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Comparative Anatomy of *Ilex paraguariensis* "Erva-Mate" and its Adulterant *Citronella gongonha* "Falso-Mate"

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HIGHLIGHTS

- *Citronella gongonha* is used as an adulteration of *Ilex paraguariensis*.
- Anatomy characters can differentiate the species *I. paraguariensis* from *Citronella gongonha.*
- Microscopy features support the quality control of erva-mate.

Abstract: *Ilex paraguariensis* (Aquifoliaceae), commonly known as “yerba mate” or “erva-mate”, is a shrub or tree native to South America. The leaves of the plant are traditionally used to prepare a caffeine-rich infusion with several medicinal properties, including antirheumatic, antithrombotic, anti-inflammatory, anti-obesity, diuretic, and hypocholesterolemic. *Ilex paraguariensis* is adulterated with several other species of *Ilex*, such as *I. brevicuspis*, *I. dumosa,* and *I. theezans*. It is also commonly adulterated with unrelated species, such as *Citronella gongonha* of Cardiopteridaceae. The latter species, commonly called “false-mate”, is one of the most frequent adulterants due to its similar habit and leaf morphology. Comprehensive studies on authentication and quality control of erva-mate are still scarce. This study provides a detailed comparative anatomy of the leaves and stems of *I. paraguariensis* and *C. gongonha* by light and scanning electron microscopy. The main distinguishing characteristics are the epidermal cell wall shape, type of stomata, crystal morphotypes, stem shape, presence or absence of glandular trichomes, and sclerenchymatous sheath. This data can help in the authentication and quality control of erva-mate raw materials.

Keywords: Adulteration; Anatomy; Aquifoliaceae; Cardiopteridaceae; Microscopy.

INTRODUCTION

The adulteration of botanical raw materials is a global problem, which can include the total or partial substitution of one plant for another, the presence of impurities above the minimum limits recommended in pharmacopeias or official compendia, or any condition that reduces the ideal quality of a given raw material. Misidentification or confusion due to nonspecific folk names or morphological similarities between different taxa can favor the adulteration or substitution of plant raw materials. In addition, plant drugs are commonly commercialized as fragmented or pulverized, devoid of diagnostic morphological features, making authentication difficult. In this situation, the microscopy technique can support the identification and quality assessment of the raw material [1].

Ilex paraguariensis A.St.-Hil. (Aquifoliaceae), popularly known as yerba-mate or erva-mate, is a medicinal plant growing naturally in northeastern Argentina, southern Brazil, and eastern Paraguay. It is used to prepare "chimarrão" or "mate", a traditional drink made from an infusion of the fragmented leaves of this species. The plant is also used in traditional medicine for its antioxidative, antirheumatic, antithrombotic, anti-inflammatory, anti-obesity, diuretic, hepatoprotection, and hypocholesterolemic properties [2,3]. These activities have been related to the high content of polyphenols [2,3], being the activity reported for the species [4]. The leaf extract has also been used in the cosmetic and food industries [5].

It has been reported that several other species of *Ilex*, including *I. dumosa* Reissek, *I. brevicuspis* Reissek, and *I. theezans* Mart., are adulterated or substituted to *I. paraguariensis* and traded as erva-mate [6]. In addition, materials of *I. paraguariensis* are frequently adulterated with those of an unrelated species, *Citronella gongonha* of the family Cardiopteridaceae. This species is commonly called falso-mate or false-mate due to its use as an adulterant to erva-mate (*I. paraguariensis*) [7]. Other vernacular names of this species include congonha, congonha-de-bugre, congonha-do-sertão, congonha-falsa, congonha-anta, inhanê and yapon [8].

Despite the prevailing confusion and adulteration issues, comprehensive studies on authentication and quality control of erva-mate are still scarce. Therefore, this study aimed to provide a detailed comparative anatomy of the leaves and stems of *I. paraguariensis* and *C. gongonha* by light and scanning electron microscopy to aid the authentication and quality control of the herbal materials.

