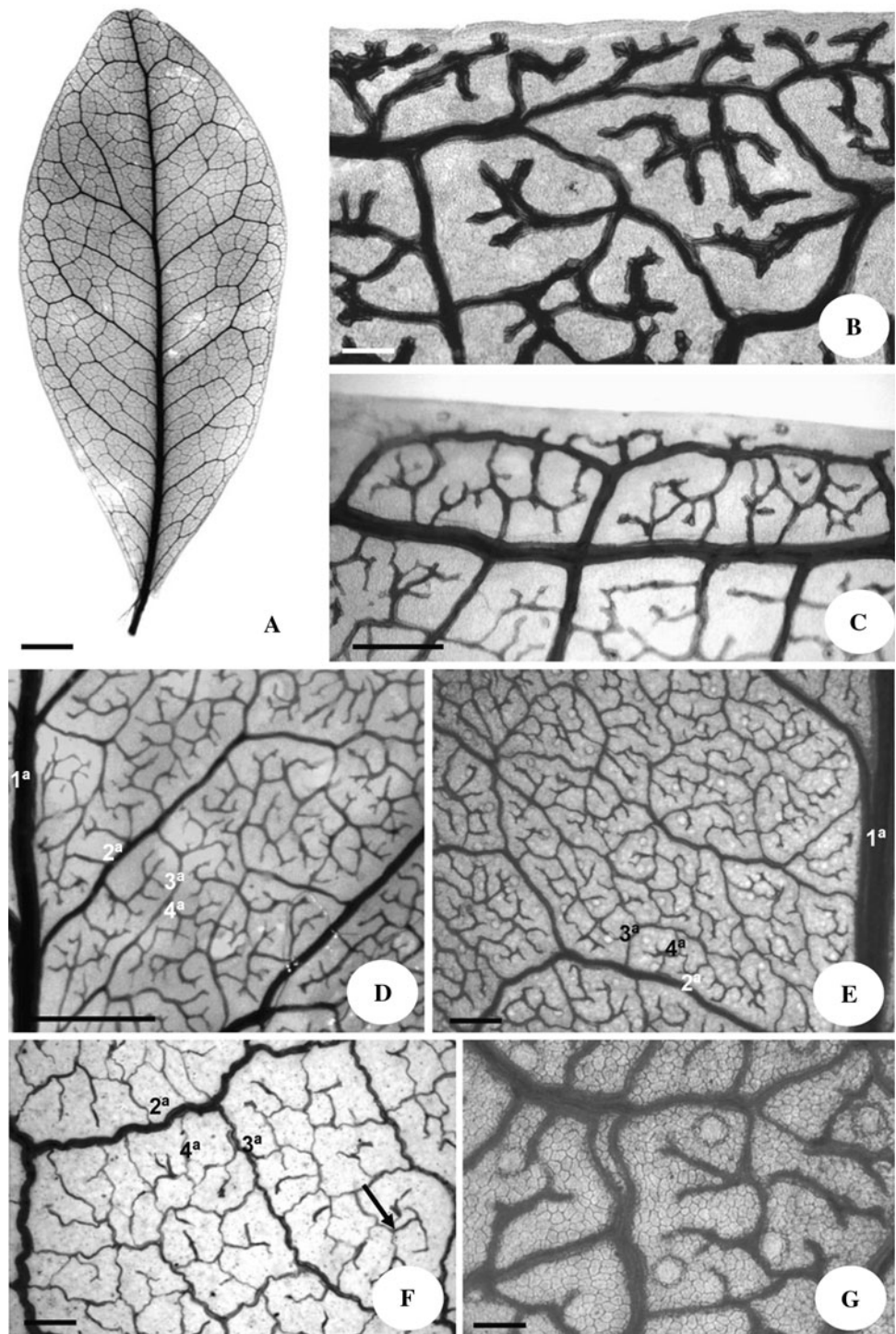
The epidermis has a papillose aspect in *E. leiocarpa* (Fig. 7a), *H. apiculata* (Fig. 7b), *B. riedelianum* and *P. alatus* and a quadrangular to rectangular aspect in *Pilocarpus* sp., *P. riedelianus*, *P. pauciflorus*, *R. echinata*, *M. flavida*, *G. trifoliata*, *P. spicatus* (Fig. 7c), *P. jaborandi*, *P. giganteus*, and *P. microphyllus*. In some species, the epidermis was partially replaced by the periderm, as in *B. riedelianum*, or completely replaced, as in *M. flavida*, which may present lenticels. Unicellular nonsecretory trichomes were observed in some species, except in

