Leaf anatomy and epicuticular waxes composition of *Clusia fluminensis* Planch. & Triana (Clusiaceae)

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Clusia fluminensis Planch. & Triana (Clusiaceae) is a Brazilian native species found in regions of high luminous intensity and water restriction. There are reports of its use in folk medicine, however, there is little literature information on its chemical composition and structural characterization. The results of the phytochemical study and biological tests conducted by our research group so far have shown that *C. fluminensis* has great potential to be used as a source of biologically active substances, making its anatomical characterization of fundamental importance. The leaf structure was analyzed through optical microscopy and scanning electron microscopy. The leaves epicuticular waxes were analyzed by GC-MS. The leaf is hypostomatic with paracytic stomata and polygonal epidermic cells with 4 to 7 sides. The epidermis is uniseriate, with cuticle, thicker in the adaxial epidermis with cuticular flanges. The dorsiventral mesophyll showed hypodermis in the adaxial surface and numerous druses all over the mesophyll and many secretory ducts in the mesophyll and midvein. The vascular system is constituted by collateral bundles involved in numerous fibers, which in the midvein are densely grouped. The highest content of epicuticular waxes was obtained from leaves collected in summer. GC-MS data of the waxes indicated a marked presence of triterpenes such as α-amyrin, β-amyrin, lupenone, epifriedelinol and friedelin.

Keywords: *Clusia fluminensis*, Clusiaceae, epicuticular waxes, leaf anatomy, secretory ducts.

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1. Introduction:

Among the wide variety of botanical families Clusiaceae Lindl. stands out by the description of its use in folk medicine as wound healing, antimicrobial, and anti-inflammatory (Langenheim, 2003; Fenner et *al.* 2006; Mohamed et *al.* 2017) and because of its great representativeness throughout the tropics (Stevens, 2001 onwards).

Clusiaceae actually comprises 14 genera (APG IV, 2016), and is chemically characterized especially by the presence of xanthones (Molinar-Toribio et *al.* 2006; Auranwiwat et *al.* 2014; Genovese et *al.* 2016), polyisoprenylated benzophenones (Porto et *al.* 2000; Marti et *al.* 2009; Sriyatep et *al.* 2014), flavonoids (Compagnone et *al.* 2008; Kaikabo et *al.* 2009; Akpanika et *al.* 2017), coumarins (Ito et *al.* 2000) and terpenes (Rukachaisirikul et *al.* 2000; Medina et *al.* 2004).

Some of the substances isolated from species of Clusiaceae were submitted to biological activity assays, as examples it can be mentioned the benzophenones guttiferone A and F and the xanthones mangostin and γ-mangostin, which showed inhibitory properties against the HIV virus (Gustafson et *al.* 1992; Chen et *al.* 1996; Fuller et *al.* 1999), the xanthones allanxanthone A, 1,5-dihydroxyxanthone, and 1,5,6-trihydroxy-3,7-dimetoxyxanthone, which showed cytotoxic activity against the human tumor cell line KB (Nkengfack et *al.* 2002), and the flavonoids fukugetin and GB-2a, which showed anti-inflammatory activity (Castardo et *al.* 2008).

The genus *Clusia*, in particular, comprises 300-400 species widely distributed in Central and South America, including trees, shrubs, hemi-epiphytes, epiphytes and lianes (Stevens, 2001 onwards; Bittrich et *al.* 2015). Some species are employed in folk medicine to treat leprosy, to heal wounds, and also as analgesics (Langenheim, 2003; Sanz-Biset et *al.* 2009).

Numerous species of *Clusia* have physiological and structural characteristics which are possibly associated with their occurrence in environments with water scarcity, such as succulent and coriaceous leaves. Regarding photosynthetic metabolism, several species present crassulacean acid metabolism (CAM) in constitutive way, or changing from C3 metabolism to CAM under drought conditions (Lüttge, 2004, 2008), which allied to the structural characteristics may restrict the excessive water loss.

The cuticle of higher plants and some bryophytes is coated with a waxy layer, comprising a mixture of hydrophobic substances, known as epicuticular wax (Koch et *al.* 2004). Many functions have been proposed for these waxes, including regulation of moisture, self-cleaning behavior, gas exchange with the atmosphere, protection from mechanical and pest damage, light reflection and protection from air pollutants (Reicosky and Hanover, 1978; Koch and Ensikat, 2008). The epicuticular waxes composition may vary depending on the season, place and age of the plant, and can also differ among different parts of the same plant (Eglinton and Hamilton, 1967).

The distribution of certain classes of constituents in the epicuticular waxes may be of taxonomic value. In fact, previous studies performed described the taxonomic significance of the proportion of alkanes and triterpenes in the waxes of *Clusia* for the separation of species in infrageneric sections (Medina et *al.* 2004, 2006).

Clusia fluminensis Planch. & Triana, popularly known as abaneiro, is a Brazilian native species found in regions of high luminous intensity and water restriction. Despite widely used as an ornamental plant, there are reports on the use of its bark and resin in folk medicine to treat diarrheas (Liber Herbarum Minor, 2014). Ethnobotanical survey published by Fonseca-Kruel & Peixoto (2004) cites the leaves of *C. fluminensis* to be used for medicinal purposes by the fishermen of the extractive reserve Marinha de Arraial do Cabo.

In terms of chemical investigation little has been done up to this moment. The hydrocarbon tricosane, the terpenoids lupenone, friedelin, α and β-friedelinol, amyrin, the steroid sitosterol, and the alcohol n-octacosanol have been isolated from the leaves of *C. fluminensis* (Nagem et *al.* 1993).

The polyisopenylated benzophenones weddellianone A, lanceolatone, clusianone and spiritone were identified and quantified by high performance liquid chromatography in the resins of the male flowers of *C. fluminensis* (Porto et *al.* 2000). However, the isolation of benzophenones from this species only happened later, when clusianone was isolated by our group from the male flowers of *C. fluminensis* through counter-current chromatography (Silva et *al.* 2012).