MATERIAL AND METHODS

Leaves and stems of *Ilex paraguariensis* were collected at the Medicinal Garden located on Uvaranas campus of the State University of Ponta Grossa (Latitude 25° 5' 23" S; Longitude 50° 6' 23" W). Samples of *Citronella gongonha* were collected on the Federal University of Paraná (UFPR) campus (Lat. 25º4'49" S; Long. 49º2'33" W). Plant material was identified by a specialist and representative specimens were registered and deposited at the UPCB Herbarium of the State University of Paraná (UPCB #30838, *C. gongonha*) and Botanical Garden of Rio de Janeiro (JBRJ #47459, *I. paraguariensis*). Access to the botanical material was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (CGEN/SISGEN – A887B90).

Leaf and stem samples were fixed in FAA (formaldehyde, acetic acid, and alcohol) for 3 days [9], then stored in 70% (*v/v*) ethyl alcohol. Transverse sections of the tissues were made free-hand using razors and double-stained in Astra blue and basic fuchsin. Colorless nail polish was used to mount the slides [1].

For scanning electron microscopy (SEM) analysis, the FAA-fixed samples were dehydrated in increasing concentrations of ethanol solutions (70%, 80%, 90%, 100%) and dried using a critical point dryer. After mounting on stubs, the samples were coated with gold using a Shimadzu IC-50 sputter coater. The samples were analyzed and imaged using a Mira 3 Tescan field emission scanning electron microscope in high vacuum mode at an accelerating voltage of 15 kV. Chemical microanalysis of the crystals was performed using an EDS attached to the SEM.

RESULTS AND DISCUSSION

The main microscopic characters of the leaves for differentiation between *Citronella gongonha* and *Ilex paraguariensis* are shown in Figure 1 and Figure 2 and summarized in Table 1 and Table 2.