P. pauciflorus, *P. giganteus*, *E. leiocarpa* (Fig. 7a), and *H. apiculata* (Fig. 7b), with glandular trichomes.

Internal to the epidermis, on both surfaces, all individuals analyzed have secretory structures, which can be cavities or secretory cells (Fig. 4d). This feature, notable in Rutaceae, is described as translucent spots on the leaves, which correspond to multicellular glandular cavities that produce aromatic essential oils (Solereder 1908; Metcalfe and Chalk 1950; Cronquist 1981). Secretory cavities show both a schizolysigenous and a combination of schizogenous and lysigenous development. The secretory cells are observed in the cortical region, xylem, phloem, and medulla. The two

Fig. 6 Views of cleared leaves.

a Camptodromous–brochidodromous type of the foliar venation in *Pilocarpus pauciflorus*. **b** Incomplete ultimate marginal venation in *Pilocarpus microphyllus*. **c** Complete ultimate marginal venation in *Pilocarpus spicatus*. **d** Tertiary and quaternary veins dichotomizing in *Pilocarpus microphyllus*. **e** Tertiary veins random reticulate and quaternary veins dichotomizing in *Helietta apiculata*. **f** Areolation imperfect in *Esenbeckia leiocarpa*. **g** Areolation incomplete in *Helietta apiculata*. 1^a primary vein, 2^a secondary vein, 3^a tertiary vein, 4^a quaternary vein. Scale bars **b**, **g** 160 μ m; **c**, **e** 7 μ m; **d** 13 μ m; **f** 3 μ m



secretory structures were observed in almost all species, except in *E. leiocarpa*, which has only secretory cells.

The petiole outlines in transverse section are concave–convex in *B. riedelianum*, *H. apiculata*, *P. spicatus*, *M. flavida*, *R. echinata* (Fig. 7d) and *P. alatus*, but plan–convex in *G. trifoliata*, *P. jaborandi*, *P. giganteus* and *P. microphyllus*, and circular in *E. leiocarpa*. The vascular

system is organized in two ways in the basal region of the petiole: closed cylinder (Fig. 7e) and open with a single cylinder (Fig. 7f), or with free vascular bundles (Fig. 7d). However, in the species that have an open vascular cylinder, the vascular cylinder is closed in the apical region. This variation in the organization of the vascular system in petioles of species of *Pilocarpus* was mentioned by

