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Comparative Leaf Anatomy of *Guettarda* Species (Rubiaceae)

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HIGHLIGHTS

- First anatomical data for *G. uruguensis* and *G. pohliana*.
- Identifies four trichome types, emphasizing their distribution and morphology in each species.
- Phenolic compounds and druse crystals were observed.
- The results contribute to the structural knowledge of the genus *Guettarda*.

Abstract: This study presents a comparative anatomical analysis of three *Guettarda* species - *Guettarda pohliana*, *Guettarda uruguensis*, and *Guettarda viburnoides* - commonly known as the "veludinho" group due to their velvety leaves. As members of the Rubiaceae family, these species are of taxonomic and pharmacognostic interest. The study focuses on the leaf anatomy, identifying four distinct trichome types and describing their distribution and structure. In particular, differences in stomatal positioning, the presence of druses, and sheath extensions were noted. *G. pohliana* and *G. uruguensis* exhibited a biconvex midrib shape, while *G. viburnoides* presented a plano-convex shape. Phenolic compounds and crystals were identified, reinforcing their taxonomic importance within the *Guettardeae* tribe. This research provides the first anatomical data for *G. uruguensis* and *G. pohliana*. These results contribute to the anatomical characterization of the *Guettarda* genus, expanding the current knowledge of these species.

Keywords: leaf anatomy; trichome; *Guettarda*; Rubiaceae; Brazilian flora.

INTRODUCTION

The Rubiaceae family, comprising approximately 650 genera and 13.000 species, is the fourth-largest among angiosperms [1]. This cosmopolitan family is particularly abundant in tropical regions, especially within the Atlantic Forest [2,3]. Members of this family are characterized by opposite leaves and interpetiolar stipules [4,5], and many species hold significant medicinal and economic value. For instance, *Coffea arabica* L. is

widely known for its caffeine content [6], while *Uncaria tomentosa* (Willd.) DC. (cat's claw) contains quinic acid alkaloids and glycosides with immunomodulatory and anti-inflammatory properties [7]. The leaf anatomy of the Rubiaceae family is marked by consistent diagnostic features, including opposite phyllotaxy, interpetiolar stipules, dorsiventral mesophyll, and paracytic (rubiceous) stomata restricted to the abaxial surface. Although anatomical data are still limited for the genus, species of *Guettarda* studied so far commonly exhibit uniseriate epidermis, calcium oxalate crystals in the form of druses, and various non-glandular trichomes. Anatomical studies have also reported the presence of bundle sheath extensions and idioblasts containing phenolic compounds and crystals [8,9,10].

Within the Rubiaceae family, the genus *Guettarda* L. belongs to the subfamily Cinchonoideae and the monophyletic tribe Guettardeae [12], comprising approximately 160 species [13]. *Guettarda uruguensis* Cham. & Schltdl., *Guettarda viburnoides* Cham. & Schltdl., are native to South America and exhibit distinct geographic distributions. *G. uruguensis* is primarily distributed in southern and southeastern Brazil, Uruguay, and northeastern Argentina, typically inhabiting dense ombrophilous forests of the Atlantic domain. *Guettarda pohliana* Müll. Arg. has a more limited range, occurring mainly in southeastern Brazil, particularly in remnants of the Atlantic Forest. In contrast, *G. viburnoides* is one of the most widespread species of the genus, found across multiple Brazilian biomes, including the Atlantic Forest, Cerrado, Caatinga, and reported in Bolivia and Paraguay. These species occur in various environments, ranging from mature forests to secondary vegetation and forest edges [14,15,16].

Species within the *Guettarda* genus are used in traditional medicine [17,18,19] and exhibit various biological activities [20,21]. Regarding the species analyzed in this study, research indicates that *G. uruguensis* [22], *G. viburnoides* [23,24], and *G. pohliana* [25] possess anti-inflammatory properties, while *G. uruguensis* [26] and *G. viburnoides* [23, 24] exhibit antioxidant activity. Antimicrobial effects have been reported for *G. uruguensis* [27] and *G. viburnoides* [28], whereas cytotoxic activity has been observed in *G. uruguensis* [26] and *G. pohliana* [29]. To evaluate the safety of these species, in vivo toxicological studies were conducted using mice treated with *G. uruguensis* [22] and *G. viburnoides* [11]. The results indicated no changes in toxicity markers, suggesting safety in the tested models and doses.