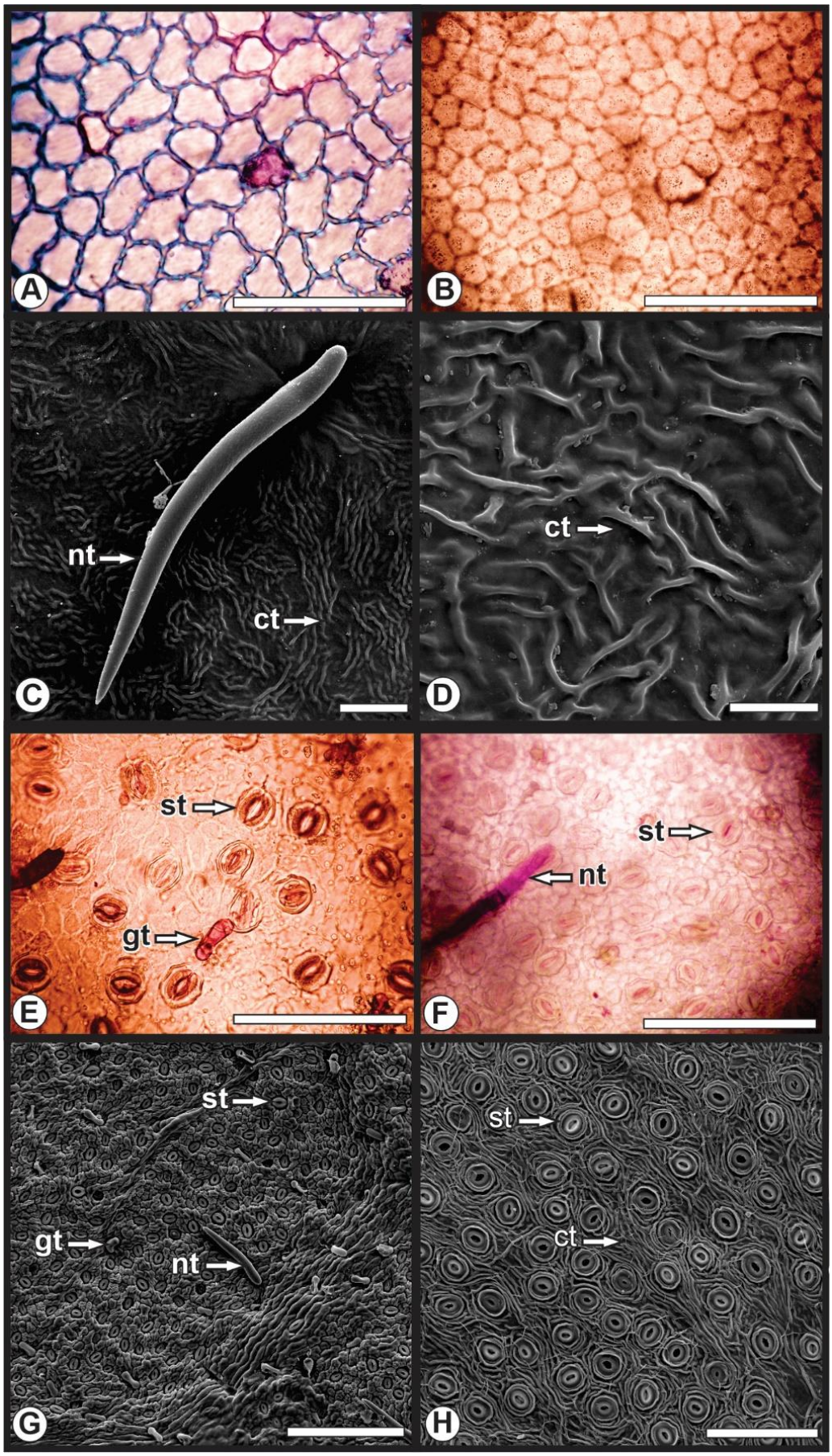

Figure 1. Anatomy of *Citronella gongonha* and *Ilex paraguariensis* leaves in frontal view. Adaxial side (A-D) and abaxial side (E-H). *C. gongonha* (A, C, E, G) and *I. paraguariensis* (B, D, F, H). ct: cuticle; gt: glandular trichome; nt: non-glandular trichome; st: stomata. Scale bar: 1 cm = (A, B), 200 µm = (I), 100 µm = (J), 50 µm = (C, D, E, G, H), 20 µm = (F).

Table 1. Leaf epidermal characteristics of *Citronella gongonha* and *Ilex paraguariensis.*

Anatomy features	***Citronella gongonha***	***Ilex paraguariensis***
Epidermal anticlinal cell walls (both sides)	Slightly wavy and thin	Straight and thin
Cuticle (both sides)	Striated	Striated
Stomata type	Anomocytic	Ciclocytic
Presence of stomata	Hypostomatic	Hypostomatic
Position of stomata in relation to the epidermis	Below	Same level
Non-glandular trichome	Simple	Simple
Glandular trichome	Capitate clavate-shaped	Absent

It has been shown that microscopic characteristics of the epidermis, especially those of the stomata and glandular trichomes types, are important for distinguishing between *C. gongonha* and *I. paraguariensis*, as demonstrated in other studies [1, 10, 11].

