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Leaf anatomy and morphology of *Eugenia rotundifolia* applied to the authentication of the "abajurú" commercially sold

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Article

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Abstract: *Eugenia rotundifolia* Casar., Myrtaceae, occurs in the sandy coastal environments of Rio de Janeiro and Espírito Santo States. To the best of our knowledge, *E. rotundifolia* was not included in ethnobotanical and nor in pharmacological research, but its leaves are sold in a public market of Rio de Janeiro City as "abajurú", the common name for *Chrysobalanus icaco* L., Chrysobalanaceae. *C. icaco*, in contrast, has been studied and its medicinal effects reported. Since *E. rotundifolia* is often sold in the public market as "abajurú," this paper describes and compares the leaf anatomy and morphology of *E. rotundifolia* with *C. icaco*, previously described in the literature, in order to provide tools for the authentication of commercialized "abajurú." Phyllotaxy; texture; color; margin; midrib prominence; stomata type; type and shape of midrib and petiole vascular bundle; the presence of marginal vein, secretory cavities, which are seen as translucent dots, and overlying cells only in *E. rotundifolia*; and the presence of trichomes, periclinal divisions in adaxial face epidermis, hypodermis and vascular bundle extensions only in *C. icaco* were found to be the most distinguishable diagnostic characters.

Keywords:
Eugenia rotundifolia
Chrysobalanus icaco
abajurú
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leaf anatomy
leaf morphology

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Introduction

Eugenia rotundifolia Casar., Myrtaceae, is a 2 m shrub that produces flowers with white corolla, and deep purple mature berries (Figure 1A). This species common name is "araponga" or "aperta-goela" (Souza & Morim, 2008; Zamith & Scarano, 2004). Its flowering occurs in March, and its fruiting occurs from May to June. Its reproductive success is extremely low in that more than 19,000 flowers are necessary in order to produce one viable seed (Silva & Pinheiro, 2007, 2009).

E. rotundifolia has a restricted distribution, occurring in the sandy coastal environments of Rio de Janeiro and Espírito Santo States (Souza & Morim, 2008). These ecosystems are also known as "restingas", and they are highly threatened by anthropic occupation (Tonhasca Júnior, 2005; Peixoto et al., 2002). Specifically, cities like Rio de Janeiro have grown over "restinga" soil, and nowadays real estate speculation has seriously imperiled this environment (Rocha et al., 2007; Araújo & Lacerda, 1987).

To the best of our knowledge, *E. rotundifolia* was not included in ethnobotanical and nor in pharmacological research. However, when Silva (2008) studied plants sold in "Mercado de Madureira," Rio de Janeiro City, it was observed that nine out of fifteen interviewed herbalists were selling *E. rotundifoli*a as "abajurú", the common name for *Chrysobalanus icaco* L., Chrysobalanaceae.

Chrysobalanus icaco is a shrub or small tree (up to 5 m tall) that also produces continuously flowers with white corolla and yellow, purple or black drupe fruits (Prance, 1972a). In Brazil, C. icaco has wide distribution, occurring all along the coast (Prance, 1972a,b). This species is commercially sold as "abajurú", "abagerú" or "guajurú" (Silva & Peixoto, 2009; Presta & Pereira, 1987). Many ethnobotanical studies have found that the aqueous extracts of its leaves and roots have medicinal benefits in the treatment of diabetes (Agra et al., 2008; Albuquerque et al., 2007; Barbosa-Filho et al., 2005; Fonseca-Kruel & Peixoto, 2004). In fact, pharmacological studies have validated the hypoglycemic properties of this plant (Lorenzi & Matos, 2002; Volpato et al., 2002; Presta & Pereira, 1987; Presta, 1986). The bark, roots and leaves are also used to treat diarrhea and leucorrhoea (Costa, 1977). Maioli-Azevedo & Fonseca-Kruel (2007) and Fonseca-Kruel & Peixoto (2004) also report the consumption of its fruits.

Therefore, considering the recent commercialization of *E. rotundifolia* leaves as "abajurú," coupled with the risks that arise from the consumption of its leaves due to a non proven or even studied medicinal property, the leaf anatomy and morphology of *E. rotundifolia* were investigated in order to provide tools for the authentication of the commercially sold "abajurú." In addition, macro and microscopic characterization of a phytotherapic is required by the Brazilian legislation (Anvisa, 2004). The *E. rotundifolia* results were compared to *C. icaco*, as described in the specialized literature, highlighting diagnostic characters and thus providing greater insight into the authentication of "abajurú" fresh and dried leaf samples commercially sold. The morphology and anatomy of *C. icaco* commercially sold leaves were also investigated in order to provide images for the comparison between both species of "abajurú", as well as some diagnostic characters not available in the literature.