In this study, we performed a comparative leaf anatomical analysis of *G. pohliana*, *G. uruguensis*, and *G. viburnoides*, commonly known as "veludinho". This popular name is likely associated with their epidermal characteristics, as their leaves exhibit dense pubescence, giving them a velvety texture. During the fruiting season, these species can be easily distinguished based on their fruit color, among other characteristics: *G. pohliana* produces red fruits, *G. uruguensis* bears purple fruits, and *G. viburnoides* has white fruits. The fruits of *G. uruguensis* are edible and can be consumed fresh or used in liqueur production. This species is classified as an unconventional food plant [30,31,32].

Although *G. viburnoides* has been previously studied morpho-anatomically [10,11], no such studies are available for *G. uruguensis* and *G. pohliana*. In *G. viburnoides*, leaf anatomy includes sinuous epidermal walls, druses and prismatic crystals, non-glandular trichomes with crystals, and secondary metabolites [11]. This study aims to identify and describe the anatomical characteristics of these species, providing the first leaf anatomical data for *G. uruguensis* and *G. pohliana*, contributing to a better understanding of the *Guettarda* genus.

MATERIAL AND METHODS

Plant material

Guettarda pohliana and *G. viburnoides* were collected in January 2013 in Campina do Monte Alegre–SP (23°32'09"S, 48°30'44"W), and *G. uruguensis* in February 2013 in Curitiba–PR, at Parque Municipal do Iguaçu (25°32'13"S, 49°13'33"W). *G. pohliana* and *G. uruguensis* are predominantly shrubs, while *G. viburnoides* may occur as a shrub or small tree [14,31]. The three species have opposite, pubescent leaves and tubular flowers, typical of the genus [4], and grow in ombrophilous forest and secondary vegetation of the Atlantic Forest biome [14]. Three adult individuals were sampled for each species, and three fully expanded leaves per individual were collected from the third to fifth node. Specimens were identified by a botanist from the Municipal Botanical Museum of Curitiba and deposited as exsiccates: *G. pohliana* (MBM297775), *G. uruguensis* (MBM386376), and *G. viburnoides* (MBM287987). Access to genetic material was authorized under registration 02001.00165/2013-47 by the Genetic Heritage Management Council (CGEN/MMA).

Anatomical studies

The samples were fixed in formalin-acetic acid-alcohol (FAA) solution [33] and stored in 70% ethanol until sectioning or processing for semi-permanent slides. Leaf sections were cut in the transverse direction. Semi-permanent slides were prepared from samples embedded in polyethylene glycol 1500 U.S.P. (PEG). For PEG inclusion, the samples were pre-infiltrated in a solution of PEG and 70% ethyl alcohol in a 1:1 ratio, in an oven at 56 °C for 12 hours, followed by infiltration with a pure PEG solution for 4 hours. Sections were cut with a thickness of 35 µm using a rotary microtome with a steel blade. The slides were stained with toluidine blue [34] or double-stained with basic fuchsin and Astra blue [35]. The sections were then mounted in glycerin [36], and sealed with colorless enamel. Histochemical tests with Sudan III [37] and ferric chloride [33] were employed to identify lipophilic and phenolic compounds. Photomicrographs were obtained using a microscope with an attached digital camera and image capture software for ultrastructural surface analysis using scanning electron microscopy [38]. The fixed samples underwent increasing ethanolic dehydration (80%, 90%, and 100%), with transfers occurring every ten minutes. These dehydrated samples were dried (Bal-Tec CPD-030). Subsequently, the samples were mounted on aluminum stubs using copper tape, gold-coated in the Balzers SCD-030 sputter coater, and observed and photographed using the JEOL JSM-6360LV scanning electron microscope.

