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Original Article

Anatomical characters and chemical profile of leaves of three species in Lauraceae family

Ricardo de Almeida Gonçalves[a], Alyson Bruno Pinheiro[b], Michele Aparecida de Oliveira[b], Roberto Tanaka do Nascimento[b], Patrícia Fernanda Rosalem[a], Vera Lúcia Garcia[c], Aline Redondo Martins[a,*]

[a] Departamento de Biologia e Zootecnia, Faculdade de Engenharia de Ilha Solteira, Universidade Estadual Paulista "Júlio de Mesquita Filho", Ilha Solteira, SP, Brazil
[b] Faculdade de Tecnologia, Capão Bonito, SP, Brazil
[c] Divisão de Química Orgânica e Farmacêutica, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, Campinas, SP, Brazil

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ABSTRACT

The Lauraceae family is widely distributed in tropical and subtropical climates, has more than 2500 species and in the taxonomic point of view, it is one of the most difficult families to identify species. The aim of this study was to analyze the leaf anatomy of three species of Lauraceae (*Ocotea indecora* (Schott) Mez, *Nectandra barbellata* Coe-Teix. and *Endlicheria paniculata* (Spreng.) J.F.Macbr.) and identify the chemical profile of essential oil extracted from the leaves by hydrodistillation method. The leaves of the three species were obtained in "Parque Estadual Intervales", Atlantic Forest, São Paulo state, Brazil. Samples of leaves were fixed, dehydrated, embedded in synthetic resin and sectioned for mounting histological slides for anatomical description of leaf tissues. The essential oil extracted by hydrodistillation method from dried leaves was analyzed by gas chromatography to establish its chemical profile. The leaves are hypostomatic, the epidermis in *E. paniculata* and *N. barbellata* present regular cells walls and irregular cells walls in *O. indecora* in both sides of epidermis. The three species present a dorsiventral mesophyll. Histochemical analyses presented lipid substances in secretory cavity and cuticle; starch, phenolic compounds and mucilage were observed in parenchyma cells of midrib and mesophyll. Ultra structural analyses demonstrated that trichomes in the species *E. paniculata* and *O. indecora* are shown only on the abaxial leaf face and species *N. barbellata* presented trichomes on both sides of the epidermis (abaxial and adaxial). *Ocotea indecora* essential oil revealed as main compound the bicyclogermacrene and *N. barbellata* the δ-cadinene. The species showed different morphological characters and different compounds of the essential oil, being these data useful for the differentiation of the species.

Introduction

Lauraceae is classified to Laurales order, presents pantropical distribution, including America, Asia, Africa and Australia (Stevens, 2001). The family is characterized as bushes or trees, rarely climbing (genus *Cassytha*), usually aromatic. It has alternate leaves, simple, without stipules, with whole edges and usually tough (Souza and Lorenzi, 2008). It has an important ecological role, since in the Atlantic Forest is one of the families that has more representatives that leads to an increase in the species richness of this biome (Quinet and Andreata, 2002).

The family Lauraceae also stands out for its economic importance in the pharmaceutical, wood and food industries (Judd et al., 1999). Some genera are used in carpentry and construction, as they offer good quality wood, other genera are widely used in the food industry (cinnamon and bay), there are also genera that have great value in the aromatic and cosmetic industry as well as in the medical industry (Marques, 2001).

According to Vieira et al. (1997), the high economic value of these species has led to their increasing exploration over the years, making them "vulnerable" or "endangered", according to the classification of the International Union for Conservation of Nature and Natural Resources (I.U.C.N.). In Brasil, several species of the family are found in the categories "vulnerable", "endangered" and "critically endangered", according to Ministerial Order No. 443/2014 of the Ministry of the Environment (Ministério do Meio Ambiente, 2014). Therefore, effective conservation mea-

* Corresponding author.
E-mail: aline@bio.feis.unesp.br (A.R. Martins).

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sures to prevent many species from extinction have become essential.

The Lauraceae is an important family of Angiosperm and one of the most complex families of the Brazilian flora for identification due to the large number of species and the use of cryptic characters to distinguish genus and species (van der Werff, 2002; Souza and Lorenzi, 2008). In some genera, it is not safe to rely on only vegetative characters for their identification (Rohwer, 1993). The genera *Nectandra* and *Ocotea* are very difficult to distinguish, only possible to identify because of the position of basal glands and anthers' shape (Coe-Teixeira, 1980).

Ceolin et al. (2009), analyzing the anatomy of the leaves of some species of the family Lauraceae verified the presence of characteristics that can be used in their taxonomy, as hypostomatic leaves with paracytic stomata and in some species characteristic smell when the leaf was split (due to the presence of oily cells in mesophyll). Coutinho et al. (2006a) described the anatomy of the leaf of *Ocotea duckei* Vattimo-Gil and concluded that some characteristics present in the leaves allowed the distinction of the species within the genus. It is possible a division of genus within the family analyzing chemical markers of the essential oil of species (Wofford, 1974; Ferreira et al., 1980).

