Leaf anatomy of *Celtis iguanaea* (Cannabaceae): a contribution to the classification and taxonomy of the species

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Key words:
- Curaçao
- Rosales
- Taxonomy
- Urticalean rosids

Abstract

Over the last two decades, the circumscription of *Celtis iguanaea* has been questioned due to the difference between the current characterization and others drawn up from specimens native to Curaçao, the type locality. This problem affects the species richness of the genus, as different species are mistakenly identified as *C. iguanaea*. Besides taxonomic challenges, this misinterpretation harms other anatomy, morphology, ontogeny and other study fields. To mitigate this problem, this article presents the first morpho-anatomical description of the leaves of *C. iguanaea*, based on specimens native to Curaçao. Herborized leaves were processed for analysis using light microscopy and scanning electron microscopy. The study identified the presence of multicellular and unicellular trichomes - the first secretory and the last non-secretory, anomocytic-type stomata, large cystoliths of silica on both leaf surfaces with apex projected beyond the epidermal surface, and dorsiventral mesophyll. Mucilaginous cells were widely distributed in the epidermis and in the cortical region of the midrib and petiole. The vascular system had collateral-type bundles. The petiole had a concave-convex shape and a single arch-shaped vascular bundle. These results can help to elucidate taxonomic problems related to *C. iguanaea* delimitation, and its congeners.

Palavras-chave:
- Curaçao
- Rosales
- Taxonomia
- Rosídeas urticóides

Resumo

Nas últimas duas décadas, a circunscrição de *Celtis iguanaea* tem sido questionada devido à diferença entre a caracterização atual e outras elaboradas a partir de espécimes nativos de Curaçao, localidade do tipo. Esse problema afeta a riqueza de *Celtis*, pois diferentes espécies são erroneamente identificadas como *C. iguanaea*. Além do desafio taxonômico, essa interpretação equivocada prejudica outras áreas do conhecimento, como: anatomia, morfologia, ontogenia, entre outras. Para mitigar esse problema, este artigo apresenta a primeira descrição morfo-anatômica das folhas de *C. iguanaea*, a qual foi realizada a partir de espécimes nativos de Curaçao. Folhas herborizadas foram processadas para análise em microscopia de luz e microscopia eletrônica de varredura. O estudo identificou a presença de tricomas multicelulares e unicelulares - o primeiro secretor e o último não secretor, estômatos do tipo anomocítico, grandes cistólitos de sílica em ambas as faces foliares - com ápice projetado além da superfície epidérmica e mesofilo dorsiventral. Células mucilaginosas estão amplamente distribuídas na epiderme e na região cortical da nervura mediana e do pecíolo. O sistema vascular possui feixes do tipo colateral. O pecíolo possui formato côncavo-convexo e um único feixe vascular em forma de arco. A partir destes resultados, espera-se contribuir para a elucidação dos problemas taxonômicos relacionados à *C. iguanaea* delimitação e seus congêneres.

Original Paper

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Introduction

Celtis L. is the most species-rich genus of Cannabaceae, encompassing 64 species with pantropical distribution (Fu *et al*. 2023). *Celtis* can be recognized by a combination of characters: individuals are lianas, shrubs or trees, monoecious; with armed or unarmed branches; estipulate leaves, where alternate, trinervate, abaxial surface is with domatia present or not; inflorescences solitary or in pairs with diclinous (= unisexual) flowers; dialysepal calyx, five sepals; apetala; five stamens opposite to the sepals; pseudomonomerous gynoecium and bicarpellate, uniovulate, with stigma entire or bifurcate; and small drupes with a pyrene (Berg & Dahlberg 2001; Zamengo *et al*. 2020; Leme *et al*. 2021).

Celtis is classified in four subgenera: *C*. subg. *Celtis* Planch. (name invalidly published according to Art. 21.3, 22.2: ICN, Turland *et al*. 2018), *C*. subg. *Mertensia* Planch., *C*. subg. *Solenostigma* (Endl.) Planch., and *C*. subg. *Sponioceltis* Planch (Planchon 1848). To circumscribe the subgenera, Planchon (1848) used the distribution of the species and morphological characters. Thus, *C*. subg. *Celtis* occurs in both the northern and southern hemispheres, *C*. subg. *Mertensia* is endemic to the Neotropical region, *C*. subg. *Solenostigma* to Oceania, and *C*. subg. *Sponioceltis* to the Caribbean. The main morphological characters are the presence (*C*. subg. *Mertensia*) or absence (*C*. subg. *Celtis*, *C*. subg. *Solenostigma* and *C*. subg. *Sponioceltis*) of thorns, and flowers with stigma lobes divided (*C*. subg. *Mertensia* and *C*. subg. *Solenostigma*) or entire (*C*. subg. *Celtis* and *C*. subg. *Sponioceltis*).

Among Neotropical species (*Celtis* subg. *Mertensia*), *Celtis iguanaea* (Jacq.) Sarg. is the most geographically widespread, ranging from Florida, USA to Argentina (Berg & Dahlberg 2001). In their last revision of *C*. subg. *Mertensia*, Berg & Dahlberg (2001) proposed 26 synonyms for *C. iguanaea*, which reduced the previously suggested diversity within the genus (Planchon 1848). The synonymizations by these authors were primarily based on the morphology of thorns (straight or curved), leaf surface texture (scabrous or smooth), leaf indumentum (glabrous or velutinous), and the size and color of the drupes. The proposal by Berg & Dahlberg (2001) has been questioned due to discrepancies in the morphological characterization of *C. iguanaea* compared to others based on native specimens from Curaçao, the type location of the species (Jacquin 1760, 1763, 1788; Lamarck 1789; Planchon 1848; Boldingh 1913).

Recent taxonomic studies have revealed that several species previously considered synonymous with *C. iguanaea* (*sensu* Berg & Dahlberg 2001) are actually distinct species, such as *C. alnifolia* (Wedd.) Miq., *C. spinosa* Spreng., and *C. spinosissima* (Wedd.) Miq. (Chamorro *et al*. 2021; Zamengo *et al*. 2020, 2023). These findings emphasize the need to revise the current classification of *C. iguanaea* (Berg & Dahlberg 2001). Incorrectly identifying specimens as *C. iguanaea* not only poses challenges in taxonomy, but also adversely affects other fields of research. These misidentifications have affected studies in anatomy (Pilati & Souza 2006; Arambarri *et al*. 2011; Barros *et al*. 2023), phylogeny (Fu *et al*. 2023), ontogeny (Leme *et al*. 2020, 2021; Pedersoli *et al*. 2019, 2020; Teixeira *et al*. 2020), and pollination (Arruda & Sazima 1988).