Materials and Methods

Study site and plant material

Eugenia rotundifolia Casar., Myrtaceae, leaf branches were collected during 2009 in the "Restinga de Grumari", which is located in the "Área de Proteção Ambiental de Grumari," in Rio de Janeiro City (23º02'94''S and 43º31'98''W), under authorization of Secretaria Municipal de Meio Ambiente nº 09/2008 (Proc. nº 14/000.808/2007). The plant material was collected from five georeferenced specimens growing in open shrub formation, one of the six vegetative communities in the "Restinga de Grumari". Only fully developed leaves exposed to the sun were analyzed. *E. rotundifolia* voucher specimens were deposited at the Universidade Federal do Estado do Rio de Janeiro Herbarium (HUNI650). This species was identified by Marcelo da Costa Souza, M.Sc. in Botany. *Chrysobalanus icaco* L., Chrysobalanaceae leaves were obtained from a commercial source in Rio de Janeiro City.

Leaf morphology

The phyllotaxy and the leaf morphologic characters size, shape, texture, color, coating, margin, venation, midrib prominence, gland presence and position, and petiole size and section were visually analyzed (Hickey, 1973).

Leaf anatomy

The leaves were fixed in formalin:acetic acid:70% ethanol (FAA, 18:1:1) and stored in 70% ethanol (Johansen, 1940). Samples were taken from leaf midrib, the intercostal region, margin, and petiole. Leaf anatomy was based on handmade transversal and longitudinal sections. These sections were clarified with 50% sodium hypochlorite commercial solution, and stained with 1% alcian blue - 1% basic fucsin in 50% ethanol (Luque et al., 1996). Epidermis was dissociated using Franklin's method (acetic acid:hydrogen peroxide, 1:1) (Johansen, 1940), and also stained according to Luque et al. (1996).

In *E. rotundifolia*, the mean density of secretory cavities (cavities.mm^{-2}) was measured through the analysis of 25 optical fields in both leaf surfaces tangenital longitudinal sections (Donato & Morretes, 2007). Measures of the diameter of secretory cavities were accomplished using a micrometric scale lamina. The observations were captured in an Olympus® BX41 light microscope.

Results and Discussion

Leaf morphology

E. rotundifolia specimens exhibit opposite phyllotaxy (Figure 1B). Adult leaves are simple; succulent; elliptic to obovate, obtuse or short obtuse-acuminate apex, and obtuse base; 5.4-6.6 cm long and 4.0-5.0 cm broad; glabrous on both surfaces; light green; and with revolute margin (Figures 1B-C, 2F). The venation is pinnate, with a marginal vein markedly seen (Figure 1C). Midrib is prominent in the adaxial surface of the leaves (Figures 1C, 3B). Glands occupy a laminar position; as a result, the leaf is densely punctuated by the presence of secretory cavities, which are seen as translucent dots (Figure 1C). The petiole is 3.7-4.7 mm long and cylindrical to plane-convex (Figure 1C).

C. icaco leaf morphology was described by Costa (1977) and Prance (1972a,b). The subject was also briefly addressed by Presta et al. (2007). According to Presta et al. (2007) and Prance (1972a,b), *C. icaco* leaves are simple; entire; alternate; coriaceous; orbicular to ovate-elliptic; retuse, rounded or with a short blunt acumen at the apex, and subcuneate at base; 2.0-8.0 cm long and 1.2-6.0 cm broad; glabrous on both surfaces; and dark green (Figure 4A,E). The primary veins are inconspicuous (Prance 1972a) and the venation is pinnate (Costa, 1977) (Figure 4A). Midrib is prominent in the abaxial surface of the leaves (Costa, 1977) (Figure 4A, F). Petioles are 2.0-4.0 mm long (Prance, 1972a) and cylindrical (Figure 4A). Neither marginal veins nor translucent dots have been recorded.

Prance (1972a) also pointed out that *C. icaco* leaves exhibit extreme variation both in size and in shape. Likewise, Souza & Morim (2008) mentioned the same in relation to *E. rotundifolia* leaves. Therefore, the most useful morphologic diagnostic characters to the authentication of the commercialized "abajurú" do not rely on the leaves quite similar shape (Figures 1C, 4A), but rather on its phyllotaxy, texture, color, margin, marginal vein, midrib prominence, and the presence of translucent dots.