The purpose of this study was to analyze and describe the leaf anatomy of three commonly found and representative tree species of the Lauraceae family (*Ocotea indecora* (Schott) Mez, *Nectandra barbellata* Coe-Teix. and *Endlicheria paniculata* (Spreng.) J.F.Macbr.) found in semideciduous seasonal forest in southeastern Brazil and design the chemical profile of the essential oil.

Material and methods

Anatomical characterization

The leaves were collected at a fragment of the preserved Atlantic Forest at Parque Estadual Intervales (P.E.I.), located in the southern region of São Paulo State, near the town of Ribeirão Grande between the coordinates S 24°12′ and 24°32′, and W 48°03′ and 48°32′. The leaves were sampled from three individuals of the same species, featuring a work in triplicate. An expert in the Lauraceae family, Prof. Dr. Pedro Luís Rodrigues de Moraes, identified the species and the plant specimens were incorporated to the UNESP Herbarium – Ilha Solteira (HISA). The herbarium vouchers are: HISA10292 (*Nectandra barbellata* Coe-Teix.), HISA10293 (*Endlicheria paniculata* (Spreng.) J.F.Macbr.), HISA10296 (*Ocotea indecora* (Schott) Mez). Three leaves of the lower part of the crown were sampled from three different individuals of each species. Foliar tissue samples were fixed in FAA 50 (Johansen, 1940) to avoid material loss due to natural degradation. After 48 h, the material was stored in 70% alcohol. In the completely expanded leaf, the middle region of the leaf was analyzed at the midrib and lamina areas. The samples were dehydrated in ethylic series, included in hydroxyethyl-methacrylate (Leica Historesin) and the blocks were cut at 8 μm thick.

The material was stained with toluidine blue 0.05% in phosphate buffer and citric acid with pH between 4.5 and 6.0 (Sakai, 1973). The slides were mounted with synthetic resin.

Histochemistry

Histochemical tests were performed in fixed material included in historesin. For lipid detection, we used Sudan IV (Jensen, 1962); for starch, iodized zinc and chloride (Strasburger, 1913); for phenolics, ferric chloride (Johansen, 1940) and for pectin substances and mucilage, Ruthenium red (Johansen, 1940). To verify the natural aspect of the organ, we assembled the cuts of the material only in water, that is, without treatment and observed them under a light microscope.

Epidermis characterization

We used the "Jeffrey solution" for chemical maceration to achieve dissociation of foliar epidermis (adaxial and abaxial surfaces). This technique consists of the immersion of leaf fragments in a mixture of acids (10% nitric acid and chromic acid 10%, 1:1) to allow the dissolution of middle lamella and the epidermis isolation (Johansen, 1940).

Photomicrographs were obtained in trinocular microscope "Photonic Bel" coupled to Moticam 1000 camera of 1.3 M Pixel.

Scanning electronic microscopy (SEM)

Three samples of fixed leaves (of each species) were dehydrated in ethylic series, dried to a critical point, fixed in aluminum bracket with carbon double-sided tape and metallized with a gold layer of 30–40 nm.

The analyses were performed using a scanning electron microscope Zeiss model LEO 435VP, operated at 20 kV with scales printed directly on the electron micrographs, at NAP/MEPA (Núcleo de Apoio à Pesquisa em Microscopia Eletrônica aplicada à Pesquisa Agropecuária) ESALQ-USP.

Extraction of essential oil

The volatile oil obtained through hydrodistillation using the Clevenger apparatus (Simões and Spitzer, 2003). It was used 100 g of dried leaves placed in a volumetric flask containing 2.5 l of distilled water during 3 h counted from the boiling.

Thin layer chromatography

The eluent used for thin-layer chromatography (TLC) was dichloromethane with silica gel 60 F254 as stationary phase (Merck art. 1055540001). After elution, the chromatogram was observed under ultraviolet (UV) light at 254 nm and 366 nm to determine if any substance showed fluorescence. The chemical developer used was anisaldehyde solution with sulphuric acid (Wagner and Bladt, 1996). The R_f and the color of the developed spots in the sulphuric acid solution of anisaldehyde were used as the evaluation parameters.

Analysis of essential oils

The essential oil extracted was analyzed by gas chromatography coupled to a mass spectrometer (GC-MS).