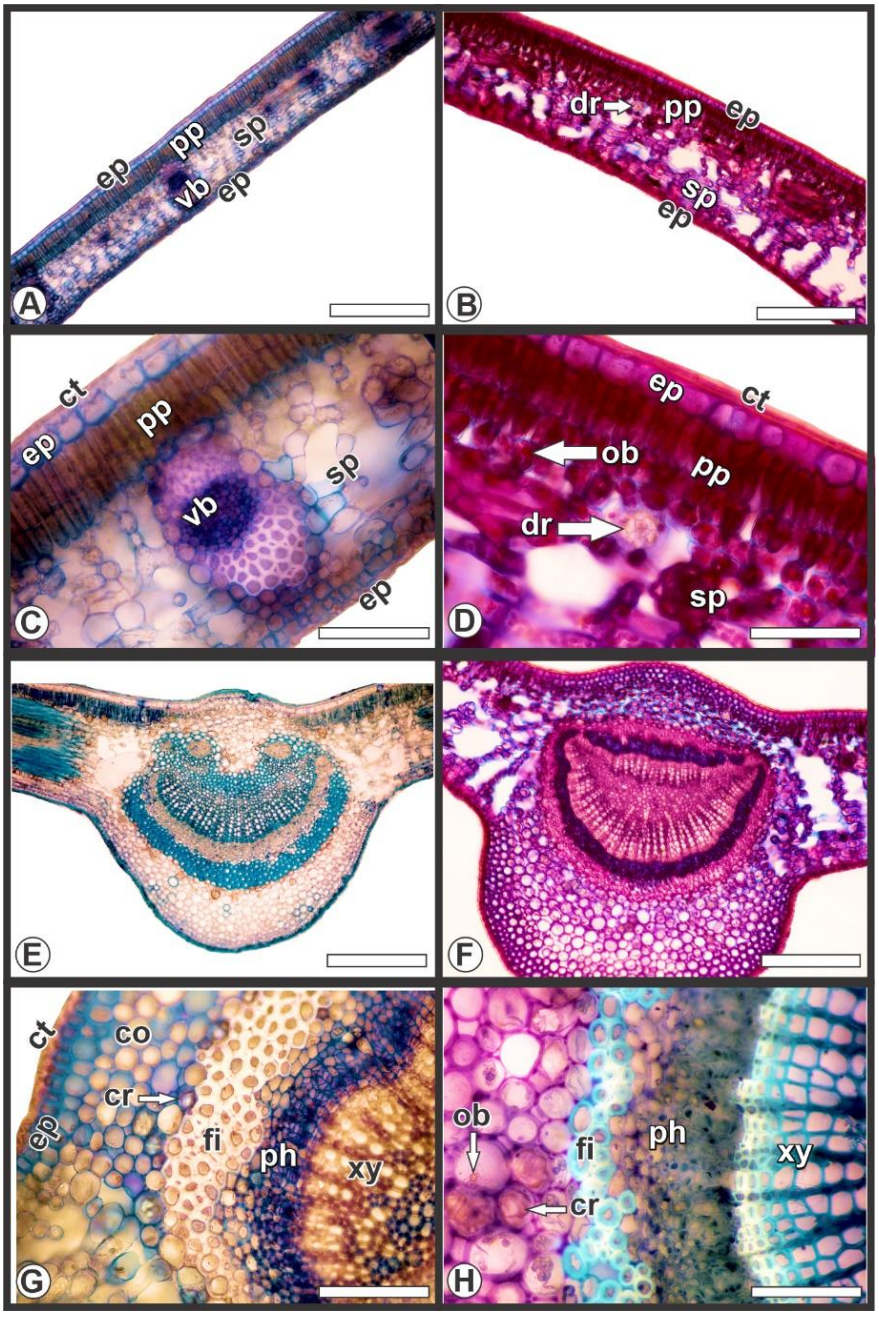

Figure 2. Leaf anatomy of *Citronella gongonha* and *Ilex paraguariensis*. Cross-sections of lamina (A-D) and midrib (E-H). *C. gongonha* (A, C, E, G) *I. paraguariensis* (B, D, F, H). co: collenchyma; cr: crystal; ct: cuticle; dr: druse; ep: epidermis; fi: fibers; ob: oil body; ph: phloem; pp: palisade parenchyma; sp: spongy parenchyma; vb: vascular bundle; xy: xylem. Scale bar: 250 µm = (A, B, E, F), 50 µm = (C, D, G, H).

Table 2. Comparative leaf anatomy of *Citronella gongonha* and *Ilex paraguariensis*.

Anatomy features	***Citronella gongonha***	***Ilex paraguariensis***
Mesophyll	Dorsiventral	Dorsiventral
Number of palisade/spongy parenchyma strata	2-3/several	2-3/several
Oil bodies in the mesophyll	Present	Present
Midrib shape in transverse section	Biconvex	Biconvex
Vascular system	One bundle in an open arc and two dorsal	Single collateral and centric disposition

Considering the anatomical features of *C. gongonha* and *I. paraguariensis* midrib, the pattern of the vascular system helps the species' differentiation. This characteristic has stood out in the identification and differentiation of medicinal species, such as species of the genus *Eucalyptus* [12], *Passiflora* [13], and *Piper* [14]. In addition to vascular system patterns, midrib shape also contributes to species differentiation as observed in the *Baccharis* genus [1,15].