RESULTS

Guettarda pohliana, *G. uruguensis*, and *G. viburnoides* exhibit morphological and anatomical characteristics consistent with the Rubiaceae family and the *Guettarda* genus. However, some characters may be considered relevant for identifying individual species.

In cross-section, the lamina exhibits a single-layered epidermis in all three studied *Guettarda* species (Figures 1a, 1b, and 1c). The cuticle is thick on the adaxial surface and thin on the abaxial surface (Figure 1d). Stomata are present on the abaxial surface (Figures 1e, 1f, and 1g), classifying the laminae of all three species as hypostomatic. They are classified as paracytic or rubiaceous. Stomata are located at the same level as the epidermal cells in *G. pohliana* and *G. uruguensis* (Figures 1a, 1b, 1e, and 1f). In *G. viburnoides* (Figures 1f, 1g), stomata project above the level of epidermal cells. The mesophyll exhibits a dorsiventral organization in all three species, with 1-2 layers of palisade parenchyma toward the adaxial surface and a reduced, poorly stratified spongy parenchyma on the abaxial side (Figures 1a–c). Phenolic compounds are detected in the mesophyll, especially in the palisade parenchyma region in all *Guettarda* species, as shown in *G. viburnoides* (Figure 1g). Along the mesophyll are small collateral vascular bundles surrounded by fibers with thick and non-lignified walls (Figures 1a–c). In *G. pohliana*, idioblasts containing druses are distributed in the palisade parenchyma (Figure 1a). All three species exhibited bundle sheath extensions.

The trichomes observed in *Guettarda* species exhibit four distinct morphological types. Type 1 trichomes are non-glandular (tector), apparently unicellular, long, and inclined on the surface, with thickened walls and internal crystals. They are present in all three species (Figures 1h, 1s). Type 2 trichomes are multicellular, erect, and long, with thinner walls that may present smooth or slightly ornamented surfaces; they also occur in all species (Figures 1j, 1r). Type 3 trichomes are smaller, non-glandular, erect, pointed, and thin-walled; they are present across all species analyzed (Figures 1k, 1l). Type 4 trichomes are apparently unicellular, inclined, with a rounded apex and thin walls, and were observed exclusively in *G. viburnoides* (Figure 1f). Trichomes occur on both adaxial and abaxial leaf surfaces, with a higher density on the abaxial surface (Figures 1m–1p); among the species analyzed, *G. viburnoides* exhibits the most dense indumentum (Figure 1q).