Leaf anatomy studies contributed to the characterization of *Celtis* genus and species (El-Alfy *et al*. 2011a; Thadeo *et al*. 2014; Shahbaz & Sharif 2017; Arogundade & Adedeji 2019; Câmara *et al*. 2020; Batool *et al*. 2024). Therefore, detailed anatomical descriptions of leaf structure with records of collection sites can be conductive to taxonomic and systematic studies of *Celtis*.

The leaf of *Celtis* can be recognized anatomically by single-layered epidermis, dorsivental structure, mesophyll formed by one layer of palisade parenchyma and spongy parenchyma, collenchyma in the midrib and cystoliths (Metcalfe & Chalk 1957; Dottori 1976; Pilati & Souza 2006; Nughes *et al*. 2013). Furthermore, species of the genus can be recognized by presenting secretory or non-secretory trichomes and by the type and distribution of stomata (El-Alfya *et al*. 2011; Shahbaz & Sharif 2017).

Cystoliths are a synapomorphy of the Urticalean rosids clade (Sytsma *et al*. 2001), which is formed by four families: Cannabaceae, Moraceae, Ulmaceae and Urticaceae. Although cystoliths have been recorded in the leaf blade of *Celtis* species (Metcalfe & Chalk 1957; Dottori 1976; Honaine *et al*. 2005; Nughes *et al*. 2013), few studies describe their chemical composition (El-Alfya *et al*. 2011; Honaine *et al*. 2005).

The previously highlighted challenge of accurately distinguishing *Celtis* species (Elias 1970; Hunziker & Dottori 1976; Romanczuk & del Pero de Martinez 1978; Nee 1984; Berg & Dahlberg 2001) is largely due to their cryptic morphological characteristics (Chamorro *et al*. 2021). For instance, after extensive morphological analysis, *Celtis ehrenbegiana* (Klotzsch) Liebm. was found to involve three distinct entities (Asmus *et al*. 2018). Recent studies have emphasized the importance of pyrenes in delineating these species (Hunziker & Dottori 1976; Sattarian & Van Der Maesen 2006; Zarafshar *et al*. 2010; Heo 2020; Zamengo *et al*. 2020; Chamorro *et al*. 2021). While pyrenes are significant for taxonomy, species such as *C. iguanaea* and *C. spinosissima* can be challenging to differentiate because of their similar pyrene profiles (Zamengo *et al*. 2020; Chamorro *et al*. 2021). In such scenarios,

leaf anatomy serves as a valuable tool to distinguish similar taxa (Alves *et al.* 2002; Carvalho *et al*. 2017; Shahbaz & Sharif 2017; Santos *et al*. 2020).

Anatomical studies of Neotropical *Celtis* species are limited, especially with regard to specific variability (Dottori 1990, 1991, 1994; Marchiori & Freitas 1993; Pilati & Souza 2006; Nughes *et al*. 2013; García *et al*. 2016; Pedersoli *et al.* 2019; Nascimento *et al*. 2022; Barros *et al.* 2023). Generally, descriptive studies are related to medicinal species, such as *C. ehrenbergiana* (Arambarri *et al*. 2011) and *C. iguanaea* (Paula *et al.* 2010; Arambarri *et al*. 2011), whereas taxonomical studies are very rare (Maiti *et al*. 2016). Therefore, our work fills a gap by describing anatomical and micromorphological analyses of *C. iguanaea* leaves from Curaçao, identifying characters that aid in the characterization of the species. Furthermore, we compare our results with other anatomical studies of specimens identified as *C. iguanaea*, as well as other *Celtis* species. This contribution holds significant importance for *Celtis* taxonomy by calling for a reassessment of the current classification of *C. iguanaea* (Berg & Dahlberg 2001).

Materials and Methods

Plant material

Dried leaves of *Celtis iguanaea* were obtained from the type locality of the species, from specimens *E.A.T. Houtepen 1* and *E.A.T. Houtepen 2* (RB herbarium numbers 815396, 815397 and barcode numbers 01443256, 01443257), are native to Curaçao in the Caribbean. The leaves were photographed under a stereomicroscope for morphological characterization. Afterwards, five herborized leaves of each voucher were processed for analysis under light microscopy (LM) and scanning electronic microscopy (SEM). The leaves were rehydrated in 1:1 solution of 70% ethanol and pure glycerin (Sosnovsky *et al.* 2017, modified), dehydrated in ethanolic series, and preserved in 70% ethanol to be processed for anatomical analysis.

Anatomical procedures

To conduct the anatomical analysis, the rehydrated leaves were cut in the middle third of the leaf blade, including the intercostal portion, main vein, leaf margin and petiole. The samples were dehydrated in an up to 95% series of ethanol and embedded in glycol-methacrylate (Historesin Leica®) according to the manufacturer's instructions. The transverse sections were sectioned at 6–10 μm thickness in a rotary microtome model RM-2145 (Leica Microsystem, Germany), stained with 0.1% toluidine blue pH 4.4 (O'Brien *et al*. 1964), and mounted with water and coverslip. The epidermis surface was analyzed by dissociation with epidermal peels prepared using Franklin solution (hydrogen peroxide and glacial acetic acid 1:1 v/v; Franklin 1945), stained in 0.25% basic fuchsin dye dissolved in 50% ethanol and mounted on slides in 50% glycerin. Photomicrographs were taken using a MoticamPro 252B digital camera coupled to a Nikon Eclipse Ci light microscope. Leaf cross sections were treated with ruthenium red (Gregory & Baas 1989) and toluidine blue (modified from O'Brien *et al.* 1964) to identify mucilage. In order to identify the composition of the crystals (druses and cystholits), we submitted the sections to 5% sulfuric acid and hydrochloric acid to dissolve calcium oxalate crystals following the methods of Strasburger (1924). However, as this method did not successfully dissolve the cystholits, we used energy dispersive X-ray spectroscopy (EDS) (Oxford Instruments, Model: Xplore) to detect their chemical composition.