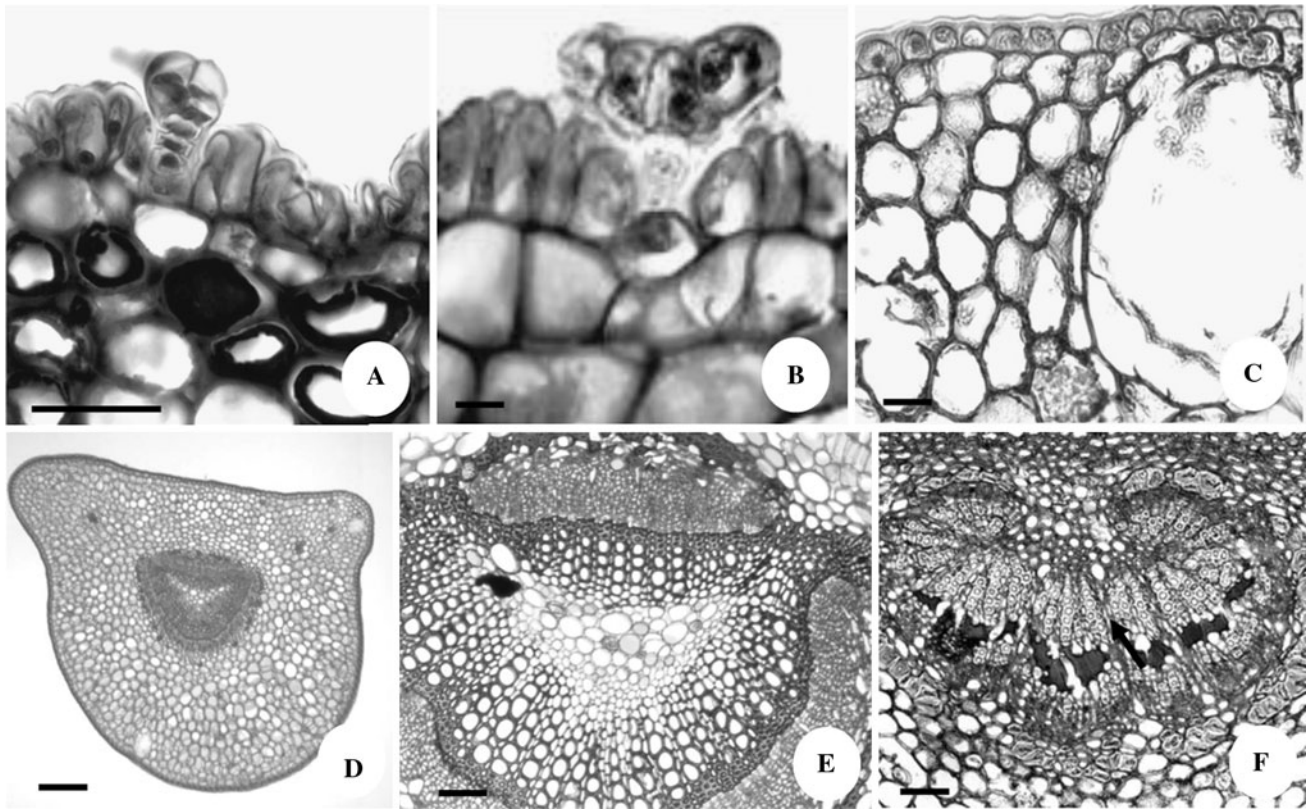


Fig. 7 Transverse section of the petiole. **a** Papillose epidermis and capitate trichome in *Esenbeckia leiocarpa*. **b** Papillose epidermis and peltate trichome in *Helietta apiculata*. **c** *Pilocarpus spicatus*. **d** Petiole

concave–convex in *Raulinoa echinata*. **e** Closed cylinder in *Esenbeckia leiocarpa*. **f** Open cylinder in *Pilocarpus spicatus*. Scale bars **a, b** 80 μm ; **c** 40 μm ; **d** 340 μm ; **e, f** 520 μm

Marquete (1981). Pericyclic fibers (Fig. 7e, f) were observed in all species of *Pilocarpus*, *R. echinata* and *M. flavida*.

Anatomically, the analyzed species showed differences that indicate the importance of leaf anatomy for taxonomic evaluation. For example, the presence of calcium oxalate druses was restricted to the *Pilocarpus* genus, which makes this an important diagnostic character at the genus level. Overall, therefore, the importance of leaf morphoanatomy, as an additional tool for taxonomy, is evident in *Pilocarpus* species, considering the following characteristics: glandular trichome with multicellular uniseriate peduncle and a unicellular apical region (*Pilocarpus* sp.); anomocytic stomata and vascular bundles arranged in an open arc in the midrib (*P. microphyllus*); epidermis in transverse section with adaxial cells > abaxial cells, and papilla-like projections on the epidermal cells of the petiole (*P. giganteus*); epidermal cells with sinuous anticlinal walls in the frontal view of the adaxial surface, and peltate trichomes (*P. alatus*); epidermal cells with sinuous anticlinal walls in the frontal view of the adaxial surface, and glandular trichomes with unicellular peduncle and unicellular apical region (*P. spicatus*); lower secondary veins more obtuse than upper secondary veins (*P. jaborandi*); random

reticulate pattern of the quaternary veins (*P. pauciflorus*), dichotomous branching of the quaternary veins, and absence of nonglandular trichome (*P. riedelianus*).

However, it is important to note that the results observed in the present study are not corroborative of studies in which leaf pattern (simple or compound) is related to such anatomical characters as the number of layers of palisade parenchyma or anticlinal walls of epidermal cells in frontal view.

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