The main microscopic characters of the petiole for differentiation between *Citronella gongonha* and *Ilex paraguariensis* are shown in Figure 3 and Table 3.

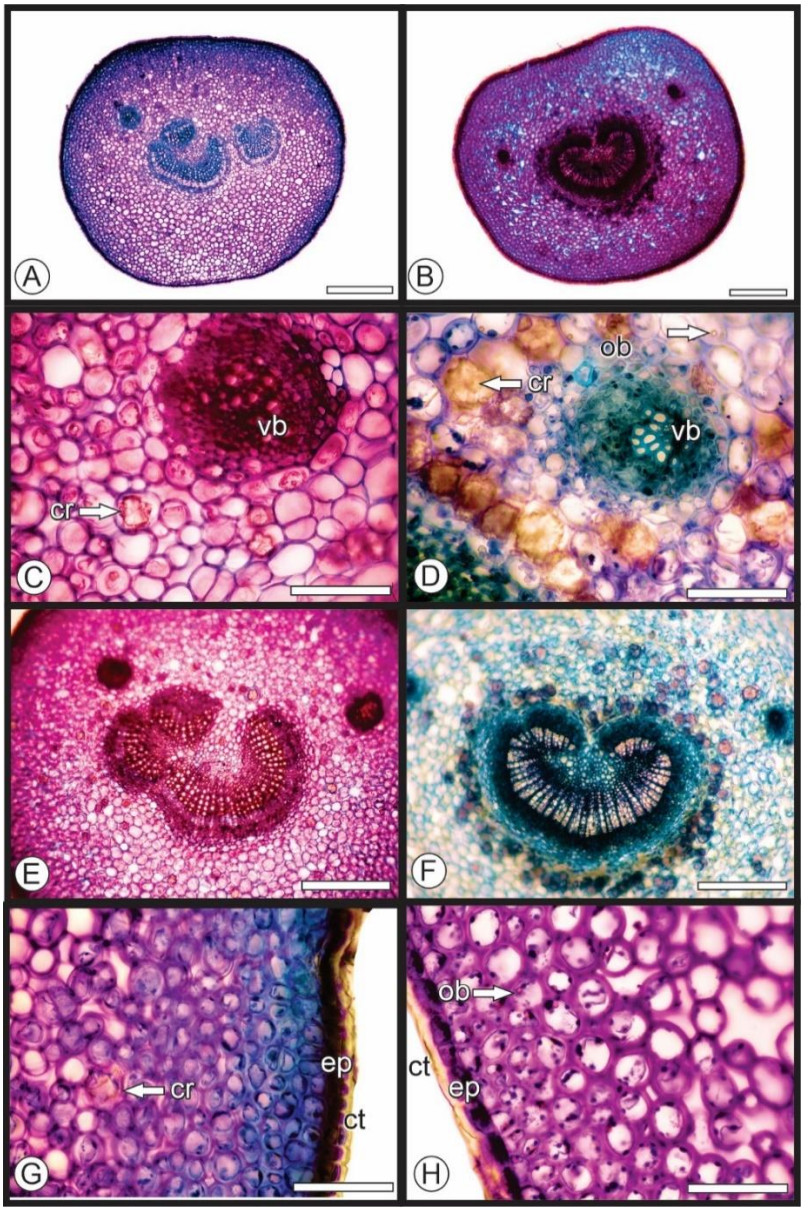

Figure 3. Petiole anatomy (cross-section) *Citronella gongonha* (A, C, E, G) and *Ilex paraguariensis* (B, D, F, H). cr: crystal; ct: cuticle; ep: epidermis; ob: oil body; vb: vascular bundle. Scale bar: 500 µm = (A, B), 250 µm = (E, F), 50 µm = (C, D, G, H).

Table 3. Anatomy characteristics petiole of *Citronella gongonha* and *Ilex paraguariensis*.