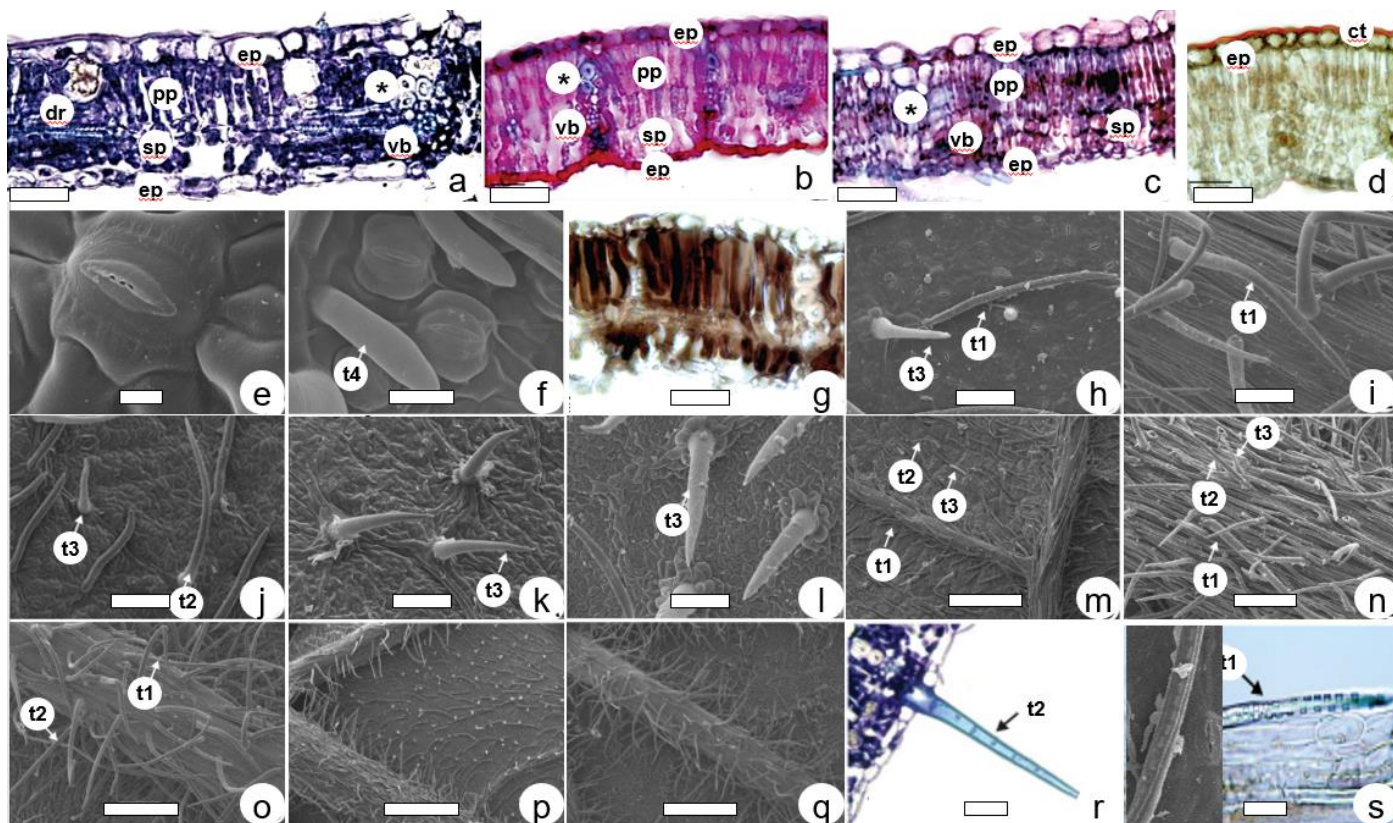


Figure 1. Leaf anatomy of *Guettarda* species. *G. pohliana* (a, h, n, p, s), *G. uruguensis* (b, d, e, j, k, m), *G. viburnoides* (c, f, g, i, l, o, q, r). In cross-section (a-d, g, r, s). In frontal view (all others). Normal light microscopy (a-d, g, r, s). Scanning electron microscopy (e, f-q). Staining with toluidine blue (a, r), staining with safranin and astra blue (b, c), test with Sudan III (d), and with ferric chloride (g). Adaxial leaf surface (k, l), abaxial leaf surface (all others). Legends: ep: epidermis; pp: palisade parenchyma; sp: spongy parenchyma; dr: druse; *: sheath extension; vb: vascular bundle; (t1): type 1 trichome; (t2): type 2 trichome; (t3): type 3 trichome; (t4): type 4 trichome. Scale bars a, b, c, g, h, i, j, k, l, r, s = 50 μ m; e = 5 μ m; f = 10 μ m; m, n = 100 μ m; o = 200 μ m; p, q = 500 μ m.

The midrib, in cross-section, shows different shapes. In *G. pohliana* (Figure 2a) and *G. uruguensis* (Figure 2b), the midrib is biconvex, with an adaxial surface prominent and tapered in *G. uruguensis*. *G. viburnoides* shows a plano-convex shape (Figure 2c). In *G. pohliana* (Figure 2d) and *G. viburnoides* (Figure 2f), the collenchyma is annular, whereas in *G. uruguensis* (Figure 2e) it is angular. The remaining fundamental tissue is parenchymatous with druses in all three species (Figure 2h). Phenolic compounds showed a positive reaction to the ferric chloride solution, observed in the midrib in the epidermis, cortex, and phloem (Figures 2h, 2i). The vascular system is represented by a horseshoe-shaped vascular bundle (Figures 2a, 2b, and 2c). Small vascular bundles occur in the medullary region, similar to *G. pohliana* (Figure 2a) and *G. viburnoides* (Figure 2c). In *G. uruguensis*, these bundles are condensed (Figure 2b). For *G. pohliana*, a vascular system surrounded by fibers was observed, with thick and non-lignified walls adjacent to the phloem (Figure 2j). All three species, *G. pohliana*, *G. uruguensis*, and *G. viburnoides*, exhibited druses.