Results

Epidermis characterization, anatomical and histochemistry characterization

The leaves of the three species are classified as hypoestomatic. *Endlicheria paniculata* (Figure 1a,b, Box 1) and *Nectandra barbellata* (Figure 1c,d, Box 1) showed epidermis with unicellular trichomes, on the adaxial face, these trichomes are located predominantly in the region of the vascular bundles and on the abaxial face, they are distributed all over the surface. *E. paniculata* and *N. barbellta* exhibit walls of the epidermis cells, with the regular contours on both sides of the leaf. In *Ocotea indecora* (Figure 1e,f, Box 1), the epidermis cell wall showed sinuous boundaries on both sides and unicellular trichomes scattered only on the abaxial face of the leaf. The stomata of the three species were characterized as paracytic-type.

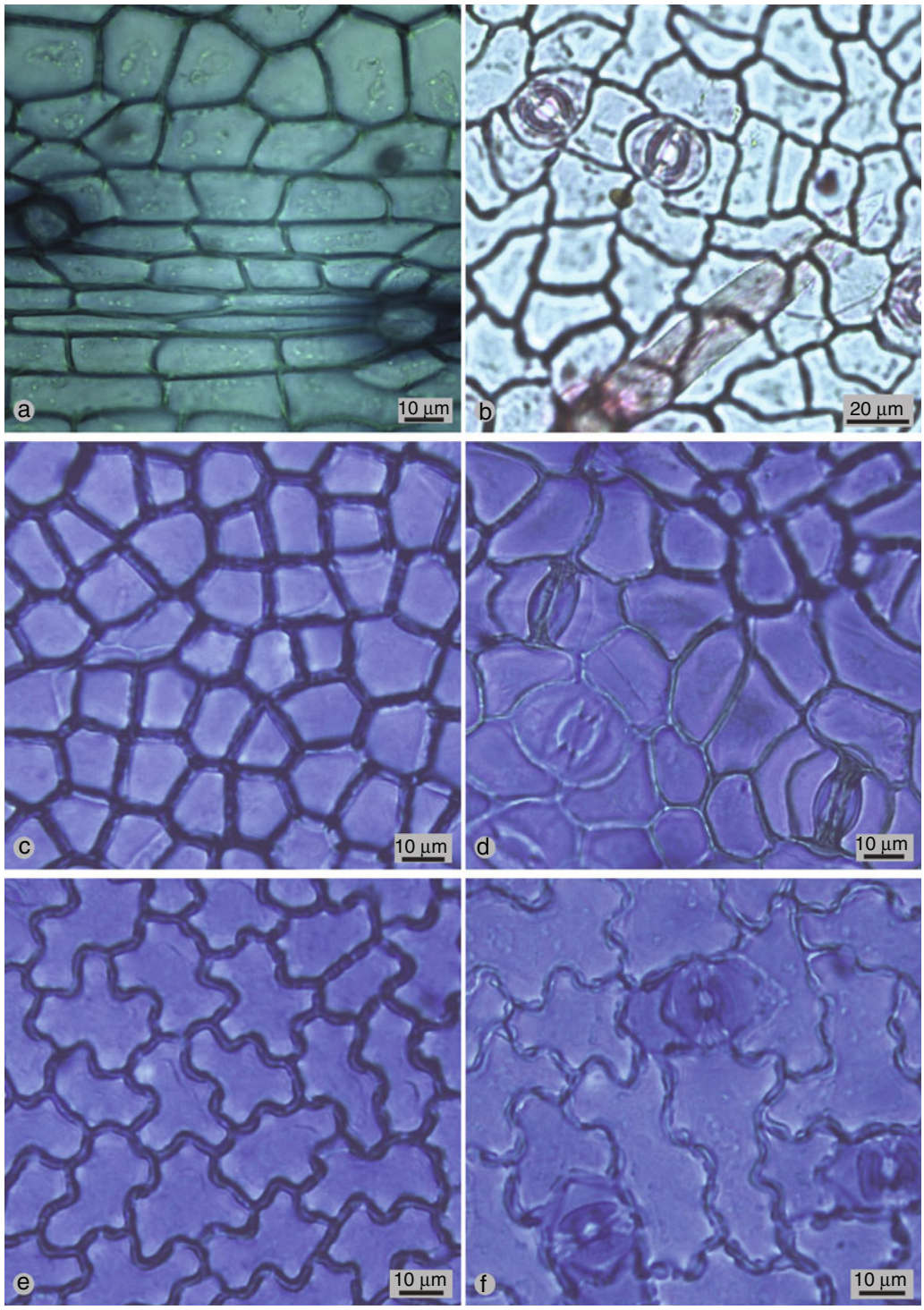

Fig. 1. Epidermis leaves in paradermic view: (a, b) *Endlicheria paniculata*; (c, d) *Nectandra barbellata*; (e, f) *Ocotea indecora*. (a, c, e) Adaxial face; (b, d, f) Abaxial face.