Previous works from our group showed that crude extracts from this plant have antioxidant activity properties against the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) and that this activity could be correlated to the presence of flavones and flavonols in the extracts (Silva and Paiva, 2012). We also isolated the triterpene lanosterol from the fruits of this species, which together with clusianone and crude extracts from different organs of *C. fluminensis* showed inhibitory effects against biological activities of *Bothrops jararaca* snake venom (Oliveira et *al.* 2014). Other results from our group showed highly significant effects of hexanic extracts from the flowers of *C. fluminensis* and clusianone against *Aedes aegypti* (Anholeti et *al.*, 2015). The effects of the hexanic extracts from the fruits and from the flowers of *C. fluminensis*, as well as their main constituents, lanosterol and clusianone, respectively, were also evaluated on hemipterans insects *Dysdercus peruvianus* and *Oncopeltus fasciatus*. The topical treatments with the hexanic extracts significantly affected the survival of *O. fasciatus* and delayed the development of both hemipterans. Moreover, lanosterol significantly reduced both the survival and development of *O. fasciatus* and *D. peruvianus*, while clusianone only reduce the survival of *D.* peruvianus (Duprat et *al*., 2017). Recently we tested crude extracts and isolated substances from *C. fluminensis* against the HSV-1 virus, finding them to be active in non-cytotoxic concentration. We also examined the effects of the extracts and isolates on the activity of the HIV-1 reverse transcriptase (Meneses et *al*., 2016).

From the data obtained in the literature about the family and the genus to which it belongs associated with the preliminary results of the phytochemical study and biological tests conducted by our research group so far we believe that *C. fluminensis* has great potential to be used as a source of biologically active substances, making its anatomical characterization of fundamental importance.

In morphological terms, the species of the genus *Clusia* are very similar, characterized by being coriaceous and succulent (Lüttge and Duarte, 2007), which makes the anatomical characterization of the leaves of *C. fluminensis* essenti*al*. The aim of this study was then to contribute to the anatomic study of *C. fluminensis*, by analyzing its microscopic leaf structure as well as the morphological and chemical composition of its epicuticular waxes.

2. Materials and methods:

2.1. Plant Material

For the present study leaves from three male individuals of *Clusia fluminensis* were collected, one at Forte Barão do Imbuhy, Jurujuba, Niterói, RJ, Brazil (rocky outcrop) and two cultivated individuals collected at Jardim Guanabara, Ilha do Governador, RJ and at metropolitan region of Niterói, RJ. Fertile branches with fruits or flowers were collected aiming for the identification of the species. The voucher was deposited at the herbarium of the Faculdade de Formação de Professores, Universidade Estadual do Rio de Janeiro (RFFP), São Gonçalo, RJ, registered under the number 9213.

2.2. Anatomical protocol

For the anatomical analysis, mature leaves were fixed in in FAA 50%, dehydrated with ascending alcohol series and embedded in paraffin (Sass, 1951). Transversal sections 12 mm thick were made with a rotary microtome. Tissues were stained with Astra Blue 1 Basic Fuchsin (Roeser, 1962). For the study of the paradermal view of the epidermis, small rectangular areas of epidermis were removed from the medial portion of the leaf blade. For the histochemical tests it was used ferric chloride to evaluate the presence of phenolic substances, and Sudan III to evaluate the presence of lipophilic substances (Johansen, 1940). The photographic registers were performed in optical microscope Zeiss PrimoStar, coupled with a digital camera AxioCam ERc 5s.

2.3. Scanning electron microscopy (SEM)

The samples were adhered onto the carbon tape coated specimen stub, sputtered with 5 nm gold to avoid the charge effect and observed in a FEI Quanta 450 operating at 5kV

2.4. Wax extraction

For the extraction of cuticular waxes, leaves from male individuals of *C. fluminensis* were collected periodically between July 2005 and March 2007, resulting in 8 collections. Whole fresh leaves (100g) were extracted by immersion for 30 seconds in 300 mL of chloroform (Juniper & Jefree, 1983). The solvent was then eliminated in a rotary evaporator. After drying and weighting, the wax and water contents were calculated. The epicuticular waxes obtained from *Clusia fluminensis* were analyzed by gas chromatography coupled to mass spectrometry (GC/MS). For this analysis it was used an AGILENT, model 6890N, chromatograph coupled to a mass detector, model 5973N, equipped with databases (Wiley Library). Helium was used as carrier gas at a flow rate of 2 ml / min, the injector 300°C, 230°C and the detector temperature gas following schedule: beginning at 150°C increasing 10°C per minute to 300°C, remaining at 300°C for 15 minutes. The volume injected was 1 µl.

3. Results

3.1. Anatomical aspects of *C. fluminensis* leaves

Petiole

The petiole outline is triangular in cross-section. The epidermis is uniseriate, the cuticle is thick, with cuticular flanges (Figure 1A). All around the petiole are observed 7-10 layers of collenchyma, followed by cortical parenchyma (Figure 1B). Idioblasts containing these calcium oxalate crystals in druses shape are observed in the cortical region (Figure 1C). Vascular structure with a single arc-shaped bundle open on the adaxial side with curved ends, nearly meeting to form a closed tube (Figure 1D).

Leaf lamina

The morphology of the waxes in both adaxial and abaxial faces is classified as smooth layer comprising a smooth coat of about 1 µm thickness separated by cracks on the surface (Figures 2A, 2B, 2C).

The leaf of *C. fluminensis* is hypostomatic (Figures 2A, 2B, 2C, 3A), with paracytic stomata (Figure 3B). In paradermal view both faces are composed of polygonal cells, with rectilinear anticlinal walls (Figures 3A, 3B). In cross-section, throughout the whole extension of the leaf, the epidermis is uniseriated (Figures 3C, 3D). The cuticle, which is thicker on the adaxial epidermis, presents cuticular flanges. In the adaxial surface it was noticed three to seven subepidermic layers without chloroplasts (Figures 3C, 3D, 3E), and with the presence of secretion drops that react positively to lipophilic and phenolic substances (Figure 3F).