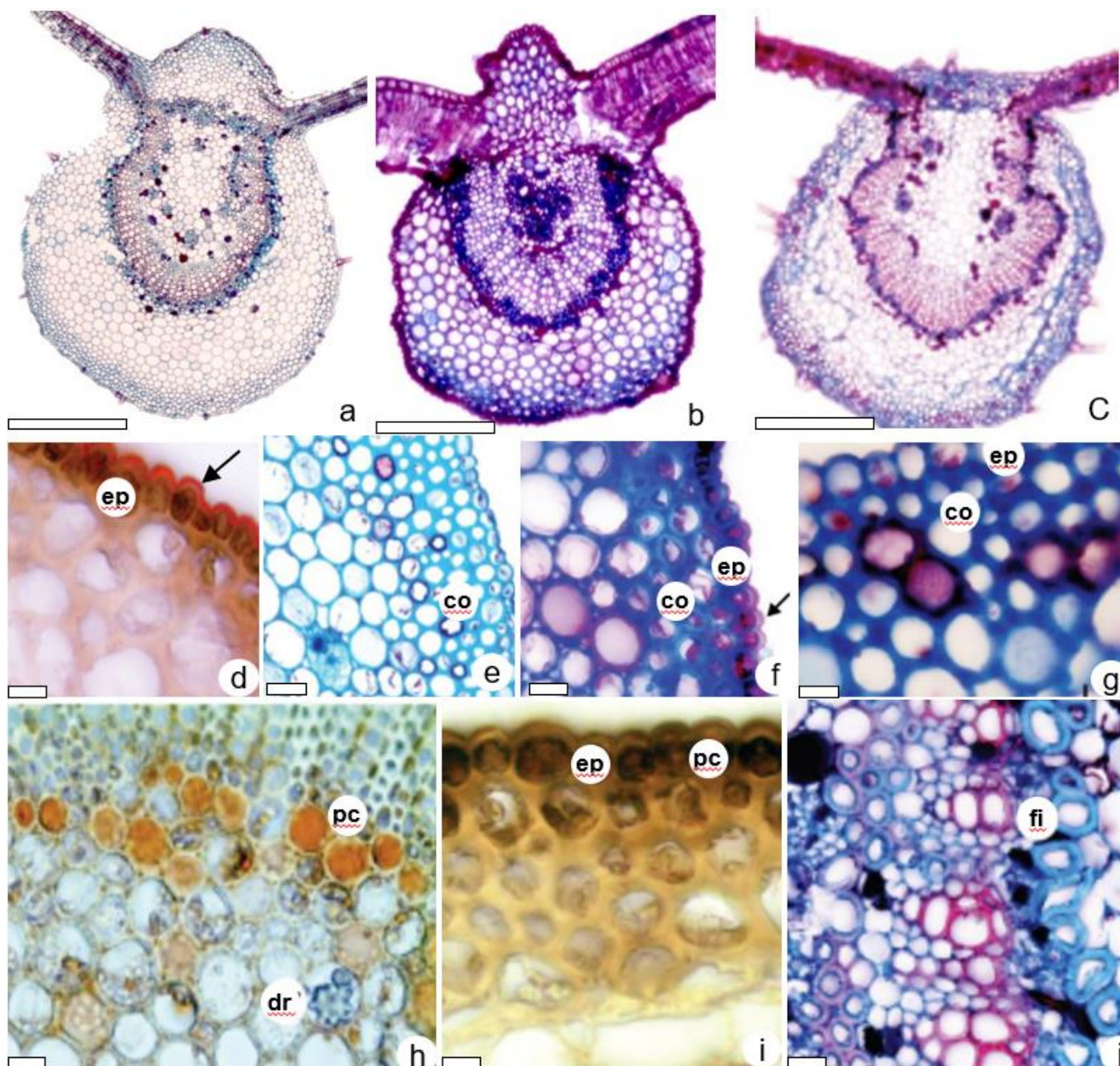


Figure 2. Anatomy of *Guettarda* - leaf midrib in cross-section. *G. pohliana* (a, d, e, h, j), *G. uruguensis* (b, f, i), *G. viburnoides* (c, g). Scale bars a, b, c= 500 µm; e= 100 µm; d, f, g, h, i, j= 50 µm. [arrow - cuticle, ep - epidermis, co - collenchyma, dr - druse, fi – fibers, pc – phenolic compounds]

The petiole, in cross-section, has a rounded outline in all three species (Figures 3a, 3b, and 3c). The epidermis is single-layered, consisting of rounded cells in cross-section, with trichomes similar to those on the blade in all three species. Subepidermally, about five layers of angular collenchyma occur in *G. uruguensis* and *G. viburnoides* and a ring-like collenchyma in *G. pohliana* (Figure 3d,3f). The remaining fundamental tissue is parenchymatous, with druses in *G. pohliana* and *G. uruguensis* (Figure 3d). The vascular tissue consists of a horseshoe-shaped collateral vascular bundle, more open, with a broad pith in *G. pohliana* and *G. viburnoides* (Figure 3a,3c) and more closed in *G. uruguensis* (Figure 3b), with a poorly delimited pith. Small vascular bundles occur in the medullary region, similar to *G. pohliana* and *G. viburnoides* (Figure 3a, 3c, 3e); in *G. uruguensis*, they are condensed (Figure 3f). Phenolic compounds showed a positive reaction to the ferric chloride solution, observed in the petiole in the epidermis, cortex, and phloem (Figure 3e, 3h, 3i).