The leaves of *N. barbellata* (Figure 2a,b, Box 1), in cross sections, presents uniseriate epidermis on both sides, covered with a thick cuticle. The mesophyll is dorsiventral and consists of three layers of palisade parenchyma and three to four layers of spongy parenchyma. Collateral type vascular bundles in different sizes were observed along the leaf mesophyll. The midrib of the leaves has a large vascular bundle surrounded by a sclerenchymatous ring followed by homogeneous parenchyma.

Endlicheria paniculata in cross sections (Figure 2c,d, Box 1) presents uniseriate epidermis on both sides, covered with a thick cuticle. The mesophyll is dorsiventral consisting of a layer of palisade parenchyma and five to seven layers of spongy parenchyma. Collateral type vascular bundles of different sizes were observed along the leaf mesophyll. The midrib of leaves has a large vascular bundle surrounded by a sclerenchymatous ring followed by homogeneous parenchyma.

The leaf of *O. indecora*, in cross sections (Figure 2e,f, Box 1), shows uniseriate epidermis, covered with a thick cuticle. The mesophyll is dorsiventral consisting of a palisade layer of seven to eight layers of spongy parenchyma. Collateral type vascular bundles of

Table 1
Compounds presents in essential oil of *Nectandra barbellata*.

Compound	MM (g mol^{-1})	% relative area	IA_{Cal}	IA_{Lit}
α-Pinene	136	2.20	933	932
β-Pinene	136	0.92	977	974
α-Copaene	204	4.94	1378	1374
(E) Cariofilene	204	9.79	1421	1417
α-Humulene	204	3.79	1454	1452
γ-Muurolene	204	1.24	1476	1478
β-Selinene	204	2.24	1485	1489
Bicyclogermacrene	204	1.60	1499	1500
γ-Cadinene	204	1.50	1513	1513
δ-Cadinene	204	11.42	1524	1522
α-Calacorene	200	1.47	1542	1544
Espatulenol	220	3.42	1577	1577
Globulol	222	0.93	1590	1590
Guaiol	222	3.20	1597	1600
<1-*epi*>Cubenol	222	4.26	1628	1628
Aromadendene epoxide <*allo*>	220	1.43	1634	1639
Muurolol<α->(=Torreiol)	222	7.56	1641	1644
Agarospirol	222	2.03	1650	1646
<α->Cadinol	222	4.43	1654	1654
Valerianol	222	2.25	1657	1656

MM = molar mass of the compound, expressed in grams per mol (g/mol); % relative area = area that compound have in the sample, expressed in percentage.

different sizes were observed along the leaf mesophyll. The midrib of the leaves has a biconvex shape and more prominent face abaxial, a large vascular bundle surrounded by a sclerenchymatous ring followed by homogeneous parenchyma.

Since *Nectandra* and *Ocotea* genera are difficult to distinguish using only morphological analysis, *N. barbellata* and *O. indecora* anatomical features (Box 1, Figures 1 and 2) were different in epidermis cells walls boundaries (*O. indecora* showed sinuous and *N. barbellata* regular ones); trichomes (*N. barbellata* present unicellular tricomes in both sides of the leaves and *O. indecora* has scattered trichomes only at abaxial face); palisade parenchyma (*N. barbellata* exhibit three layers and *O. indecora* just one); spongy parenchyma (three to four layers for *N. barbellata* and eight for *O. indecora*) and the midrib (convex for *N. barbellata* and biconvex for *O. indecora*). Regarding of the histochemical tests, *N. barbellata* has positive reaction for midrib idioblasts while *O. indecora* not.

Large secretory cells (idioblasts) were observed between the palisade parenchyma cells for the three species and at the midrib region for *N. barbellata* and *E. paniculata* (Box 2, Figure 2). These cells were evidenced by histochemical tests because their lipid content, as well as, a thick cuticle that covers the entire surface of the leaf epidermis. Starch grains were found only for parenchyma cells around the midrib region (*E. paniculata)* and phenolic compounds were detected for idioblasts in the midrib region and mesophyll cells. Pectin was found on the epidermal walls, midrib collenchyma (*E. paniculata* and *O. indecora*) and mucilages cells in parenchyma (*N. babellata*). The histochemical tests showed, supported by Sudan IV, important groups of lipid substances and it was emphasized in chemical analysis of essential oil.

Scanning electronic microscopy (SEM)

Ultra structural analyses showed that trichomes in the species *E. paniculata* and *O. indecora* are shown only on the abaxial leaf face and species *N. barbellata* presented trichomes on both sides of the epidermis (abaxial and adaxial).