Leaf anatomy

In *E. rotundifolia*, the cuticle is thick, especially on leaf adaxial surface (Figures 2D-F, 3A-B). Epidermis is one-layered (Figure 2E), and it is composed of cells with curved and thick anticlinal walls (Figure 2A-B). Stomata are anomocytic (ranunculaceous) and are present only in abaxial face epidermis (Figure 2B).

Leaf anatomy of many *Eugenia* species has been studied in order to identify diagnostic characters among its morphologically similar species. The taxonomic study of Fontenelle et al. (1994), for instance, investigated the anatomy and micromorphology of eleven *Eugenia* species, searching for such characters. The authors observed mainly anomostaurocytic stomata in *E. rotundifolia*. In terms of stomata type, the result obtained in the present study (anomocytic stomata) agrees with the description of Metcalfe & Chalk (1950) for *Eugenia.*

The anatomy of *C. icaco*, as described by Osornio et al. (2002), reveals the occurrence of a thin cuticle covering a single-layered epidermis (Figure 4D-E). Epidermal cells possess straight anticlinal walls. Many of them also possess recurrent periclinal divisions in adaxial leaf surface (Figure 4D). Paracytic (rubiaceous) stomata are present only in abaxial face epidermis (Figure 4C). In relation to *C. icaco* epidermis, this study results agree with this description, except in relation to the anticlinal walls of epidermal cells, which were found to be curved (Figure 4B-C) like in *E. rotundifolia* (Figure 2A-B). This work also add to this description the occurrence of a two-layered hypodermis in the intercostal region only in adaxial leaf surface (Figure 4D-E), which was also mentioned by Metcalfe & Chalk (1950).

E. rotundifolia has a thick cuticle and curved anticlinal walls in epidermal cells, while *C. icaco* has a thin cuticle and straight or curved anticlinal walls in epidermal cells. Environmental conditions may account for the difference, as proposed by Pyykko (1966) and Watson (1942). Fontenelle et al. (1994) observed sinuous anticlinal walls in epidermal cells of *E. rotundifolia* growing in the "Restinga de Maricá", attesting to the plasticity of this character.

Fontenelle et al. (1994) and Osornio et al. (2002) both describe the occurrence of unicellular trichomes only in young leaves of *E. rotundifolia* and *C. icaco*, respectively. In this study, trichomes or scars were recorded for *E. rotundifolia*, however, a few trichomes and scars were detected in the abaxial surface of *C. icaco* leaves, which is also in agreement with Osornio et al. (2002) and Metcalfe & Chalk (1950).

Mesophyll is dorsiventral in *E. rotundifolia* (Figures 2D, 2F, 3A), although it is often isobilateral to centric in *Eugenia* (Metcalfe & Chalk, 1950). Palisade mesophyll possesses up to four cell layers, and spongy mesophyll possesses up to 18 cell layers in this species (Figures 2D-F, 3A). Cardoso et al. (2009) investigated Myrtoideae leaf anatomy, searching for characters seen in its genera and subtribes. They observed the occurrence of dorsiventral mesophyll in 44 species, five of them belonging to *Eugenia.*

C. icaco mesophyll is also dorsiventral; however, Osornio et al. (2002) describe only two layers of palisade mesophyll and five or six layers of spongy mesophyll (Figure 4D-E). Such observations indicate that *C. icaco* most likely exhibits a thinner mesophyll as a consequence of its inferior number of layers. In this study, approximately the same number of layers is also observed in *C. icaco* chlorenchyma; however, as pointed out by Metcalfe & Chalk (1950), the differentiation between palisade and spongy mesophyll is not always clear. Nevertheless, environmental conditions may affect mesophyll thickness and development. Plants grown under intense light and high temperatures, such as the specimens of *E. rotundifolia* selected for this study, usually have thickened mesophyll and a more developed palisade mesophyll (Dickison, 2000).

In the intercostal region, *E. rotundifolia* vascular bundles are collateral (Figure 3A). They possess fibers in their polar region and are surrounded by a parenchymatic sheath (Figure 3A). Small groups of fibers, which are isolated from vascular bundles, also occur among chlorenchyma cells (Figure 3A). Osornio et al. (2002) did not mention the vascular bundle type; however, in this study collateral vascular bundles are also observed in the intercostal region of *C. icaco* leaves. Sclerenchymatous fibers may surround vascular bundles (Metcalfe & Chalk, 1950), which are associated with parenchymatic bundle sheath extensions (Osornio et al., 2002) (Figure 4D). Collenchyma and sclerenchyma cells may also occur in the sheath extensions of vascular bundles (Osornio et al., 2002).