The mesophyll is typical of a dorsiventral leaf blade, composed of chlorophyllous parenchyma differentiated into palisade and spongy parenchyma (Figure 3C). The palisade parenchyma consists of five to nine cell layers (Figure 3C, 3D, 3E), and the spongy parenchyma comprises several layers with small intercellular spaces (Figure 3C, 3G). The vascular system consists of collateral vascular bundles surrounded by numerous fibers. Numerous druses are observed all over the mesophyll (Figure 3G).

The midrib showed plane-convex contour (Figure 4A). 3 to 4 layers of subepidermic angular collenchyma were observed in the abaxial surface (Figure 4B). The cortical parenchyma showed several layers of cells, and numerous idioblasts with druses, phenolic and lipophilic substances (Figures 4B, 4C). The vascular system is densely grouped in a system composed by up to thirty bundles. The whole group is surrounded by a sheath of fibers that involves the central cylinder (Figure 4D, 4 F).

Secretory ducts

It was noticed the presence of secretory ducts in the entire length of the leaf, in cortical parenchyma, chlorenchyma regions of the petiole and midvein, and in the palisade parenchyma. Variations were noticed in the number of bundles that compose the vascular cylinder and in the cylinder shape according to the leaf region (basal, medium or apical). In the medulla region it was noticed the presence of thick walled cells and numerous secretory ducts, which were also observed in the hypodermis, and all over the mesophyll. These structures showed epithelium with intact and well-defined cells, characterizing its schizogenous nature. Small variations in the diameter were observed. An outstanding presence of substances of lipophilic and phenolic nature was revealed inside the secretory ducts (Figures 1C, 3F, 4F).

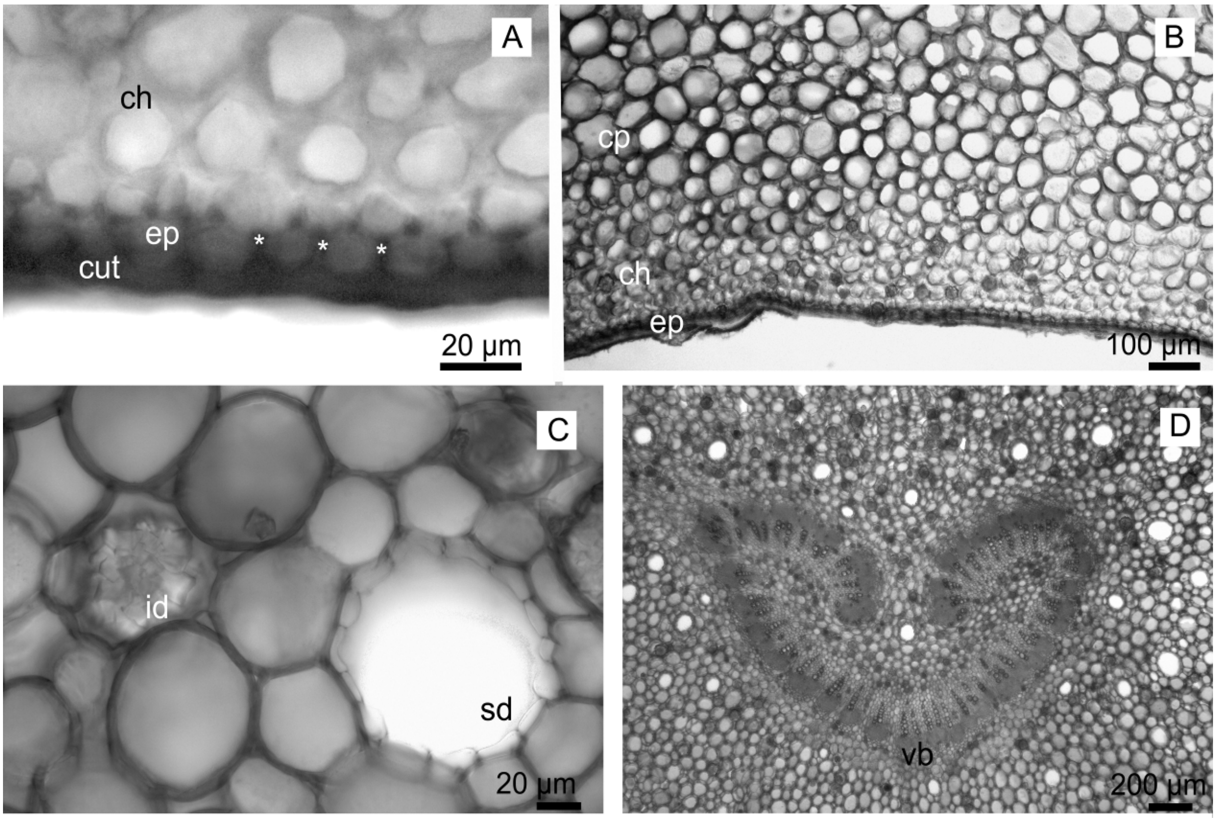

Figure 1. Petiole of *Clusia fluminensis* in cross sections. **(A)** Abaxial face of the petiole epidermis, showing the epidermal cells and the cuticle showing cuticular flanges; **(B)** Abaxial face of the petiole, with the epidermis and underlying tissues to her, chollenchyma and fundamental parenchyma; **(C)** Secretory structures in the parenchyma of the petiole: idioblasts presenting drusen and secretory canals; **(D)** Overview of the petiole vascular cylinder. ch: Chollenchyma; cp: Cortical parenchyma; cut: Cuticle; ep: Epidermis; id: Idioblasts; sd: Secretory ducts; vb: Vascular bundles.