The cuticle of *N. barbellata* (Figure 3a,b) and *E. paniculata* (Figure 3c,d) on both adaxial and abaxial faces was classified as smooth. The cuticle of *O. indecora* (Figure 3e,f) was classified as smooth on the abaxial face and plate-like on the adaxial face. The SEM analysis in this study revealed a wide variety of fungi on both sides of the leaf epidermis of three species.

Chromatographic profile

Preliminary analysis on thin-layer chromatography (Figure 4) showed spots of blue and pink coloration with R_f 0.34; 0.37; 0.47 and 0.79 (Wagner and Bladt, 1996). Comparing with some literature, the R_f are calculated for linalool (R_f = 0.30–0.33, displayed in blue) and eugenol (R_f = 0.47), common compounds to the Lauraceae family, but not confirmed in our study in the CG/ES analysis.

Analysis of essential oils

Tables 1 and 2 described the compounds of essential oils extracted from the leaves of *N. barbellata* and *O. indecora*, respectively. The *E. paniculata* species did not show satisfactory yield of essential oil. The oil of *N. barbellata* had 70.62% of its composition described and the major compound are were δ-cadinene, representing 11.42% of the oil analyzed. The essential oil of *O. indecora* had 99.99% of its composition described and the major compound bicyclogermacrene, representing 29.79% in the aliquot.

Discussion

Moraes and Paoli (1999) described the specie *E. paniculata* as hypoestomatic, showing trichomes on both sides of the epidermis with cells of polygonal adaxial face, almost straight anticlinal walls, and paracitic stomata in abaxial face of epidermis. Ceolin et al. (2009) studied *Nectandra lanceolata* Nees & Mart. and described it as hypoestomatic leaves with paracitic stomata, hairy leaves and yellow trichomes on the abaxial face, epidermis cells with straight walls on both sides. Coutinho et al. (2006b) describes the epidermis of *Ocotea gardneri* (Meisn.) Mez with wavy-walled and anticlinal cells, the leaf is hypoestomatic and the stomata paracytic. Occurrence of epidermal cells with sinuous contours is mentioned for the family (Metcalfe and Chalk, 1988). According to Ceolin et al. (2009) *Ocotea pulchella* (Nees & Mart.) Mez has epidermis cells with sinuous walls in the abaxial face and straight walls in the adaxial face.

Some authors describe characteristics similar to other species of the Lauraceae family. Marques et al. (2004) described leaves of *Beilschmiedia rigida* (Mez) Kosterm. as hypoestomatic, with paracitic stomata type, uniseriate epidermis and mucilage cells in the mesophyll and the midrib. Farago et al. (2005) describes *Ocotea puberula* (Rich.) Nees leaf anatomy as uniseriate epidermis, covered by thick cuticle and trichomes, secretory cells in the mesophyll, collenchyma angular and sclerenchymatic sheath at midrib. Duarte and Oliveira (2006) observed hypoestomatic leaf for *Laurus nobilis* L., uniseriate epidermis, cuticle moderately thickened, paracitic type stomata, tector trichomes, dorsiventral mesophyll, idioblasts and biconvex midrib.

According to Metcalfe (1987), the presence of secretory oily cells and mucilage is common in at least 20 genera of the Lauraceae family and these cells are usually spherical with suberized walls, yellowish content and often with translucent dots on the leaves. Simple trichomes, not flattened and unicellular, are also common for the family (Fahn, 1990). Blum (1999) state that substances, such as phenolic compounds, are able to inhibit absorption of water and nutrients through roots in sensitive plants, one of the criteria established by these substances as evidence of allelopathy.

Metcalfe and Chalk (1979) shows that the species *Laurus nobilis* has thicker and uniform cell walls, which is the most common type in the midrib of leaves of this species. Plants of *Ocotea* genus presents external a thick cuticle at periclinal walls and cuticular flanges at anticlines walls (Toledo et al., 2004). The same was observed in *Ocotea catharinensis* Mez, which also features uniform anticlinal walls at adaxial epidermal cells, with sinuous in some