In *E. rotundifolia*, many idioblasts containing druses are observed in chlorenchyma, mostly in spongy mesophyll (Figure. 2C-E). They are either isolated or clustered in rows. Prismatic crystals and druses are often identified in both *Eugenia* and Myrtaceae. They were observed in *Eugenia uniflora* by Alves et al. (2008), in *Eugenia brasiliensis* by Donato & Morretes (2007), and also in the work of Cardoso et al. (2009). Druses were also identified in *C. icaco* mesophyll by Osornio et al. (2002), mainly in palisade mesophyll. Like in *E. rotundifolia*, in *C. icaco* the crystals may occur either isolated or clustered in rows.

E. rotundifolia midrib is prominent in the adaxial leaf surface (Figure 3B). This character in *E. rotundifolia* may be used in *Eugenia* diagnosis, since studies involving this genus and Myrtaceae leaf anatomy reported that midrib is often abaxial surface prominent in its species (Alves et al., 2008; Donato & Morretes, 2007). The midrib possesses a single arc-shaped bicollateral vascular strand with incurved ends, partially surrounded by fibers (Figure 3B-C). Surrounding the vascular strand, approximately fifteen layers of a parenchyma with thick-walled cells and therefore similar to collenchyma occur (Figure 3B-C). In fact, according to Fontenelle et al. (1994), in *Eugenia* midrib is mainly supported by collenchyma. These last authors also mentioned the occurrence of thick-walled cells in the ground parenchyma of *E. copacabanensis, E. ovalifolia, E. schottiana* and *E. sulcata*. Druses and prismatic crystals occur in this ground parenchyma and in phloem parenchyma, respectively (Figure 3C-E). Many of them occur clustered in rows (Figure 3D).

Costa (1977) mentioned that the midrib is abaxial surface prominent in *C. icaco* leaves (Figure 4F), in contrast to *E. rotundifolia* midrib (Figure 3B). *C. icaco* midrib possesses a single arc-shaped amphicribal vascular strand surrounded by fibers, which may discontinue the phloem tissue (Figure 4F). Like in *E. rotundifolia,* approximately fifteen layers of a ground parenchyma surround the vascular strand (Figure 4F). Many druses also occur in ground parenchyma (Osornio et al., 2002) (Figure 4F-G). Despite Osornio et al. (2002) did not mention the occurrence of crystals clustered in rows in *C. icaco*, its occurrence in the vicinities of the vascular system is observed in this study.

In *E. rotundifolia,* the petiole possesses a single arc-shaped bicollateral vascular strand with ends more incurved than in the midrib (Figure 3E). Cardoso & Sajo (2004) observed a similar tissue organization in eight other *Eugenia* petioles. Fibers may be observed around the strand; however they are less frequent when compared to midrib strand (Figure 3E). Approximately twenty layers of a parenchyma with thick-walled cells similar to a collenchyma occur in the petiole outer region as well (Figure 3E-F). Druses (Figure 3F) or prismatic crystals were also detected in this petiole ground parenchyma.

C. icaco petiole possesses a single cylindrical amphicribal vascular strand (Figure 4H). As in *E. rotundifolia* (Figure 3E), in *C. icaco* a small number of fibers may occur around the vascular strand (Figure 4H). Also around the *C. icaco* vascular strand occur approximately 25 layers of a ground parenchyma (Figure 4H). Many druses and some prismatic crystals occur both in *C. icaco* medullary and ground parenchyma (Figure 4H-I).