For SEM analysis, samples were dried in stove apparatus, mounted on a metal support with carbon adhesive tape, and coated with gold for 360 s using a Denton Vacuum Desk III instrument (Moorestown, Nova Jersey, EUA). Electron micrographs were obtained with a JEOL JSM - 6380 Scanning Electron Microscope (SEM) operated at an accelerated voltage of 10 kV.

Results

Leaf blade

Young leaves of *Celtis iguanaea* possessed toothed margin and a midrib with inconspicuous domatia (Fig. 1a,b), while mature leaves had entire margin and visible domatia (Fig. 1c,d).

Anatomically, *Celtis iguanaea* leaves were covered by a single-layered epidermis composed of common epidermal cells, stomata, secretory and non-secretory trichomes, and crystal idioblasts (Figs. 2; 3). In the frontal view, the common epidermal cells had straight or curved anticlinal cell wall on both surfaces (Fig. 2a-d). In the midrib region, the common epidermal cells had straight anticlinal walls, with an elongated shape (Fig. 2a-b).

Anomocytic stomata type occurs on both leaf surfaces in different distributions (Fig. 2c-e). On the adaxial surface the stomata occurred near the midrib region and on secondary veins, whereas on the abaxial side, they were distributed throughout the surface (Fig. 2b,d). In cross-section, the epidermis was covered by a thin cuticle layer and showed mucilaginous cells (Fig. 2f).

Two types of trichomes were observed: unicellular non-secretory trichomes, and pluricellular secretory trichomes (Fig. 3a-e). The length of the non-secretory trichomes varied from short and spine-like to long and filiform, both having strongly thickened cell walls (Fig.

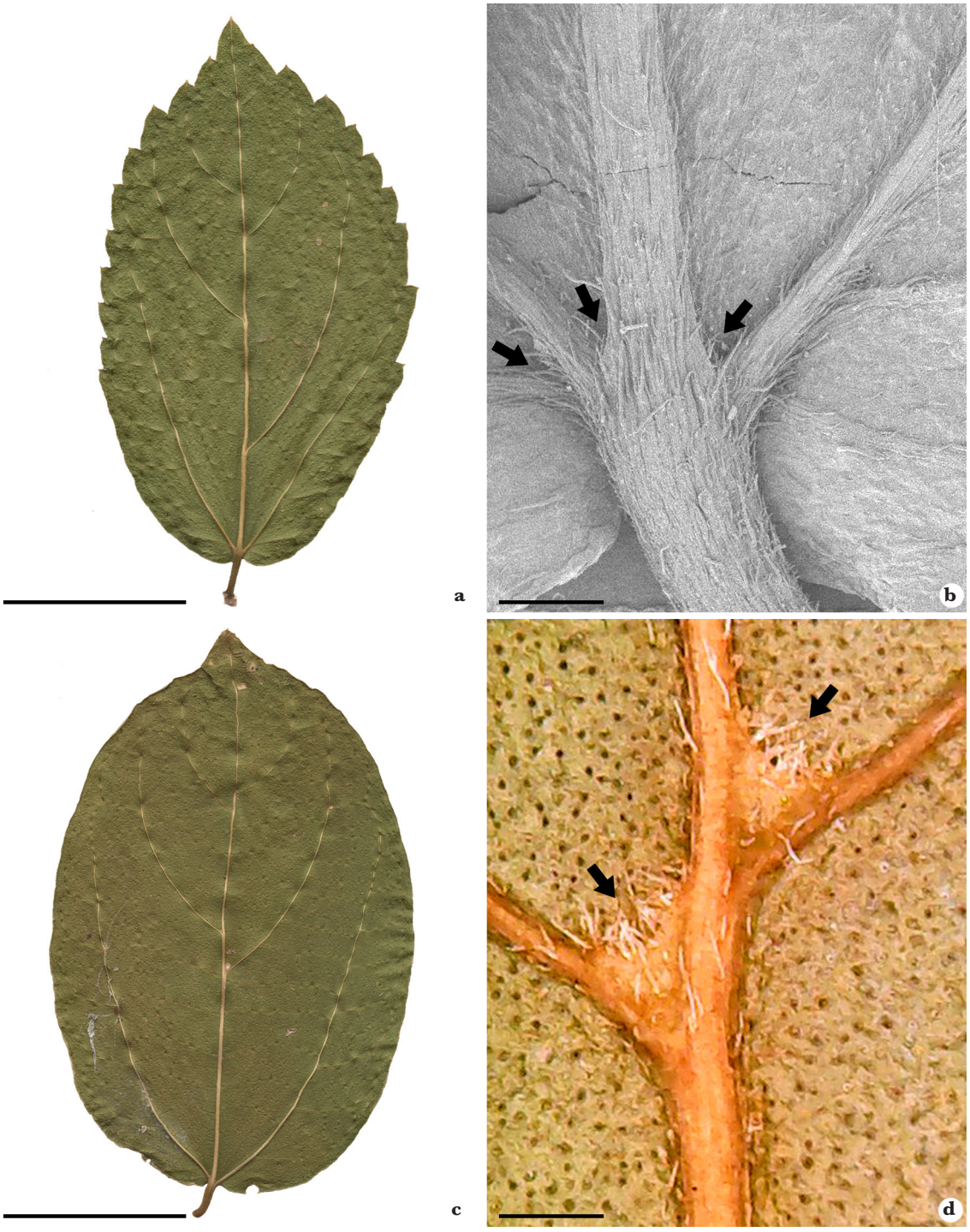

Figure 1 – a-d. Leaves and domatias in the abaxial face of *Celtis iguanaea* (Cannabaceae) – a. young leaf, abaxial surface; b. base of the young leaf, inconspicuous domatias (black arrow) (SEM); c. mature leaf, abaxial surface; d. domatias of mature leaf (black arrow) (photograph). Scale bars: a = 1 cm; b, d = 1 mm; c = 3 cm.