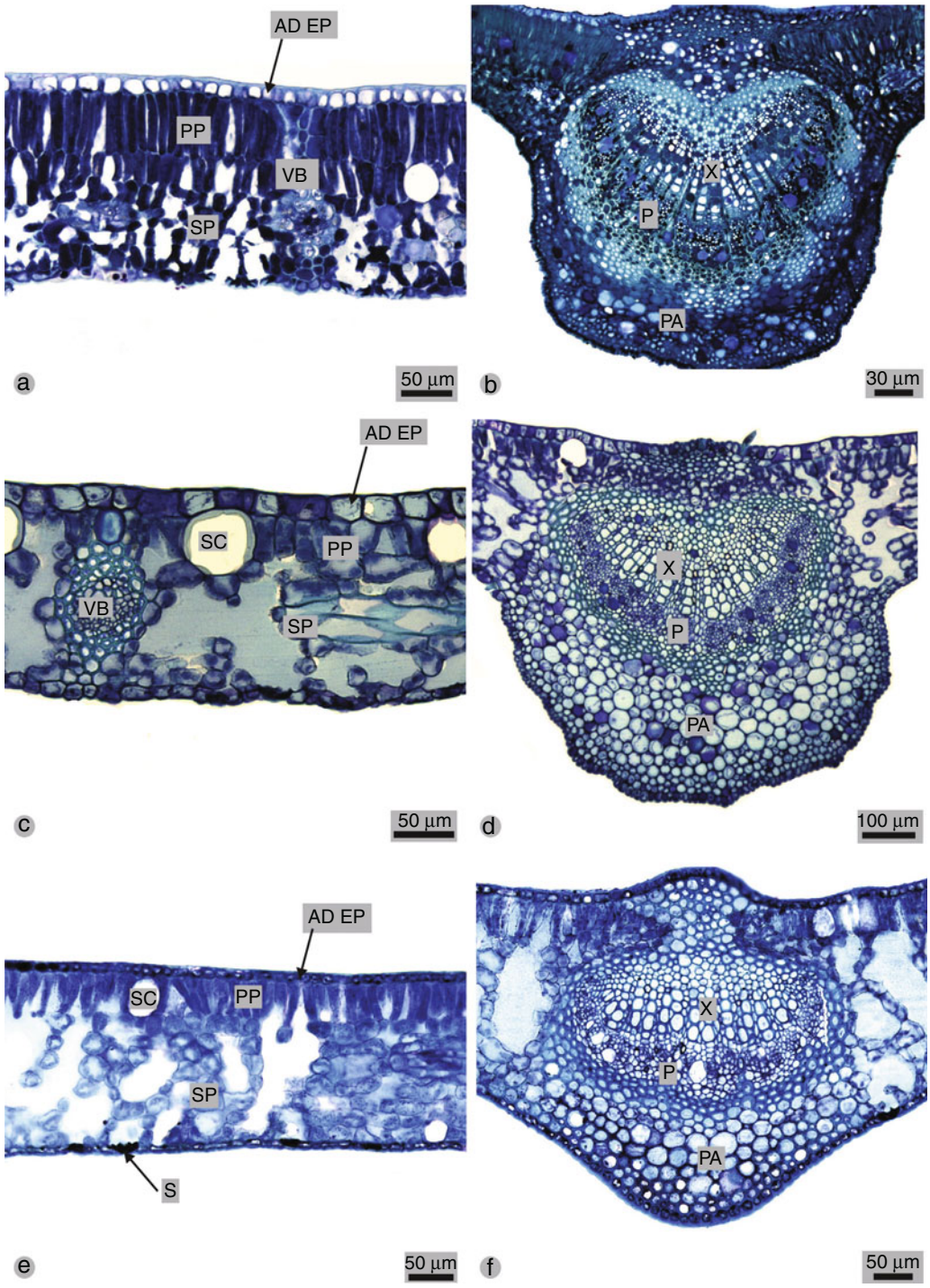

Fig. 2. Transversal cross leaves. (a, b) *Nectandra barbellata*; (c, d) *Endlicheria paniculata*; (e, f) *Ocotea indecora*. (a, c, e) Mesophyll; (b, d, f) Midrib. SC: Secretory cells; S: Stomata; AD EP: Adaxial Epiderm; P: Phloem; VB: Vascular Bundles; PA: Parenchyma; SP: Spongy Parenchyma; PP: Palisade Parenchyma; X: Xylem.

local (Moraes and Paoli, 1999). The Lauraceae family exhibit many fungi species in the leaf surface, that confer a rust appearance in the epidermis (Mendes et al., 1998).

Regarding to essential oil composition the qualitative analysis demonstrated eight compounds commonly for both species. Three chemical substances are unique for *O. indecora* and twelve chemical compounds for *N. barbellata*. One of these twelve unique substances was δ-cadinene, the major compound of *N. barbellata*. Alcântara et al. (2013) studied the essential oil of leaves of *Sextonia rubra* (Mez) van der Werff and described that the α-pinene and β-pinene are its major constituents. Alcântara et al. (2010b) analyzed the genera *Aniba* and *Licaria*, Lauraceae genus, and observed that β-caryophyllene is the most abundant compound in oil from the leaves of *Licaria martiniana* (Mez) Kosterm. and this metabolite influences the aroma and presents biological activities. Chaibub et al. (2013) studied the chemical composition of *Spiranthera odoratissima* A. St.-Hil., Rutaceae, and found that among the major compounds, δ-cadinene (representing 13.40% of oil) and the bicyclogermacrene (representing 14.73% of the oil) in tests for the antimicrobial activity. Silva et al. (2014) studying chemical compounds of *Piper arboretum* Aubl., Piperaceae, reported that its essential oil is composed mostly of bicyclogermacrene (28.7%).