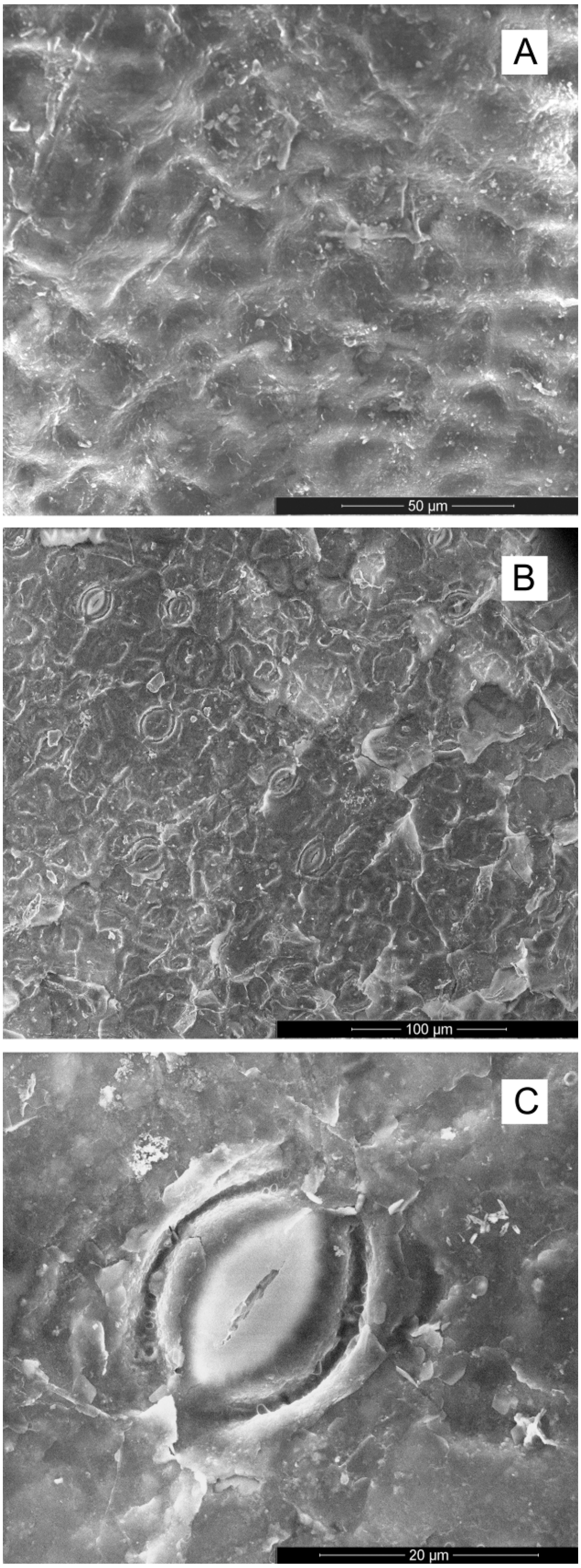

Figure 2. Surface epidermis of *Clusia fluminensis*. (A) Adaxial surface of the epidermis; (B) Abaxial surface of the epidermis, with stomata; (C) Stomata detail of the abaxial surface of the epidermis.