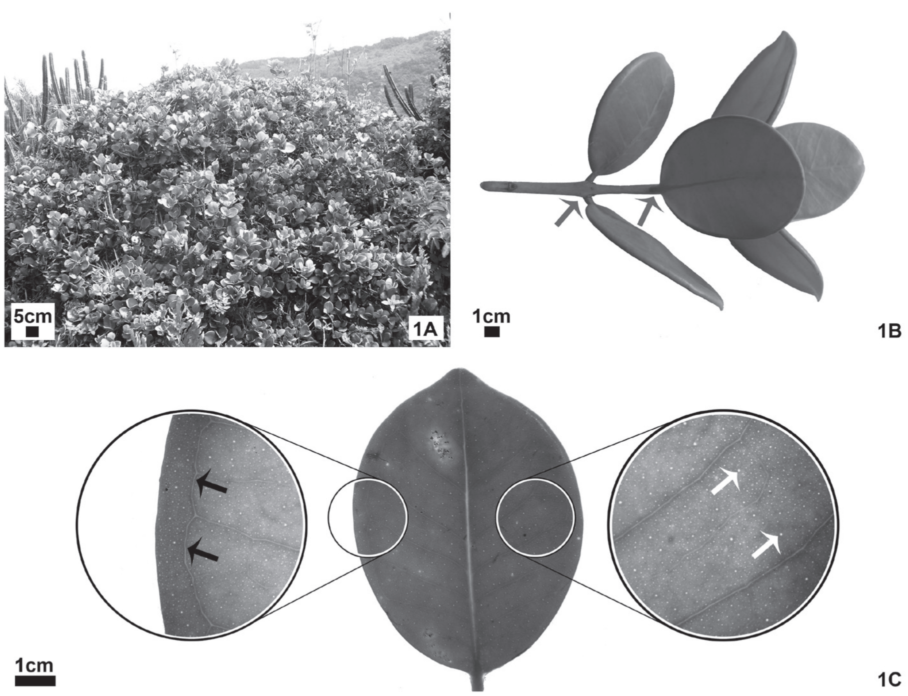

Figure 1. Aspects of *Eugenia rotundifolia* morphology. A: Habit of a "Restinga de Grumari" specimen. B: Vegetative branch showing opposite phyllotaxy (grey arrows). C: Leaf aspect showing marginal vein (black arrows) and secretory cavities seen as translucent dots (white arrows).

Pellucid glands formed by two layers of an epithelium which secretes aromatic and/or resinoid compounds are a noteworthy character of Myrtaceae (Judd et al., 2009). These structures, termed secretory cavities, were detected in many species, such as *Accara elegans* (Cardoso & Sajo, 2006), *Eugenia ovalifolia* (Defaveri, 2006), *Eucalyptus grandis* (Santos et al., 2008), *Gomidesia martiana* (Cardoso et al., 2009), *Myrcia torta* (Gomes et al., 2009), and *Psidium cattleyanum* Sabine (Arruda & Fonetenelle, 1994), among others. In *E. rotundifolia*, secretory cavities appear to be randomly distributed in mesophyll, up to three cells below the epidermis (Figure 2C-F and 3B), and they accumulate a yellow-greenish secretion. They exhibit a circular section, with a diameter of 115.0±32.1 µm and occur in higher density in adaxial leaf surface: 9.1±3.9 cavities. mm^{-2}, against 5.6±1.8 cavities/mm^{-2} in abaxial surface (Figure 2C-F).

Epidermal cells appear randomly distributed in *E. rotundifolia* (Figure 2A-B). However, when located directly over secretory cavities in the adaxial epidermis, they may be distinct in size, shape, and staining. Around these last cells, termed overlying cells, epidermal cells occur concentrically. These overlying cells are possibly related to the elimination of secretions mainly composed by essential oils accumulated in the secretory cavities (List et al., 1995). Secretory cavities have not been reported in *C. icaco* leaves (Osornio et al., 2002).

In conclusion, the anatomical and morphological characters that can authenticate fresh and dried "abajurú" leaf samples sold in the public marketplace are summarized in Table 1. It also mentions diagnostic characters related to both species habit and distribution in Brazilian territory. Although these characters are not this