3a-c). The multicellular secretory trichomes were of the capitate type, composed of a uniseriate peduncle consisting of 2–5 tabular cells, with an elongated multicellular secretory head (Fig. 3d,e). The secretory trichomes generally occurred on the abaxial surface in the region covering the midrib (Fig. 3a).

Lithocysts were detected in the epidermis on both leaf surfaces containing a large cystolith of approximately circular-to-elongate form, with a rough surface (Figs. 2f; 4; 5a-d). The cystoliths formed a tiny apex projecting towards the leaf surface and forming a small protrusion visible in frontal view (Fig. 3f). Traditionally used histochemical tests performed with acids did not result in the dissolution of these structures, indicating that they were not made of calcium carbonate or calcium oxalate. Next, EDS analyses were performed, identifying high silica concentration in the cystoliths (Fig. 4a-h), visible in the graph (Fig. 4b), as well as by image (Fig. 4c). In addition to silica, carbon, oxygen and calcium were also identified in significant quantities (Fig. 4b,d-g). The high concentration of silica and oxygen indicates that the main component of the cystolith is silicon dioxide, marked in blue-green on the EDS image (Fig. 4h). The presence of gold originated from the coating of the Denton Vacuum Desk III instrument.

The mesophyll of *C. iguanaea* was dorsiventral (Figs. 2f; 5a-c). The palisade parenchyma was composed of one layer of elongated cells, interrupted by lithocysts (Figs. 2f; 5a-c). The spongy parenchyma presented up to four cell layers with rare idioblasts containing druse crystals (Figs. 2f; 5a-c). The vascular system consisted of collateral vascular bundles (Fig. 2f). The leaf margin flexed downwards, and the epidermal cells were round in the cross section, and more voluminous compared to the others, while also storing mucilage (Fig. 5b).

In the midrib the cortical region of the was formed by angular collenchyma underlying the epidermis, consisting of 3–5 cell layers restricted to the abaxial face (Fig. 5d). Under the adaxial epidermis, the annular collenchyma was restricted to a small central region (Fig. 5d). The space between the collenchyma and the main vascular bundle was filled by six layers of regular parenchyma formed by voluminous polygonal cells. Mucilaginous cells and idioblasts with druse crystals were detected in this region, mainly in the abaxial side (Fig. 5d). The vascular system consisted of a single collateral vascular bundle with a flat concave shape surrounded by small groups of fibers in the abaxial side (Fig. 5d).

Petiole

The adaxial surface of the petiole was concave (Fig. 5e). The epidermis was single-layered, with few stomata on the adaxial surface. The cortical region was occupied by four to six layers of angular collenchyma, and internally by six layers of regular parenchyma. Mucilaginous cells and idioblasts with druse crystals were observed in the cortical region, dispersed around of the vascular system (Fig. 5e). The vascular system was composed of a single collateral vascular bundle, with a semi-circular shape and ends facing the center (Fig. 5e).

Discussion

Our study revealed distinctive features of the leaves of *Celtis* species, including single-layered epidermis with stomata predominantly on the abaxial surface, lithocysts, unicellular and multicellular trichomes, and dorsiventral mesophyll. Some characters of *C. iguanaea* leaves diverged from previously published descriptions, suggesting that leaf anatomy could contribute to a more accurate identification of specimens of this species.

The leaves of *Celtis* species have a single-layered epidermis, composed of common epidermal cells with a variable, straight-to-sinuous contour (Metcalfe & Chalk 1950; El-Alfy *et al*. 2011b; García *et al*. 2016). For some taxa, the contour of the epidermal cell wall may vary according to the growth conditions of the plant, as observed in *C. ehrenbergiana* (*sensu* Berg & Dahlberg 2001), which presents cells with sinuous walls when exposed to sunlight, compared to those that have epidermal cells with straight walls (Nughes *et al*. 2013). The influence of light conditions on cell contour needs to be further studied to confirm the potential use of this attribute in taxonomic studies. In the leaves of *C. iguanaea* evaluated here, we observed a predominance of straight-to-curved anticlinal walls, corroborating the variations cited for the genus.

The epidermis leaves of *C. iguanaea* from Curaçao is amphistomatic and have a single-layered epidermis as cited by studies describing specimens from other localities (Pilati & Souza 2006; Paula *et al*. 2010). Although stomata occur on both surfaces, on the adaxial surface the stomates are restricted to the midrib. Amphistomatic leaves, with more stomata on the abaxial side, have been described for several species of *Celtis* (García *et al*. 2016; Arambarri *et al*. 2011; Maiti *et al*. 2016). The presence of stomata only on the abaxial surface of the leaf blade is reported for *C. australis* (El Alfi *et al*. 2011). The most frequently described stomata types are paracytic, anisocytic and anomocytic (Metcalfe & Chalk 1950; El Alfy *et al*. 2011; García *et al*. 2016; Maiti *et al*. 2016). In our samples of *C. iguanaea* the anomocytic type was observed.