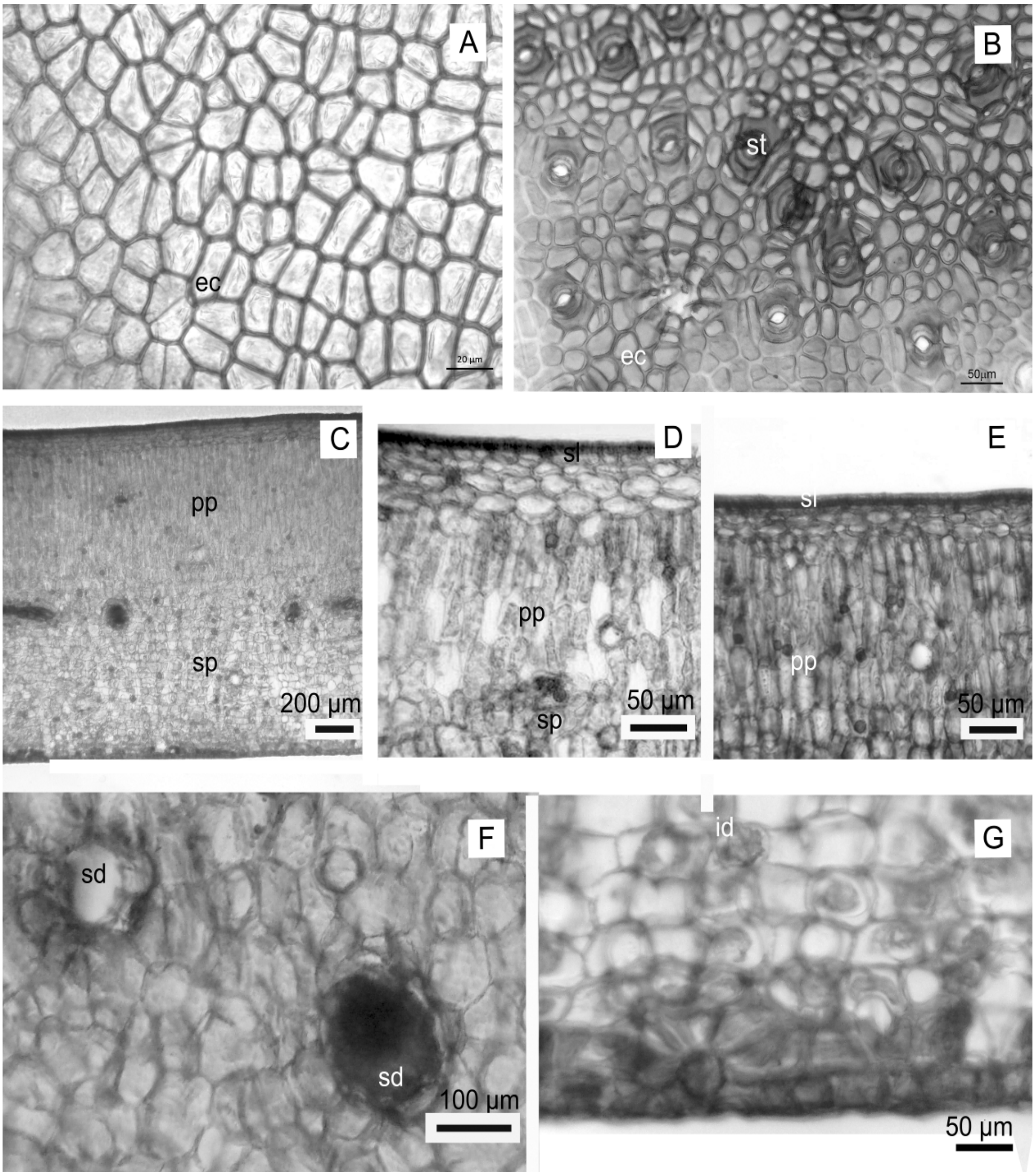

Figure 3. Mesophyll of *Clusia fluminensis*. (A) Adaxial face of epidermis paradermic section, in the intercostal space; (B) Abaxial face of epidermis of abaxial face in paradermic section, in the intercostal space; (C) Overview of the mesophyll; (D) Section of main vein in histochemical test with Sudan III, showing lipids in the palisade parenchyma; (E) Section of main vein in histochemical test with ferric chloride, showing phenols in the palisade parenchyma; (F) Secretory structures in the spongy parenchyma of the mesophyll; (G) Idioblasts containing drusen in the spongy parenchyma mesophyll. ec: Epidermal cells; st: Stomata; pp: Palisade parenchyma; sp: Spongy parenchyma; sl: Subepidermal layers; sd: Secretory duct; id: Idioblasts.

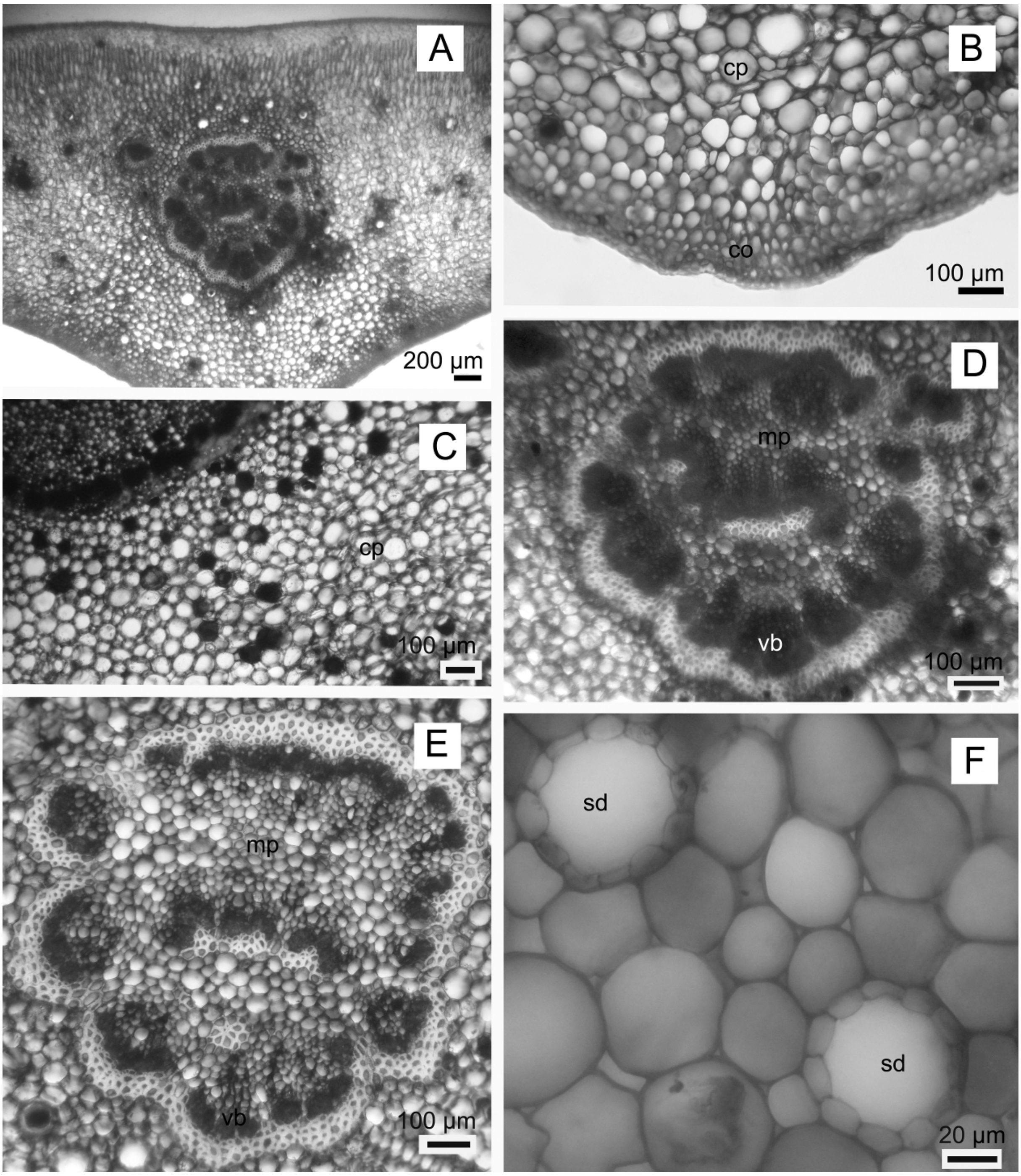

Figure 4. Midrib of *Clusia fluminensis*. (A) Overview of the midrib; (B) Abaxial face of the midrib, with the epidermis and underlying tissues; (C) Section of midrib in histochemical test with ferric chloride, showing phenols in the cortical parenchyma; (D) Section of midrib in histochemical test with Sudan, showing lipids in medullary parenchyma and surrounding the vascular cylinder; (E) Vascular cylinder of the midrib; (F) Secretory structures in the fundamental parenchyma of the midrib. cp: Cortical parenchyma; ch: Chollenchyma; mp: medullary parenchyma; vb: Vascular bundles; sd: Secretory duct.