Anatomy features	***Citronella gongonha***	***Ilex paraguariensis***
Shape in cross-section	Flat-convex	Flat-convex
Crystals	Present	Present
Non-glandular trichome	Simple	Simple
Glandular trichome	Capitate-clavate shape	Absent
Vascular system	One central C-shaped and two small ones	One central in an open arc with invaginated ends and two small ones
Oil bodies	Present	Present

According to several authors [1, 13, 16], petiole shape and vascular system patterns can be considered reliable anatomical markers in species identification. This study found that the petiole vascular system pattern could separate *I. paraguariensis* from *C. gongonha*.

The main microscopic characters of the stem for differentiation between *Citronella gongonha* and *Ilex paraguariensis* are shown in Figure 4 and Table 4.

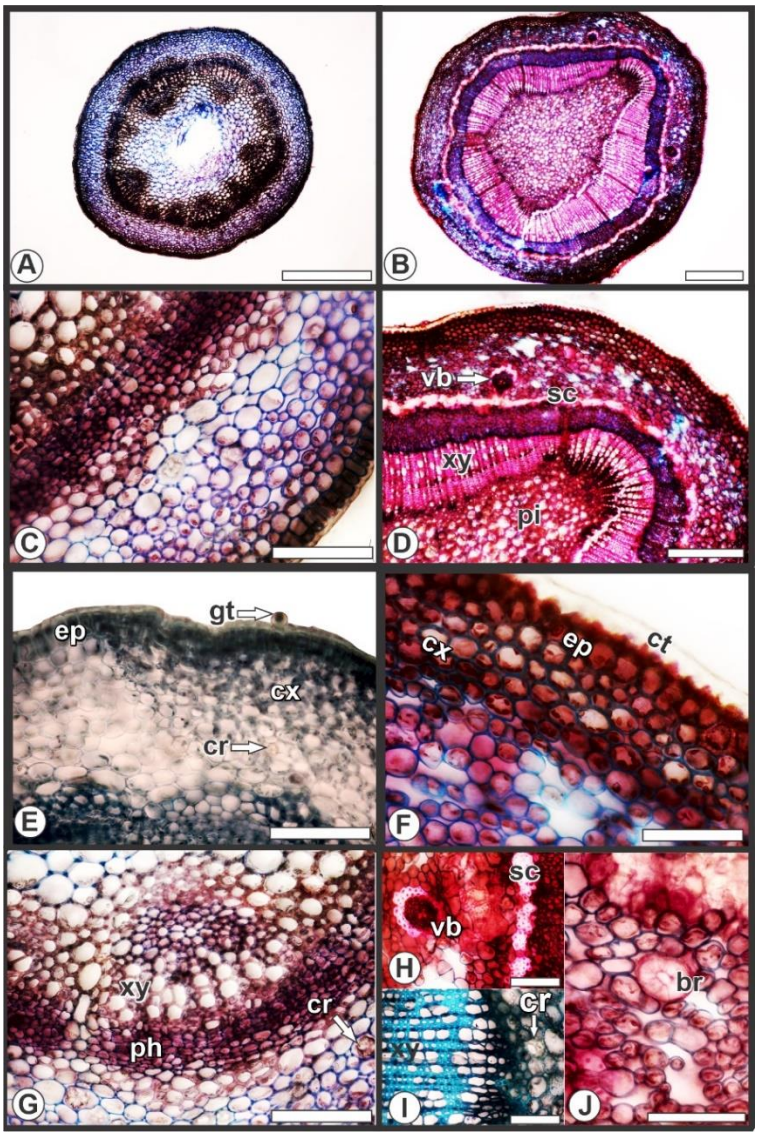

Figure 4. Stem anatomy (cross-section) *Citronella gongonha* (A, C, E, G) and *Ilex paraguariensis* (B, D, F, H, I, J). br: brachysclereid; cr: crystal; ct: cuticle; cx: cortex; ep: epidermis; gt: glandular trichome; ph: phloem; pi: pith; sc: sclerenchymatous sheath; vb: vascular bundle; xy: xylem. Scale bar: 500 µm = (A, B), 250 µm = (D), 50 µm = (C, E, F, G, H, I, J).