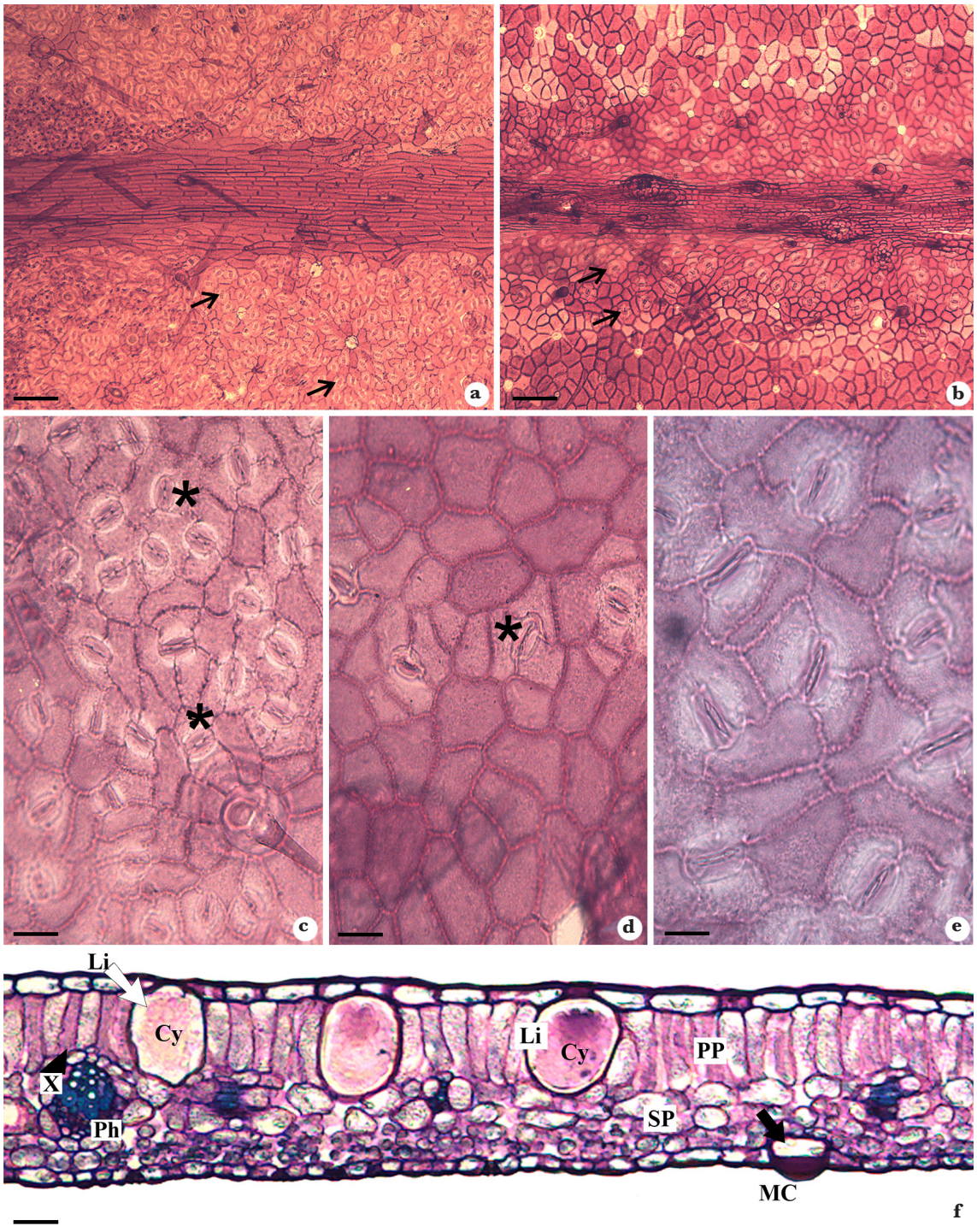

Figure 2 – a-f. Epidermis of *Celtis iguanaea* (Cannabaceae) under light microscopy – a. epidermis on the abaxial face, stomata in all surfaces (black arrow); b. epidermis on the adaxial surface, stomata near of the midrib (black arrow); c. note the stomata (*) scattered throughout the epidermis on the abaxial surface; d. few stomata (*) in the epidermis of the adaxial surface; e. anomocytic stomata; f. cross section of the leaf. Note epidermis with lithocysts (white arrow) and mucilaginous cell (black arrow); collateral vascular bundles with phloem and xylem. Symbols: Cy = cystolith; Li = lithocyst; MC = mucilaginous cell; Ph = phloem; PP = palisade parenchyma; SP = spongy parenchyma; X = xylem. Scale bars: a, b = 100 μm; c, d, e, f = 10 μm.

The leaf anatomy of *Celtis* species can be very similar. Nevertheless, certain characters, particularly trichomes, are useful to differentiate these species. Non-secretory trichomes are the most frequently described for *Celtis* species and they can be micropapilate or smooth (Tobe & Takaso 1996). Non-secretory trichomes in *C. iguanaea* are unicellular and smooth, and may be short and prickle-like or long (as in the present study), whereas the trichomes of "*C. iguanaea*" from Goiás (Brazil) have unicellular and pluricellular non-secretory trichomes (Paula *et al*. 2010). Furthermore, Paula *et al*. (2010) did not observe secretory trichomes. In fact, the specimen analyzed by Paula *et al*. (2010) was different from the *C. iguanaea* from Curaçao.

Although cystolytic trichomes (*i.e.*, trichomes presenting cystoliths at the base) often occur in Cannabaceae and are frequently used in forensic examinations of *Cannabis sativa* L. (Dayanandan & Kaufman 1976) or *Celtis* species (Gangadhara & Inamdar 1977; Maiti *et al*. 2011; Shahbaz & Sharif 2017), we did not observe this trichome type in *C. iguanaea* in the present study.

In a revision of secretory trichomes in Urticalean species, Nascimento *et al*. (2022) cite two types of secretory trichomes for *Celtis* species: 1) filiform long (uniseriate and more than four cells long) for *Celtis* sp., *C. pubescens* Spreng. (*sensu* Torres & Luca 2005), *C. boninensis* Koidz., *C. sinensis* Pers., and *C. spinosa* Spreng. (*sensu* Torres & Luca 2005), and 2) capitate (uniseriate stalk and pluricellular head) (*C. pubescens sensu* Torres & Luca 2005). However, in our samples of *C. iguanaea*, we found only one type of secretory trichomes, *i.e.*, *C. iguanaea* did not show the trichome secretor filiform described by Nascimento *et al*. (2022). Our samples of *Celtis iguanaea* presented capitate secretory