Table 2
Compounds presents in essential oil of *Ocotea elegans*.

Compound	MM (g mol^{-1})	% relative area	IA_{Cal}	IA_{Lit}
α-Pinene	136	5.50	933	932
β-Pinene	136	11.41	977	974
Limonene	136	2.24	1028	1024
<γ->Muurolene	204	3.87	1480	1478
Bicyclogermacrene	204	29.79	1496	1500
Espatulenol	220	11.16	1576	1577
Caryophyllene oxide	220	6.04	1582	1582
Guaiol	222	4.71	1596	1600
Aromadendene epoxide <allo>	220	4.51	1633	1639
Agarospirol	222	5.64	1648	1646
Valerianol	222	15.12	1656	1656

MM = molar mass of the compound, expressed in grams per mol (g/mol); % relative area = area that compound have in the sample, expressed in percentage.

Analyzing metabolic activities studies, Alcântara et al. (2010a) identified twenty compounds with inhibitory acetylcholinesterase activity for stems and leaves of *Rhodostemonodaphne parvifolia* Madriñán through essential oils extraction of techniques. Barbosa-Filho et al. (2008) studied the effect of *Ocotea duckei* oil on cardiovascular system in rats and observed that it causes hypotension and bradycardia in individuals.

Conclusion

The results show that it is possible to distinguish between the three species of the study due to the shape of the stomata, the number of layers of the spongy parenchyma and due to the difference between the essential oil compounds.