Table 4. Anatomy of stems of *Citronella gongonha* and *Ilex paraguariensis*.

Anatomy features	***Citronella gongonha***	***Ilex paraguariensis***
Shape in cross-section	Circular	Circular to oval
Epidermis	Unilayered	Unilayered
Crystals	Present	Present
Non-glandular trichome	Simple	Simple
Glandular trichome	Capitate clavate-shaped	Absent
Brachysclereids	Absent	Present
Sclerenchymatous sheath	Absent	Present

Considering stem microscopy, the presence of glandular trichomes, the presence of brachysclereids, and sclerenchymatous sheath attached to the phloem can differ in both species. The presence of a sclerenchymatous sheath is a good anatomical marker in species differentiation as observed in *Mikania* species [18].

The crystal morphotypes (Table 5) found in the leaves and stems of *Citronella gongonha* and *Ilex paraguariensis* can also be used to differentiate the species, especially when they are fragmented or pulverized. Both species have druses (Figure 5b, d, e, f). Amorphous crystals (Figure 5a) were found only in *C. gongonha*, whereas *I. paraguariensis* had prismatic crystals (Figure 5b, c). Crystal macropattern can be used to differentiate and identify species, as observed in a recent study by Raeski and coauthors [19].

Using EDS (energy-dispersive X-ray spectroscopy), it was possible to verify that both species contain calcium oxalate crystals. It is important to highlight that *Citronella gongonha* possesses amorphous crystals on the epidermis and in the EDS analysis, manganese was found in its composition, beyond calcium oxalate. Several studies have been developed focusing on crystals, evaluating and differentiating crystals by type and chemical composition [15, 16, 19]. FESEM photographs and EDS analyses are shown in Figure 5 and Figure 6, respectively.