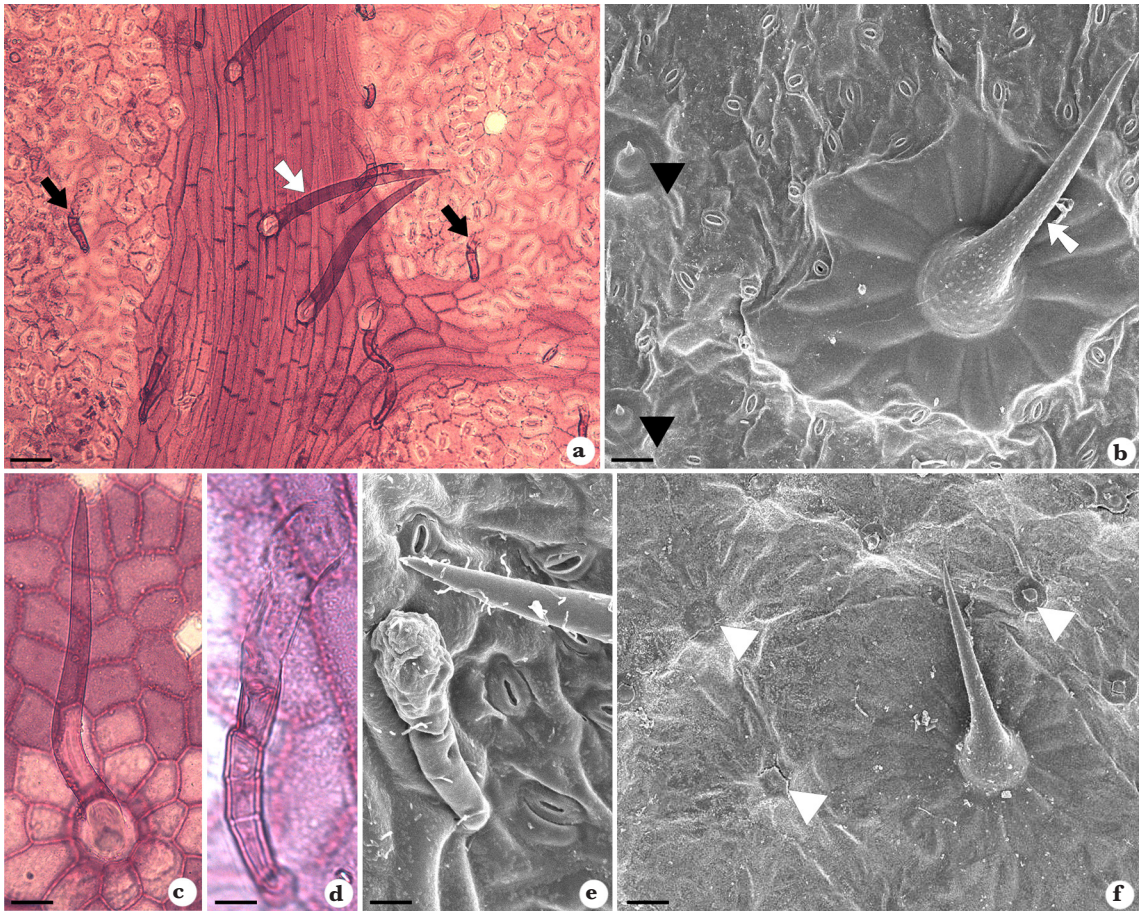

Figure 3 – a-f. Trichomes and cystolith protrusion of *Celtis iguanaea* (Cannabaceae) in light microscopy (a, c, d) and scanning electronic microscopy (b, e, f) – a. midrib with non-secretory (white arrow) and secretory trichomes (black arrow) on the abaxial surface of the leaf; b. non-secretory trichomes short (black arrowhead) and long (white arrow); c. non-secretory trichomes long; d-e. secretory trichomes; f. protrusion of the cystolith of the lithocyst (white arrowhead). Scale bars: a = 100 μm; b, d, e, f = 10 μm; c = 50 μm.