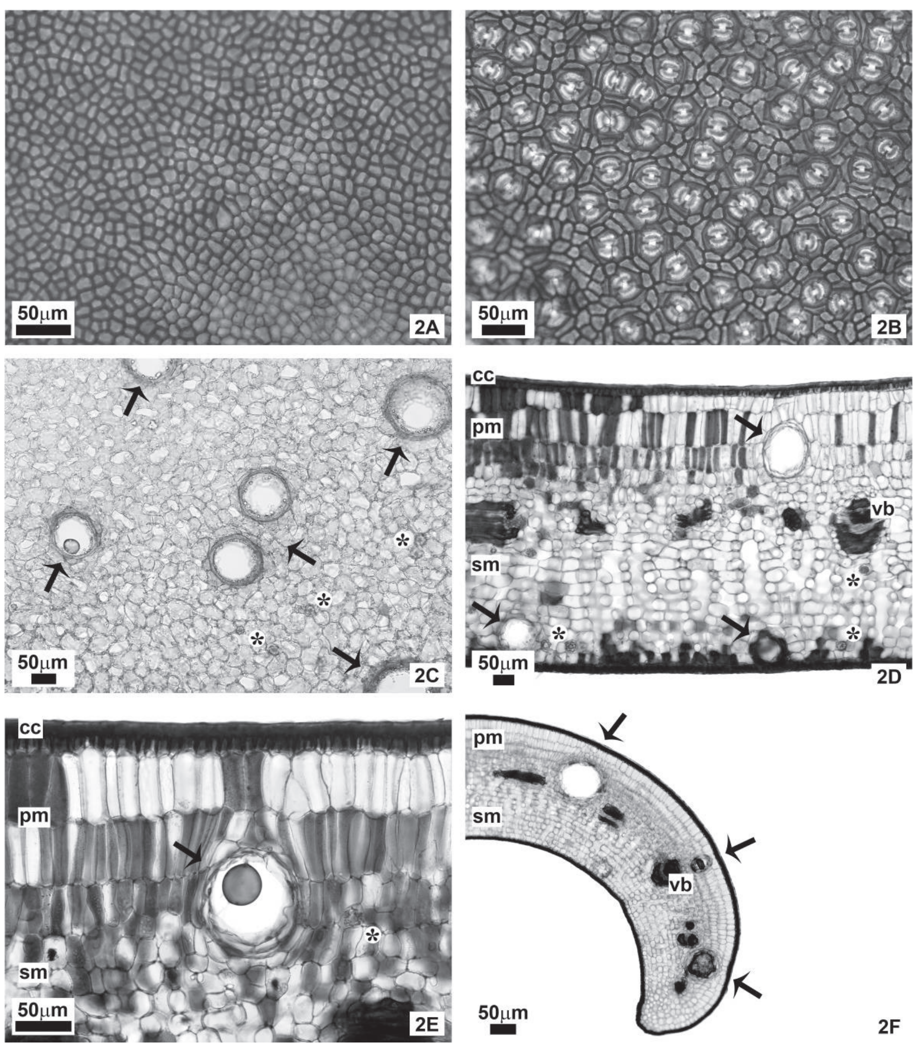

Figure 2. Aspects of *Eugenia rotundifolia* leaf anatomy. A and B: Adaxial (A) and abaxial (B) leaf epidermis. C: Abaxial leaf surface in tangential longitudinal section. D and E: Intercostal region in transversal section showing palisade and spongy mesophylls, and secretory cavities, one filled with essential oil. F: Leaf revolute margin in transversal section. Legend: cc: cuticle; pm: palisade mesophyll; sm: spongy mesophyll; vb: vascular bundle; black arrows: secretory cavities; asterisks: druses.