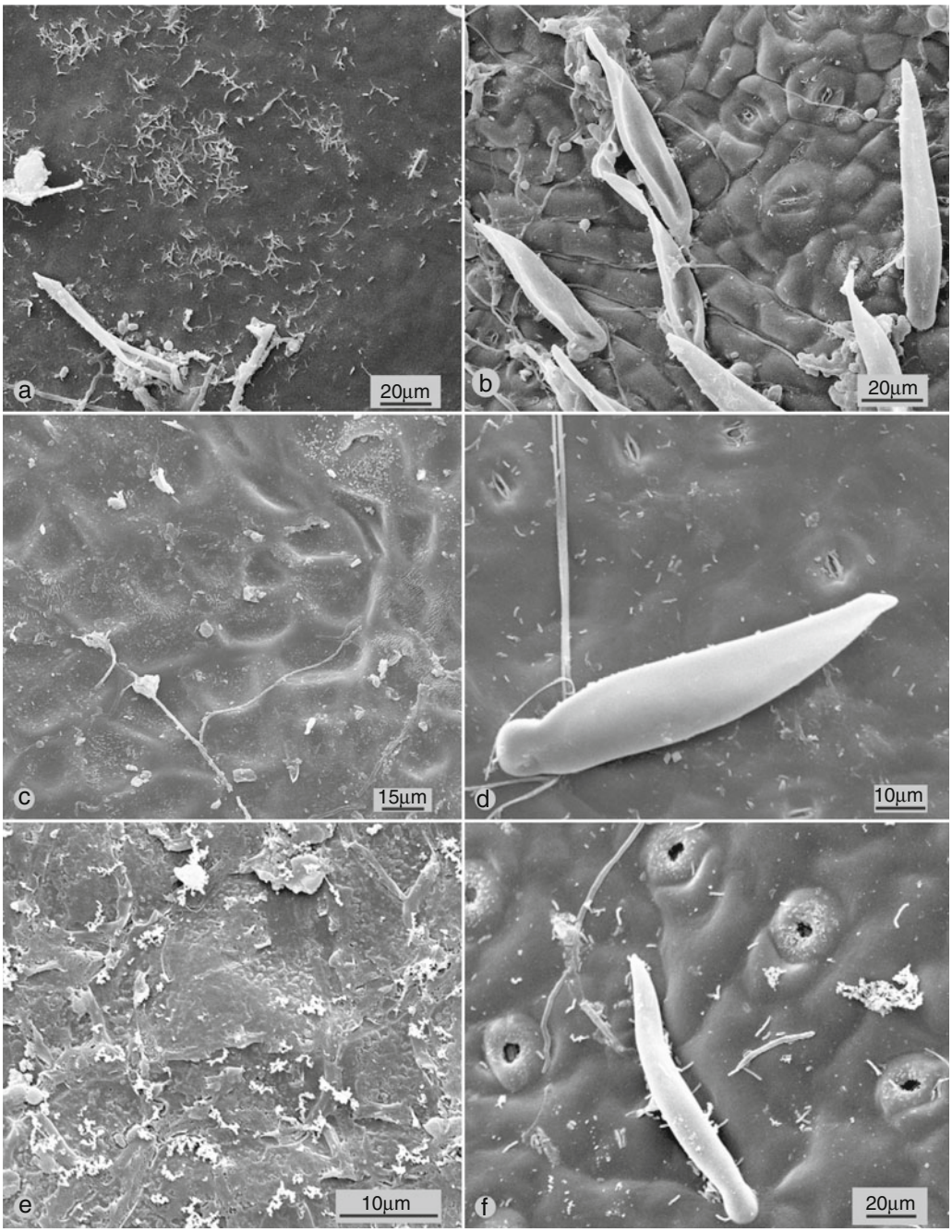

Fig. 3. Eletromicrograph of leaves surface. (a, b) *Nectandra barbellata*; (c, d) *Endlicheria paniculata*; (e, f) *Ocotea indecora*. (a, c, e) Adaxial surface. (b, d, f) Abaxial surface, with detail of the trichomes and stomata. (e) Detail of the cuticle of the adaxial face in plates.

Box 1: Anatomical differences of *Endlicheria paniculata, Ocotea elegans* and *Nectandra barbellata*.

	Endlicheria paniculata	*Nectandra barbellata*	*Ocotea elegans*
Epidermis cell wall	Regular boundaries	Regular boundaries	Sinuous boundaries
Stomata type	Paracytic	Paracytic	Paracytic
Tricomes	Adaxial and abaxial	Adaxial and abaxial	Abaxial face
Palisade parenchyma	One layer	Three layers	One layer
Spongy parenchyma	5–7 layers	3–4 layers	7–8 layers
Midrid	Convex at the abaxial side	Convex at the abaxial side	Biconvex, prominent in abaxial side
Secretory cells	Midrib and mesophyll	Midrib and mesophyll	Mesophyll

Box 2: Histochemistry tests of *Endlicheria paniculata, Ocotea elegans* and *Nectandra barbellata*. The tests used were Sudan IV for the detection of lipidic substances, Ferric Chloride for to evidence phenolic compounds, Iodine Zinc Chloride for starch and Red Ruthenium for to mucilage and pectic substances.

	E. paniculata	*O. elegans*	*N. barbellata*
Sudan IV	+ (M./PA.)	+ (P.P./M.)	+ (P.P./M.)
Ferric Chloride	+ (M.)	+ (M.)	+ (M.)
Iodine Zinc Chloride	+ (M./PA.)	+ (M.)	+ (M.)
Red Ruthenium	+ (M.)	+ (M.)	+ (P.P.)

+ = Positive reaction to the test; M. = Midrib; PA. = Parenchyma; P.P. = Palisade Parenchyma.

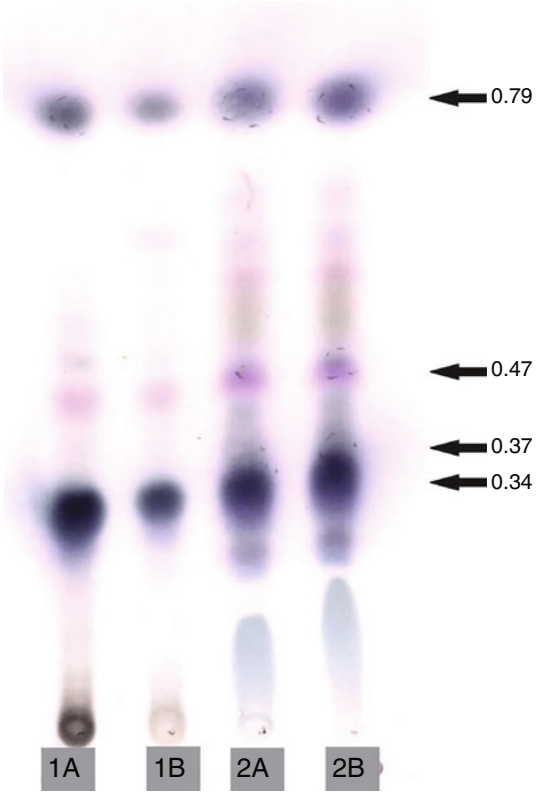

Fig. 4. Thin-layer chromatography of essential oil compounds. (1A, 1B) *Ocotea indecora* essential oil. (2A, 2B) Essential oil of *Nectandra barbellata*. Mobile phase: Dichloromethane. The numbers on the side correspond to the R_f of the main spots.

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Authors' contributions

RAG, ABP, MAO, RTN and ARM contributed in collecting sample, running the laboratory work, analysis the data and writing the section related to information on the anatomy of the leaves of the three species. PFR contributed with the description of the leaves cuticle using SEM. VLG contributed in chemical description of the essential oil extracted of the leaves. RAG and ARM designed the study, contributed to critical reading and final editing of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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