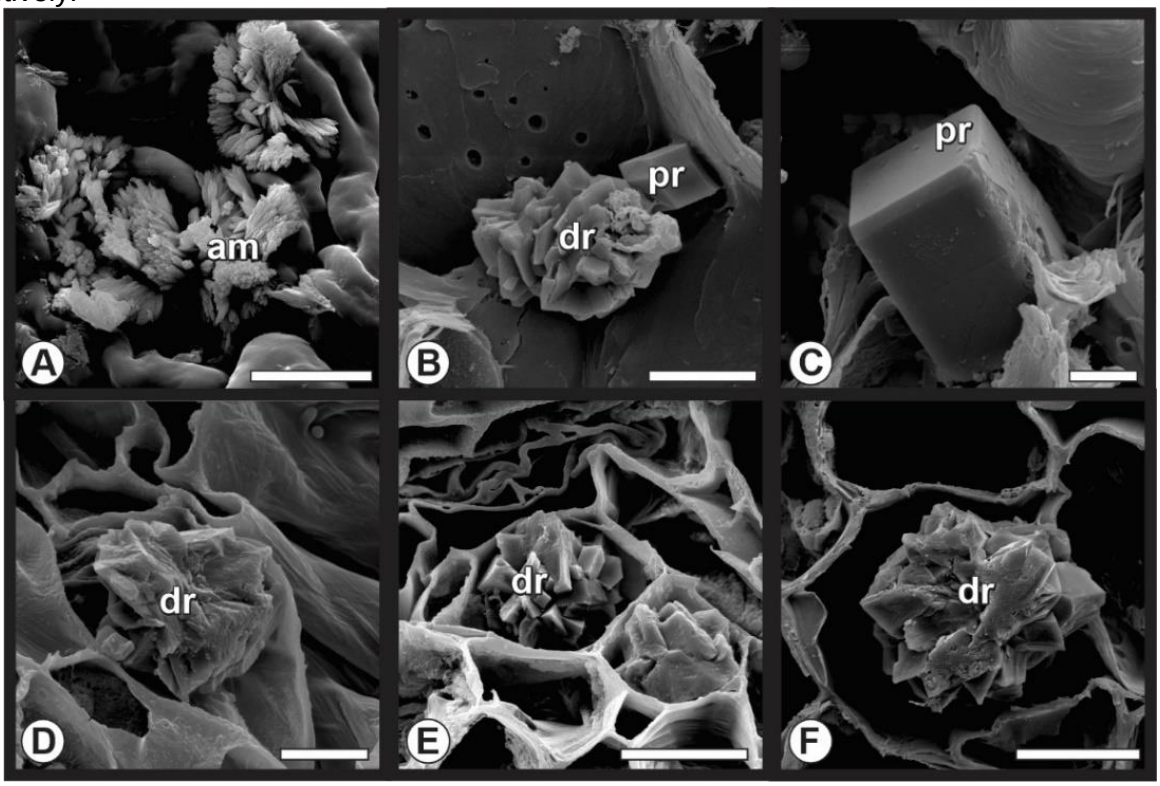

Figure 5. SEM (scanning electron microscopy). Crystals in leaf and stem tissues of the species *Citronella gongonha* (A, D) and *Ilex paraguariensis* (B, C, E, F). am: amorphous; dr: druse; pr: prismatic crystal. Scale bar: 20 µm = (E, F), 10 µm = (A, B, D), 5 µm = (C).

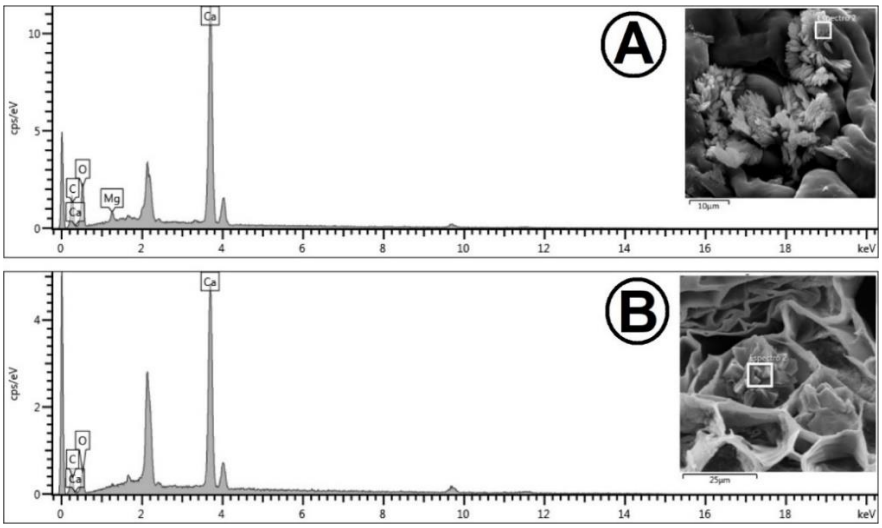

Figure 6. EDS (energy-dispersive X-ray spectroscopy) spectra of crystals present in *Citronella gongonha* (A) and *Ilex paraguariensis* (B). The unidentified peaks near 2 keV correspond to gold (Au) used in the metallization process of the material for SEM analysis.

Table 5. Type of crystals present in leaves and stems of *Citronella gongonha* and *Ilex paraguariensis*.

Type of crystal	***Citronella gongonha***	***Ilex paraguariensis***
Druse	Present	Present
Amorphous	Present	Absent
Prismatic	Absent	Present

CONCLUSION

The anatomical characteristics observed in this study can help differentiate *Ilex paraguariensis* (erva-mate) from *Citronella gongonha*, even when the materials are fragmented or powdered. The main anatomical characteristics of *C. gongonha* are the presence of anomocytic stomata, glandular trichomes, and the vascular system with one bundle in an open arc and two dorsal in the midrib, and one central C-shaped and two small ones in the petiole, as well as amorphous crystals on the leaf epidermis.

The anatomical markers for *I. paraguariensis* include the presence of ciclocytic stomata, the absence of glandular trichomes, the midrib vascular system with a single collateral bundle and the petiole with a central heart-shaped bundle and two small ones, the presence of brachysclereids, sclerenchymatous sheath and prismatic crystals in the leaves and stems.

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

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