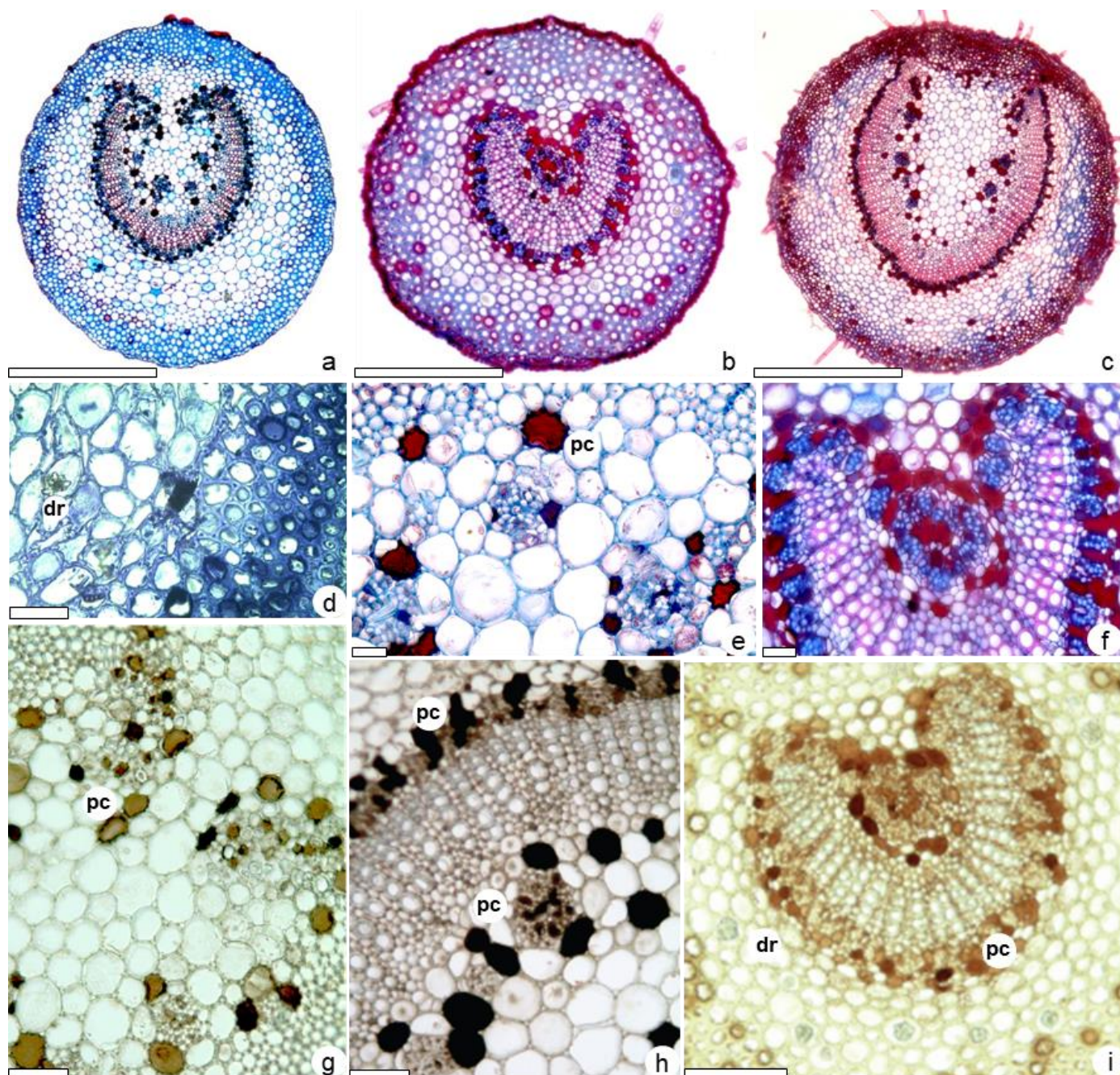


Figure 3. Anatomy of *Guettarda* - petiole in cross-section. *G. pohliana* (a, d, h, i), *G. uruguensis* (b, e, g, i). *G. viburnoides* (c, f). (e, g) Details of the medullary parenchyma showing phenolic compounds (pc). Scale bars a, b, c = 500 μ m; d, e, f, g = 100 μ m; h, i, j = 50 μ m. dr - druse, pc – phenolic compound

DISCUSSION

The anatomical characteristics observed in *G. pohliana*, *G. uruguensis*, and *G. viburnoides* align with common traits reported for Rubiaceae species, particularly within the *Guettarda* genus. The presence of hypostomatic leaves with paracytic stomata, dorsiventral mesophyll, and both unicellular and multicellular covering trichomes reinforces their placement within the family [39,40,41].

One of the most notable findings in this study is the presence of bundle sheath extensions in all three species, supporting previous reports that suggested this characteristic as a potential taxonomic marker for *Guettarda* [10]. Our observations confirm that these structures, formed by fibers surrounding the vascular bundles, occur consistently across the studied species, strengthening their relevance for systematic classification.

Regarding epidermal structures, the differences in stomatal position provide distinguishing features among the species. While *G. pohliana* and *G. uruguensis* have stomata at the same level as the epidermal cells, *G. viburnoides* exhibits raised stomata [10]. This variation may be associated with adaptations to different microclimates, as suggested in other Rubiaceae species [36].