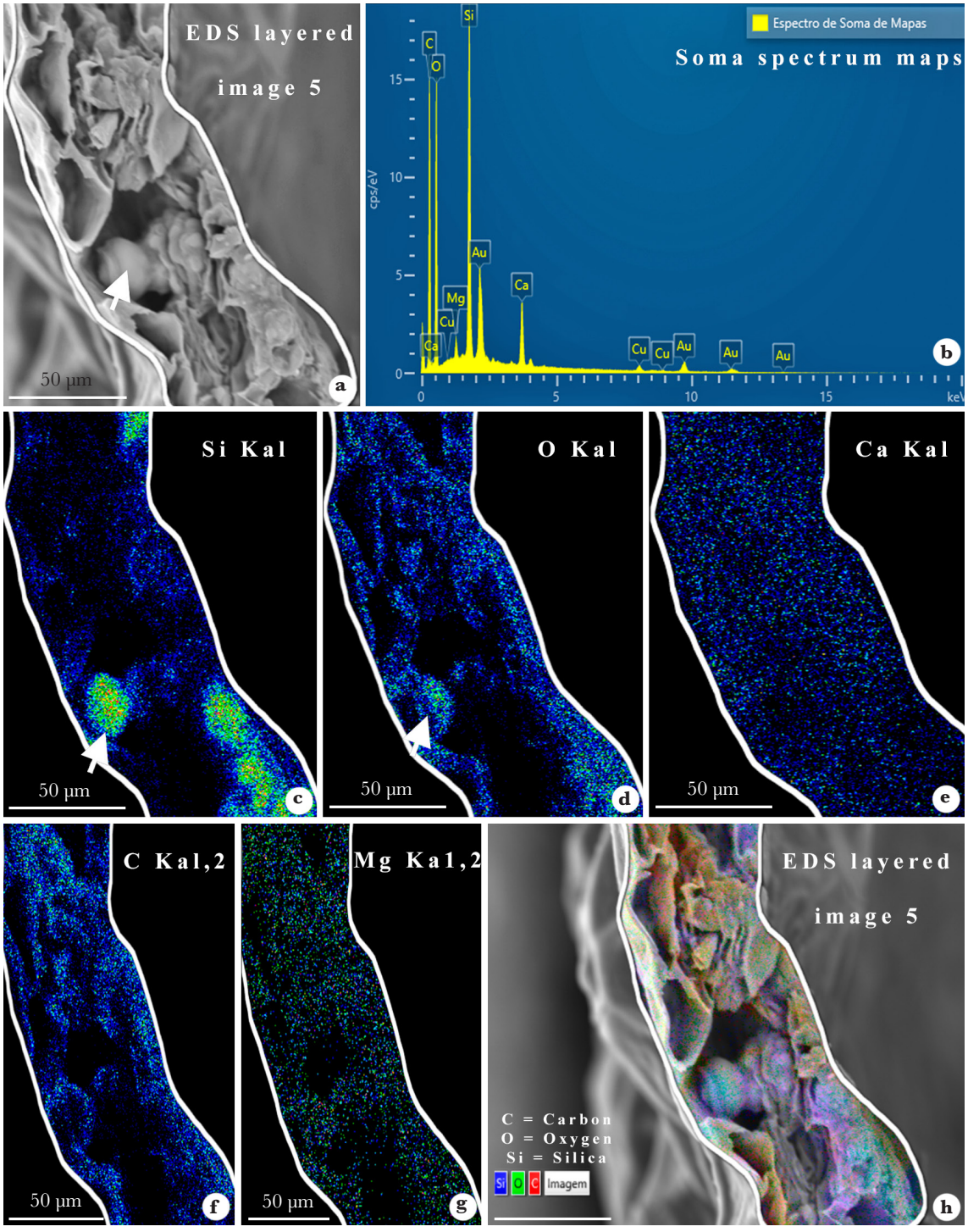

Figure 4 – a-h. EDS analysis – a. cross section of *Celtis iguanaea* leaf in SEM, the cystolith indicated by the arrow; b. graph of the main chemical components identified in the leaf; c. detection of silica in green in the leaf; d. detection of oxygen in green; e-g. detection of Ca, C and Mg in green, note the uniform distribution of these components throughout the mesophyll; h. EDS image in layered showing the presence of O, Si and C. Symbols: Ca = calcium; C = carbon; O = oxygen; Si = silica; Mg = magnesium; Cu = copper; Au = gold.

trichomes with uniseriate stalk and multicellular head, similar to those described in *C. pubescens* (Nascimento *et al*. 2022). Other studies describing the leaf anatomy of *C. iguanaea* did not cite secretory trichomes neither in seedlings (Pilati & Souza 2006), nor in leaves (Paula *et al*. 2010).

Lithocysts are epidermal idioblasts that usually contain calcium carbonate or silica and store calcium or carbon dioxide (Honaine *et al*. 2023). The presence of cystoliths in leaves appears to be a condition shared by the entire Urticalean clade (Sytsma *et al*. 2002). Cystoliths may vary in location, frequency, and dimensions in the leaf epidermis, on one or both sides of the leaf, and these variations can be used to distinguish some species. In a comparative study, Shahbaz &

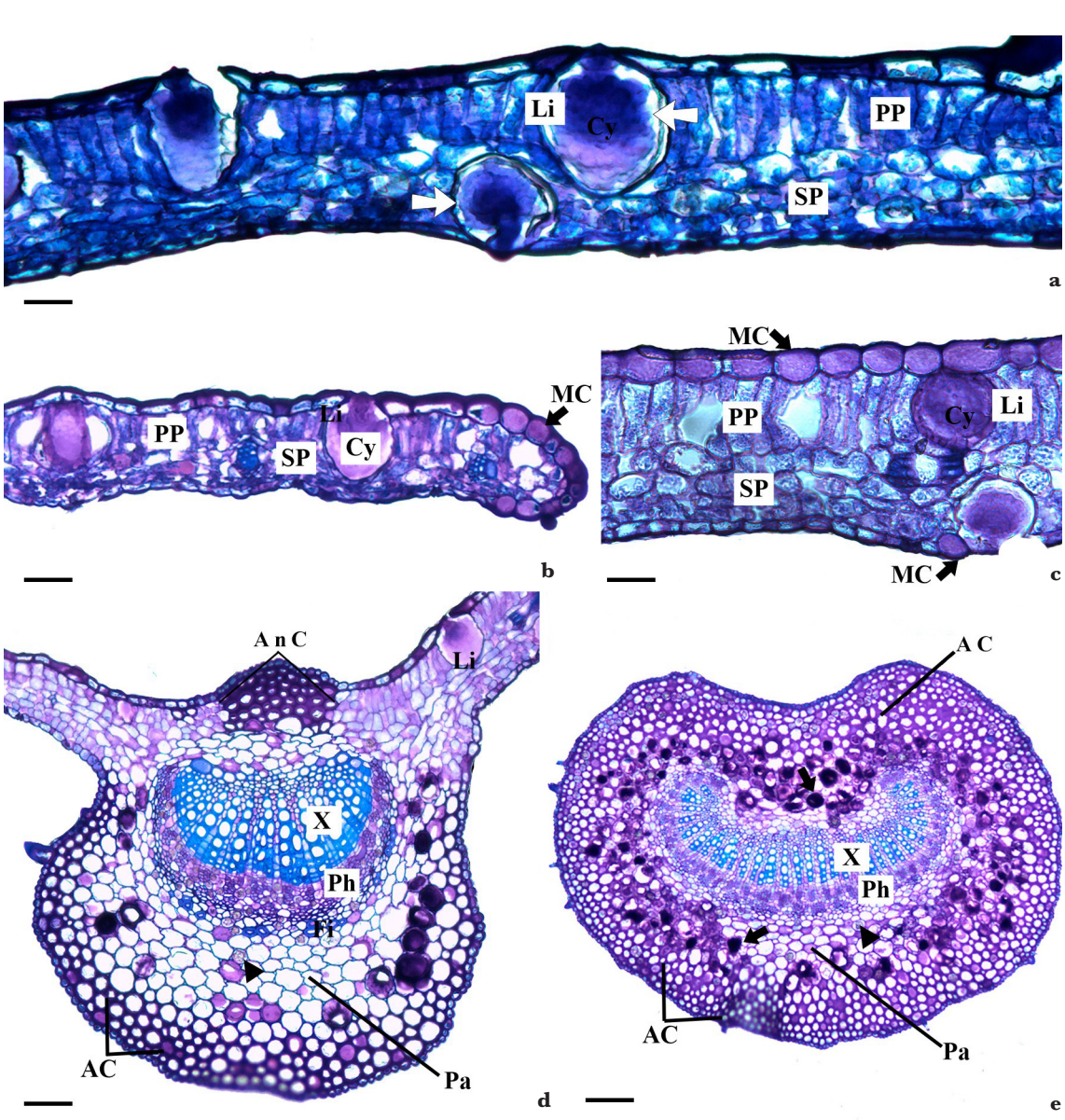

Figure 5 – a-e. Anatomy of the leaf blade of *Celtis iguanaea* (Cannabaceae) under light microscope – a. dorsiventral mesophyll. Note the lithocyst on both face (white arrow); b. leaf edge with mucilaginous cells in the epidermis (black arrow); c. mucilaginous cells (black arrow); d. midrib of the leaf. Note angular collenchyma in the cortical region and annular collenchyma restricted to the central region below adaxial epidermis; e. petiole. Symbols: AC = angular collenchyma; AnC = annular collenchyma; Cy = cystolith; Dr = druses; Fi = fiber; Li = lithocyst; MC = mucilaginous cells; Ph = phloem; PP = palisade parenchyma; SP = spongy parenchyma; X = xylem. Scale bars: a = 50 μm; b = 100 μm; c, e = 10 μm; d = 100 μm.