3.2. Chemical aspects of epicuticular waxes from the leaves of *C. fluminensis*

There were no major changes in water content in the leaves of *C. fluminensis* during the two-year study. The highest content of epicuticular waxes was obtained in sample VII (Table 1), from leaves collected in summer, a period of intense light incidence.

Table 1. Water and epicuticular waxes contents in leaves of *Clusia fluminensis.*

Entry	Date of collection	Fresh leaves weight	Dried leaves weight	Water percentage in fresh leaves	Water content	Epicuticular waxes weight	Epicuticular waxes content
I	07/2005	100.060g	21.264g	78.75%	3.70	0.144g	0.0068
II	09/2005	100.150g	19.995g	80.03%	4.01	0.055g	0.0027
III	11/2005	101.040g	19.114g	81.08%	4.28	0.153g	0.0080
IV	01/2006	100.883g	20.619g	79.56%	3.89	0.062g	0.0030
V	03/2006	100.014g	21.366g	78.64%	3.68	0.110g	0.0051
VI	09/2006	100.480g	22.860g	77.25%	3.40	0.083g	0.0036
VII	12/2006	100.180g	19.083g	80.95%	4.25	0.166g	0.0087
VIII	03/2007	100.633g	19.310g	80.81%	4.18	0.047g	0.0024

The epicuticular waxes obtained from *C. fluminensis* were analyzed by GC-MS and the mass fragmentation profiles of the substances identified in the samples were compared with equipment's database (Wiley's database) and literature data.

Analysis of the data obtained from gas chromatography did not show a great metabolic diversity in the epicuticular waxes depending on the time of collection. The mass spectra of the substances present in the waxes indicated the presence of alkanes and a marked presence of triterpenes such as α-amyrin, β-amyrin, lupenone, epifriedelinol and friedelin.

The analysis of the chromatograms indicated as the major component in the samples, except in sample V (Table 1), the substance with a retention time of 21.89 min. The mass spectrum of this substance showed a molecular ion of 428 Da, and fragmentation patterns similar to those provided by the database of the equipment for the triterpene epifriedelinol.

Sample III showed the highest percentage of epifriedelinol (Figure 5A), however the percentages of this substances didn't have a great change in samples I-III and VI-VIII (Figure 6B). The lowest levels of epifriedelinol were identified in samples IV and V. In sample V this substance triterpene was present as the second major component, representing 23.98% of the composition, while the substance with the retention time of 22.14 min, and mass fragmentation similar to that of the triterpene friedelin appeared as the major component representing 58.37% of the composition.

The wax from sample I was the only one that showed the presence of steroids, represented by sitosterol and stigmasterol.