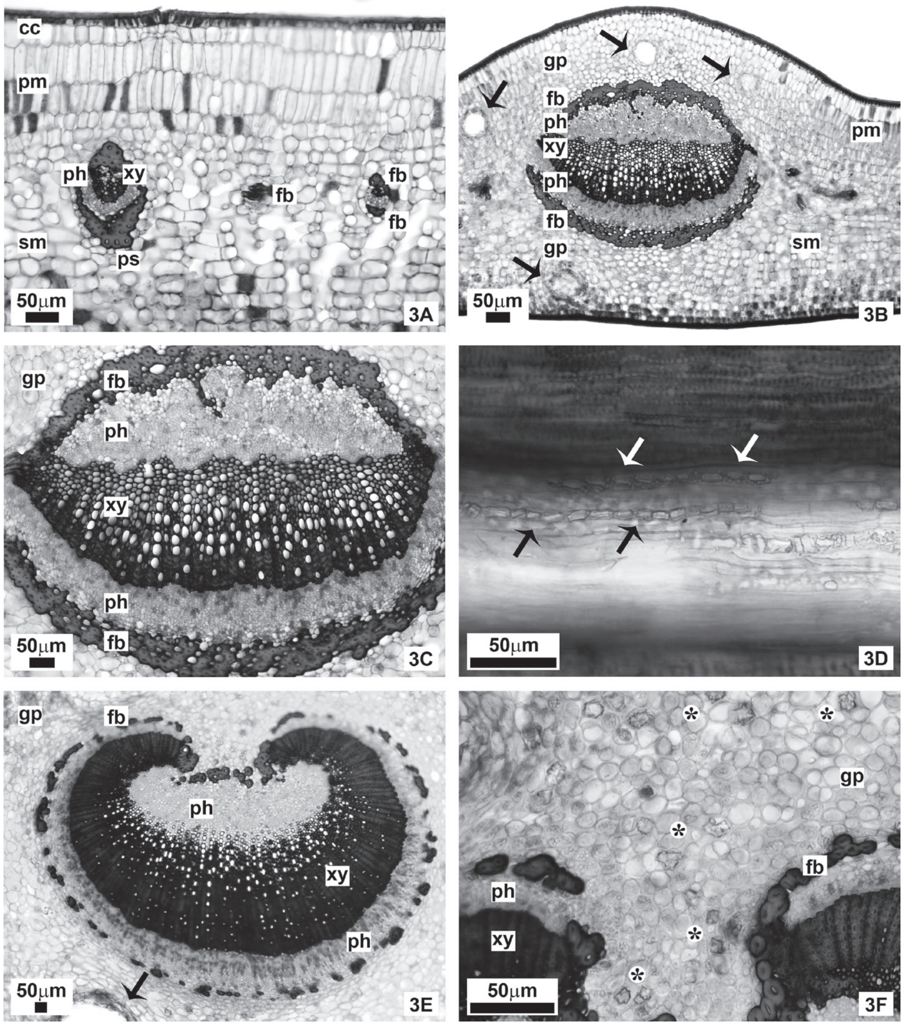

Figure 3. Aspects of *Eugenia rotundifolia* leaf anatomy. A: Intercostal region in transversal section showing vascular bundles. B and C: Midrib in transversal section showing secretory cavities (black arrows), adaxial leaf surface prominence, and the bicollateral vascular strand. D: Midrib in longitudinal section showing crystals clustered in rows (white and black arrows). E and F: Petiole in transversal section showing druses and a secretory cavity in ground parenchyma. Legend: asterisks: druses; cc: cuticle; pm: palisade mesophyll; sm: spongy mesophyll; ps: parenchymatic sheath surrounding vascular bundle; gp: ground parenchyma; fb: fibres; xy: xylem; ph: phloem.

research focus and do not contribute to the commercially sold "abajurú" authentication, since only leaves are sold, they were included in the Table 1 because they may support collectors in choosing the correct specimens.

Morphological diagnostic characters include opposite phyllotaxy in *E. rotundifolia* and alternate phyllotaxy in *C. icaco,* succulent and light green leaves in *E. rotundifolia* and coriaceous and dark green leaves in *C. icaco*, revolute margin in *E. rotundifolia* and entire margin in *C. icaco*, adaxial leaf surface prominent midrib in *E. rotundifolia* and abaxial leaf surface prominent midrib in *C. icaco*, and the presence of a markedly seen marginal vein and translucent dots only in *E. rotundifolia* (Table 1).

Among the anatomical diagnostic characters, were found anomocytic stomata in *E. rotundifolia* and paracytic stomata in *C. icaco*, bicollateral arc shaped (with incurved ends) vascular strand in *E. rotundifolia* midrib and amphicribal arc shaped vascular strand in *C. icaco* midrib, and bicollateral arc shaped (with incurved ends) vascular strand in *E. rotundifolia* petiole and cylindrical amphicribal vascular strand in *C. icaco* petiole. Periclinal divisions in adaxial face epidermis, a few trichomes, hypodermis, and bundle sheath extensions were only found in *C. icaco*, while the presence of secretory cavities and overlying cells was only observed in *E. rotundifolia* (Table 1). Since cuticle and mesophyll thickness, as well as the anticlinal walls of epidermal cells, may be influenced by environmental conditions, these diagnostic characters should be used carefully in the authentication of samples (Table 1).