Sharif (2017) identified smaller and less frequent lithocysts in *C. tournefortii* Lam. compared to *C. australis*. For *C. occidentalis* L., silica cystoliths have been reported, occurring predominantly on the adaxial surface (El-Alfy *et al*. 2011a, 2011b). In the samples of *C. iguanaea* evaluated here, cystoliths were observed on both sides of the leaf, forming tiny projections towards the adaxial surface as seen in the frontal view (Fig. 3f), possibly constituting an additional anatomical taxonomic character for *C. iguanaea*.

Celtis iguanaea has lithocysts on both leaf surfaces, another characteristic that differs from the specimen studied by Paula *et al*. (2010), which possessed lithocysts only on the adaxial leaf side. Cystoliths vary in location, frequency, and dimensions in the leaf epidermis and can be observed on one or both sides of the leaf. These variations could represent additional taxonomic information. In a comparative study, Shahbaz & Sharif (2017) found that lithocysts in *C. tournefortii* were smaller and less frequent than in those in *C. australis*. Pilati & Souza (2006) described cystoliths on both sides of *C. iguanaea* seedling leaves (eophyll).

Cystolith may be composed primarily of calcium carbonate (Metcalfe 1985; Watt *et al.* 1987), soluble with weak acidic solution, but the presence of silica as silicon dioxide or calcium silicate has also been found forming the phytolith, or at least the initial stalk-like protrusion that connects the cystolith to the outer cell wall (Metcalfe 1985; Watt *et al.* 1987, Gal *et al.* 2012). Silica was also identified in the cystoliths of *C. tala* Gillies *ex* Planch. (Honaine *et al.* 2005), *C. australis* and *C. occidentalis* (El-Alfya *et al*. 2011). The chemical composition was evaluated by energy-dispersive X-ray spectroscopy (EDS or EDX), confirming silica as the main component of the photoliths observed in the large epidermal cells in *C. iguanaea* (this study).

While *C. iguanaea* and other specimens native to South America differed in some characters, they had similar dorsiventral leaf blades with a layer of palisade parenchyma, idioblasts with druse, lithocysts and three layers of spongy parenchyma - as described from specimens from the Brazilian states of Goiás, Mato Grosso do Sul and Paraná (Pilati & Souza 2006; Paula *et al*. 2010). The vascular bundle parenchyma sheath of *C. iguanaea* has also been described for specimens from Argentina (Arambarri *et al*. 2011). Dorsiventral mesophyll and a vascular system consisting of collateral vascular bundles are features cited for the genus (Metcalfe & Chalk 1950; García *et al*. 2016; Maiti *et al*. 2016).

The midrib of *Celtis iguanaea* leaves is biconcave, with a vascular bundle surrounded by cortical parenchyma with druse crystals, collateral vascular bundle in the convex plane shape, phloem with druses and a strip of fibers facing the abaxial part. Similar characteristics have been observed in the specimens studied by Paula *et al*. (2010).

Regarding the leaf margin, Paula *et al*. (2010) described the specimens from Goiás state (Brazil) as having similar morphology, with small vascular bundles surrounded by regions of collenchyma. However, in the samples evaluated in the present study, the leaves present margins flexed towards the abaxial region, and vascular bundles surrounded by a parenchyma sheath.

Similar to the specimens studied by Paula *et al*. (2010), *Celtis iguanaea* has petioles with a concave-convex outline, uniseriate epidermis, a cortical region with angular collenchyma and crystalliferous idioblasts containing forming druses. Despite these similarities, the samples of *C. iguanaea* evaluated in the present study have stomata and mucilage idioblasts dispersed both in the cortical region and the vascular system, characteristics not mentioned previously (Paula *et al*. 2010).

Even though the *Celtis iguanaea* specimen from Curaçao has anatomical similarities with other species native to South America (Pilati & Souza 2006; Paula *et al*. 2010; Arambarri *et al*. 2011; Bento 2019; Nascimento *et al*. 2022), significant differences were observed, as presence of only a type of secretory trichome, absence of filiform uniseriate secretory trichome and cystoliths with protrusions. Our results support recently raised questions (Chamorro *et al*. 2021; Zamengo *et al*. 2020, 2023b) about the current circumscription of *C. iguanaea* (Berg & Dahlberg 2001), and we emphasize the need for a new revision of this species. Our work could also support correct identification of other *Celtis* species. From the comparative analysis of leaf anatomy with other species, we were able to highlight some characteristics that we consider relevant for the recognition of *C. iguanaea*: common epidermal cells with straight-to-curved walls, anomocytic stomata; amphistomatic leaves; stomata on the adaxial surface of the leaf blade distributed exclusively in the regions over veins; lithocysts containing silica cystoliths with circular-to-elongate shape, rough surface, occupying up to half the thickness of the leaf; secretory trichomes of the capitate type, with pluricellular uniseriate peduncle, and multiseriate head; spine-shaped to filiform non-secretory trichomes ; mucilaginous idioblasts in the epidermis, mesophyll, and cortical region of the midrib and petiole.

Although many specimens of *Celtis* have been identified as *C. iguanaea* in several regions of Brazil, these specimens probably refer to other species of *Celtis*, such as *C. alnifolia* (Wedd.) Miq., *C. clausseniana* (Wedd.) Miq., *C. serratissima* Zamengo, Torres, Gaglioti & Romaniuc and *C.*

spinosissima (Wedd.) Miq. (Zamengo *et al*. 2020). We emphasize the importance of further studies of the leaf anatomy of other, mainly Neotropical *Celtis* species, focusing on trichome types, cystolith shape and composition, stomata types and distribution, and presence of mucilage cells to support the correct taxonomic classification of several species synonymized by Berg & Dahlberg (2001).

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Data Availability Statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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