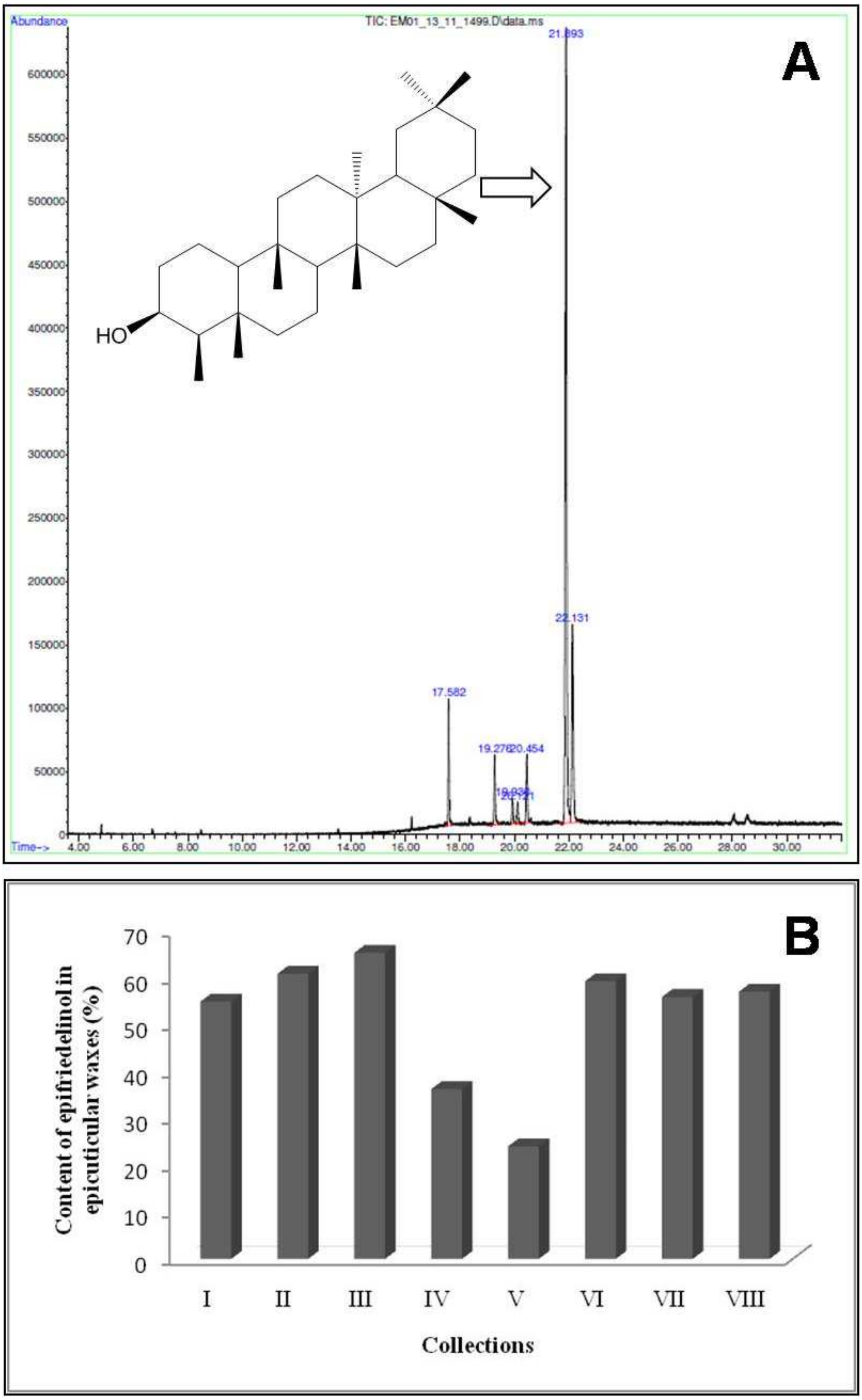

Figure 5. (A) Chromatogram of epicuticular waxes obtained from collection III and chemical structure of the major component, epifriedelinol; (B) Variation in the percentage of epifriedelinol in the epicuticular waxes of *Clusia fluminensis*. Collections performed in: (I) 07/2005; (II) 09/2005; (III) 11/2005; (IV) 01/2006; (V) 03/2006; (VI) 06/2006; (VII) 09/2006; (VIII) 12/2006; (IX) 03/2007.

3. Discussion

3.1. Anatomical aspects of *C. fluminensis* leaves

In *C. fluminensis* the stomata occur at the same level of the other epidermic cells and at the abaxial surface of the leaf blade, characterizing the leaves as hypostomatic. According to Boeger and Wisniewski (2003), this is the most common pattern of stomatal distribution for terrestrial plants, which represents a protection against the microenvironmental conditions, such as higher temperature in the adaxial surface due to intense solar exposure when it comes to sunny leaves (Lleras, 1977), and the higher humidity level in the abaxial surface when compared to the adaxial surface (Smith and McClean, 1989).

According to Lambers et *al.* (1998), under conditions of water restriction and high temperatures the stomata will be frequently closed and the plant survival may depend on the residual water lost from leaf surfaces, determined in different levels by the cuticular transpiration rate. The fact that the stomata are closed during the day ends up to decrease the CO_2 uptake rate, however, many *Clusia* species present CAM photosynthetic metabolism in constitutive way, or changing from C3 metabolism to CAM under drought conditions (Lüttge 2004, 2008).

Clusia fluminensis is a frequent plant on rocky outcrops and “restinga” vegetation and, therefore is subjected to high luminous intensity, high temperatures and water restriction, which explains its hypostomatic leaves and the presence of the thicker cuticle on the adaxial surface than in the abaxial surface. One of the biggest challenges for terrestrial plants is to develop a barrier against uncontrolled water loss which needs to be efficient and translucent for photosynthetically active radiation and the cuticle meets these requirements well (Riederer & Schreiber, 2001). According to Larcher (2000) this difference in cuticle thickness is commonly considered as prevention against transpiration. In leaves that develop under high light incidence the cuticle is thicker as demonstrated by Scarano, 2009 e Roças et *al.*, 2001. According to Riederer & Schreiber, 2001, in fact, this is the explanation usually presented, especially when discussing the adaptation of xerophytes to their habitat. However, according to the author, although the argument is coherent, there is no experimental evidence to support it. Cuticular permeability cooperates to reduce the loss of uncontrolled water during stomatal closure. However, the available quantitative results described in literature do not allow to evaluate the contributions of strictly cuticular and residual stomatal transpiration to the total loss of water in leaves of plants subjected to drought stress. The authors report that the results of other studies corroborate the elaboration of the two-way hypothesis for the diffusion of solutes and water through the cuticle of the plant, where the first one would allow the passage through the amorphous phase of the cuticular wax, being accessible only for lipophilic solutes, while the second path, consisting of pores, would allow the passage of soluble organic compounds in water and inorganic ions. Also according to the authors, the fact that the water molecule is small in size and free of charge allows it to cross the cuticle mainly through the lipophilic pathway while a small fraction of the water can diffuse through the polar pores.

New studies involving the analysis of the chemical composition and physical structure of the cuticle can help in the understanding of its functioning, determining the barrier properties of the cuticle.

Furthermore, due to environmental characteristics, the epicuticular waxes of *C. fluminensis* can also be involved in the diminution of the risk of injury due to excessive radiation by reflecting and attenuating sunlight (Tomaszewski, 2004).

In structural terms, the epicuticular waxes from the leaves of *C. fluminensis* consist of continuous coverings and can be called smooth layers. This type of wax usually has less than 1 µm thick and does not show a prominent surface sculpturing (Barthlott et *al.* 1998). Wiedemann et al (1998) observed in *C. fluminensis* wax slightly exculpated as observed in this study.

According to Juniper & Jeffree 1983, the increase in the amount of incident light is related to the occurrence of epidermal cells with flat outer periclinal walls and various forms of epicuticular wax deposition, which confer to the epidermis important reflexive features that can prevent photo-oxidation.

As discussed by Fahn (1990), the sub-epidermal layers originated both from protoderm or from the fundamental meristem (hypodermis), when constituted by large cells with thin walls are generally related to the water storage in succulent plants. However, according to Feller (1996), in coriaceous leaves, there are small cells with thick walls, considered as a protection for the leaf photosynthetic tissue when the metabolic rate is limited by the nutrients scarcity, and highly luminous intensity. A varied number of hypodermis layers was cited for *Clusia* species (Metcalfe and Chalk 1950; Borland et *al.* 1988; Guimarães et *al.* 2013).