Trichome diversity is another key distinguishing factor among the species. The four trichome types identified in this study corroborate previous descriptions for *G. viburnoides*, with *G. pohliana* and *G. uruguensis* also exhibiting a similar range of structures. However, the exclusive presence of Type 4 trichomes in *G. viburnoides*, characterized by a rounded apex and thin walls, suggests a potential diagnostic trait for species identification. Trichomes containing prismatic crystals were previously described in *Guettarda viburnoides* [10,11], and their presence was confirmed in this study, especially in Type 1 trichomes. The detection of calcium oxalate crystals within certain trichomes reinforces their protective role, a feature commonly observed in Rubiaceae [13,12]. The presence of phenolic compounds and druses in the mesophyll and midrib of all three species aligns with previous phytochemical studies, which have reported the abundance of secondary metabolites in *Guettarda* [10,11]. The histochemical detection of phenolics in the epidermis, cortex, and phloem of the petiole further supports their role in defense mechanisms and suggests their importance in taxonomic differentiation. Cells containing phenolic compounds (positive for ferric chloride) and lipid content (positive for Sudan) were detected previously in *Guettarda viburnoides* [10,11]. This finding aligns with the established chemical profile of the *Guettarda* genus, which is known for its richness in secondary metabolites, including alkaloids, iridoids, triterpenes, phenolic acids, and flavonoids [42, 43, 44, 45]. In the *Guettarda* genus, phenolic compounds have been isolated. In *G. pohliana*, 5-caffeoylquinic acid, 4,5-dicaffeoylquinic acid [25,29], and shikimic acid were identified [20]. In *G. uruguensis*, quercetin was identified, along with long-chain esters of p-coumaric acid, including eicosanyl p-coumarate, triacontyl p-coumarate, and dotriacontyl p-coumarate [22]. *G. viburnoides* shows quercetin-3-O- β -D-galactopyranoside, quercetin-3-O- β -D-glucopyranoside, grandifloroside [23] and secoxiloganin [24]. The presence of these phytochemical groups highlights the importance of continued research within this genus.

In terms of vascular structure, the horseshoe-shaped vascular bundles found in the petiole of all species are consistent with descriptions for Rubiaceae [40]. The variation in vascular bundle organization between *G. uruguensis* (more condensed) and the other two species (more open, with a broader pith) may indicate ecological adaptations that warrant further investigation.

The anatomical traits described in this study, summarized in Table 1, provide valuable taxonomic markers for distinguishing *Guettarda* species. The combination of stomatal position, trichome types, vascular bundle arrangement, and bundle sheath extensions contributes to species differentiation. Furthermore, the detection of phenolic compounds and druses reinforces the importance of anatomical and histochemical data in taxonomic classification.

Table 1. Anatomical characteristics observed in *Guettarda* species.

Anatomical Characteristic	<i>G. pohliana</i>	<i>G. uruguensis</i>	<i>G. viburnoides</i>
Stomatal position	Epidermal level	Epidermal level	Raised above epidermis
Stomatal type	Paracytic	Paracytic	Paracytic
Mesophyll	Dorsiventral	Dorsiventral	Dorsiventral
Druses in mesophyll	Present	Present	Present
Phenolic compounds	Present	Present	Present
Bundle sheath extensions	Present	Present	Present
Trichome Type 1	Present	Present	Present
Trichome Type 2	Present	Present	Present
Trichome Type 3	Present	Present	Present
Trichome Type 4	Absent	Absent	Present
Midrib shape	Biconvex	Biconvex (tapered)	Plano-convex
Pith (petiole)	Broad	Poorly defined	Broad
Crystals in petiole	Druses	Druses	Druses

CONCLUSION

The anatomical characterization of *G. pohliana* and *G. uruguensis*, presented here for the first time, contributes to the structural knowledge of the genus *Guettarda* and provides descriptive data that may be useful for future investigations involving Brazilian Rubiaceae species. The findings indicate that *G. pohliana*, *G. uruguensis*, and *G. viburnoides* share common anatomical traits; however, distinct differences were observed. In summary, the three *Guettarda* species can be distinguished by specific anatomical traits. *G. viburnoides* exhibits raised stomata and exclusive Type 4 trichomes with the highest trichome density. *G. uruguensis* shows a tapered midrib, a narrow and poorly defined petiolar pith, and condensed vascular bundles. In contrast, *G. pohliana* presents a broad, well-delimited pith and a well-developed palisade parenchyma. These features contribute to the anatomical differentiation within the genus.

These results contribute to a deeper understanding of the taxonomic relationships and diversity within the *Guettarda* genus.

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Data Availability Statement: Research data are available in the body of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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