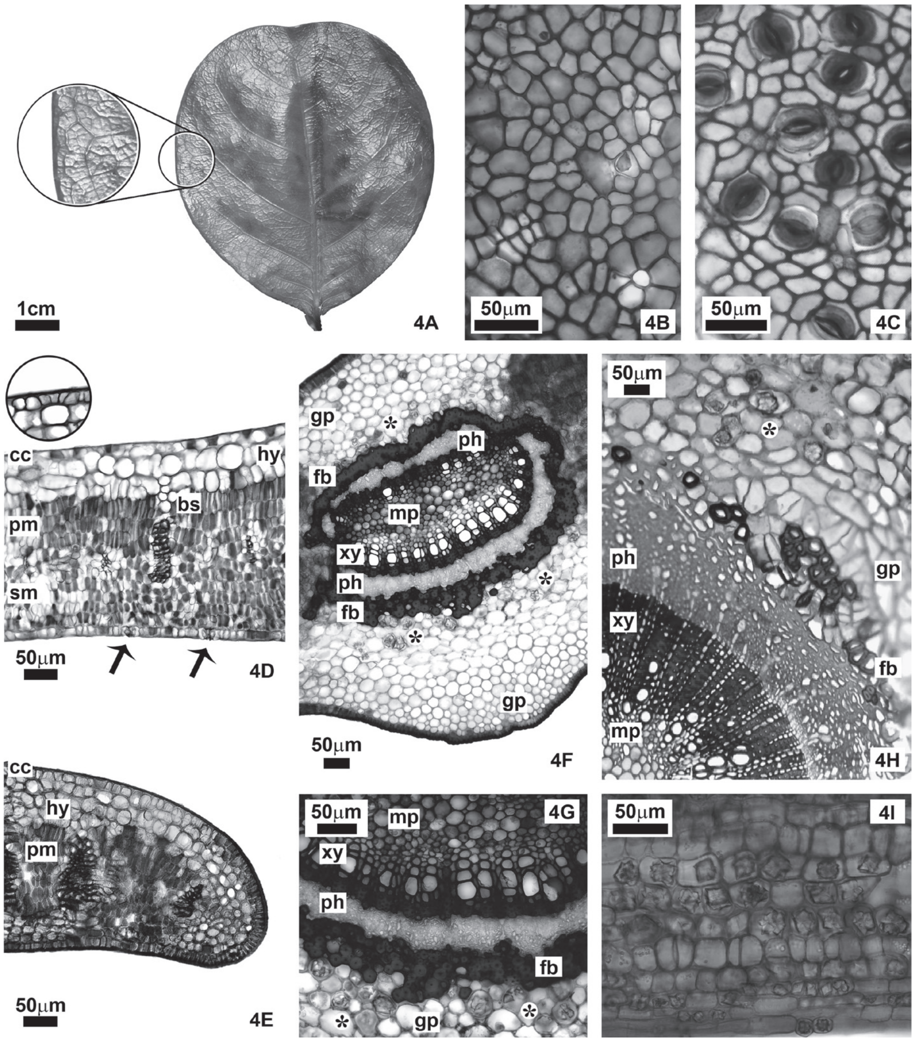

Figure 4. Aspects of *Chrysobalanus icaco* leaf morphology and anatomy. A: Leaf aspect, highlighting the venation. B and C: Adaxial (B) and abaxial (C) leaf epidermis. D and E: Intercostal region (D) and leaf entire margin (E) in transversal section highlighting the periclinal divisions in adaxial epidermis. F and G: Midrib in transversal section showing the abaxial surface prominence. H: Petiole in transversal section showing a quarter of the amphicribal vascular strand. I: Petiole in longitudinal section showing the crystals clustered in rows. Legend: asterisks: druses; cc: cuticle; pm: palisade mesophyll; sm: spongy mesophyll; hy: hypodermis; bs: bundle sheath extension; gp: ground parenchyma; mp: medullary parenchyma; fb: fibres; xy: xylem; ph: phloem.

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Table 1. Comparison of *Eugenia rotundifolia* and *Chrysobalanus icaco* distribution, habit, and morphologic and anatomic characters.

		Eugenia rotundifolia	***Chrysobalanus icaco***
	Distribution	Restricted to Rio de Janeiro and Espírito Santo "restingas"[a]	Wide, occurring throughout the coastal region[b,c]
	Habit	Shrub (up to 2m) [a,*]	Shrub or small tree (up to 5m)[b]
Morphology	Phyllotaxy	Opposite*	Alternate[b,d]
	Leaf texture	Succulent*	Coriaceous[b,d]
	Leaf color	Light green*	Dark green[b,d]
	Margin	Revolute*	Entire[b,d]
	Midrib	Adaxial leaf surface prominent*	Abaxial leaf surface prominent[c]
	Marginal vein	Present*	Absent*
	Translucent dots on leaves	Present*	Absent*
Anatomy	Cuticle	Thick*	Thin[*,e]
	Epidermal cells anticlinal walls	Curved*	Curved or straight[*,e]
	Periclinal divisions in leaf adaxial face epidermis	Absent*	Present[*,e]
	Stomata	Anomocytic[*,f]	Paracytic[*,e]
	Trichomes or scars	Present (young leaves)[g]	Present[*,e]
	Hypodermis	Absent*	Present[*,f]
	Mesophyll	Dorsiventral*	Dorsiventral[*,e]
	Palisade mesophyll	Up to 4 cell layers*	2 cell layers[*,e]
	Spongy mesophyll	Up to 18 cell layers*	5-6 cell layers[*,e]
	Vascular bundle type (intercostal region)	Collateral*	Collateral*
	Bundle sheath extensions	Absent*	Present[*,e]
	Midrib and petiole vascular strand type	Bicollateral*	Amphicribal*
	Midrib and petiole vascular strand shape	Arc-shaped, with incurved ends*	Arc-shaped and cylindrical, respectively*
	Druses	Present*	Present[*,e]
	Prismatic crystals	Present*	Present*
	Crystals clustered in rows	Present*	Present*
	Secretory cavities	Present*	Absent[*,e]
	Overlying cells	Present only in adaxial epidermis*	Absent*

[a]Souza & Morim (2008); [b]Prance (1972a,b); [c]Costa (1977); [d]Presta et al. (2007); [e]Osornio et al. (2002); [f]Metcalfe & Chalk (1950); [g]Fontenelle et al. (1994); *character seen in this study.

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