In *C. fluminensis* it was observed sub-epidermic layers constituted by thick walled cells and the presence of lipophilic and phenolic substances, indicating that this tissue acts in the storage of water and special metabolites, besides a possible role in the protection of photosynthetic structures (Feller, 1996).

The mesophyll of *C. fluminensis* is characterized by five to nine layers of palisade parenchyma, and twenty to thirty layers of spongy parenchyma, which results in the thick character of the leaf. This is a common feature in species of *Clusia*, as observed in *C. lanceolata* (Guimarães et *al.* 2013), *C. obdeltifolia* (Silva et *al.* 2014), *C. rosea* (Zambrano et *al.* 2014). In those cells are found little drops that react positively to the lipophilic and phenolic character.

Numerous calcium oxalate crystals in druses shape are observed all over the mesophyll, hypodermis and fundamental region of the midvein. Sometimes the idioblasts containing these crystals are being projected to the epidermis (both adaxial and abaxial), so that in paradermic sections there is the impression that the crystals are in the epidermis, however the cross sections allow to see clearly that they are actually adjacent to its surface. As mentioned by Franceschi and Nakata (2005), the calcium oxalate crystals have as a function to regulate the calcium levels, act in the detoxification of heavy metals, besides the plant defense against herbivory (related to the crystals in acicular shape). As in *C.*

fluminensis it was not observed the presence of needle-like crystals, the two first functions may be related. According to Gasparotto Junior et *al.* (2005) the special metabolites from *Calophyllum brasiliense* Cambess., previously belonging to Clusiaceae, may be present in the leaves isolated or grouped with calcium oxalate crystals, which could explain the great presence of those crystals along the leaf blade of *C. fluminensis*.

The whole leaf blade is crossed by numerous secretory ducts which vary in number and diameter with content that reacts positively to lipophilic and phenolic substances. Those ducts are delimited by epithelial cells, without traces of lysis, which indicates their schizogenous origin and confirm the data obtained from Curtis and Lersten (1990) for *Hypericum* species (previously Clusiaceae), and the data obtained by Gasparotto Junior and co-workers (2005) for *Calophyllum brasiliense* Cambess.

The presence of lipophilic and phenolic substances was observed in several tissues that compose the leaf blade, such as the parenchyma and the hypodermis. The secretory ducts also contribute to the massive production of those substances, which is justified by its wide occurrence. Such observations corroborate the data obtained from the literature for the chemical composition of *Clusia* species.

3.2. Chemical aspects of epicuticular waxes from the leaves of *C. fluminensis*

As previously shown, there were no major changes in water content in the leaves of *C. fluminensis* during the two-year study. This pattern reflects the adaptive capacity of the species, even in conditions of prolonged drought, as happened in sample VIII (table 1). This profile is consistent with the fact that many species of *Clusia* change metabolism from C3 to CAM under drought conditions (Lüttge, 2004, 2008).

The highest content of epicuticular waxes was obtained from the leaves corresponding to sample VII, followed by sample III, which also happened in summer, in similar weather conditions. This characteristic may be related to the role of epicuticular waxes in light reflection on the leaf surface (Reicosky and Hanover, 1978). Although samples IV and V were also obtained from leaves collected in summer, *Clusia fluminensis* individuals were presenting flowers and the plant could be changing metabolism due to the reproductive period, resulting in a lower content of epicuticular waxes. Both samples II and VI were obtained from leaves collected in September, during the beginning of spring, after a period of lower temperatures and cloudy weather, which may explain the lower waxes content, since the light incidence was less intense.

Plant waxes are a composed of a mixture of hydrophobic substances, which may include long-chain aliphatic and cyclic compounds such as fatty acids, aldehydes, alcohols, ketones, alkanes, alkyl esters, triterpenes, tocopherols, etc. The wax composition is not a constant character of a given plant nor of its tissues, and dynamic changes may occur depending on factors such as environmental conditions or ontogenetic development (Tomaszewski and Zieliński 2014).

Literature data indicated the presence of alkanes and triterpenes in the waxes of species of *Clusia* and described the taxonomic significance of the proportion of such metabolites in the waxes for the separation of species in infra-generic sections (Medina et *al.* 2004, 2006). All samples showed a higher proportion of triterpenes over alkanes, the same feature was observed for *Clusia coclensis* and *Clusia cretosa* (Medina et *al.* 2006).

The percentages of the major component of *C. fluminensis* waxes, epifriedelinol, didn't have a great change from collections I-III and VI-VIII. However, in sample V friedelin appeared as the major component. It is interesting to notice that by the time collections IV and V were performed the plant was in the flowering period, which could again be affecting its metabolism, contributing to lower levels of epifriedelinol. Epifriedelinol and friedelin have demonstrated microbicidal activity against fungi and bacteria (Kuete et *al.* 2007; Tamokou et *al.* 2009; Kannathasan et *al.* 2015), and their presence in the waxes from the leaves of *C. fluminensis* may be related to the role of waxes in plant defense against pathogen attack. Also, the presence of high levels of friedelane triterpenes in the epicuticular waxes of *C. fluminensis* may contribute to add medicinal value to the leaves of this species, since it indicates an antimicrobial potential that can be exploited for the development of pharmaceutical formulations for topical use, for example.

4. Conclusions:

In a conclusion we demonstrated that the anatomical features found in *C. fluminensis* leaves correspond to the general characteristics described for Clusiaceae and related families. The data obtained from the chemical investigation of the epicuticular waxes showed a high content of triterpenes, especially epifriedelinol. Analysis of the data did not show a great metabolic diversity in the epicuticular waxes depending on the time of collection, however there was a change in the major component during the flowering period. The results shown are relevant for the characterization of this species, contributing to the study of the genus *Clusia* and the family Clusiaceae.

Competing interests
The authors declare that they have no competing interests.

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