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Original article

Anatomy and volatile oil chemistry of *Eucalyptus saligna* cultivated in South Brazil

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ABSTRACT

Eucalyptus saligna Sm., Myrtaceae, commonly known as Sydney blue gum, is often confused with several other species in the genus. The leaf volatile oils of the species have been reported to have antimicrobial, insecticidal, nematicidal, repellent and cytotoxicity properties. The present work provides anatomy as well as volatile oil chemistry of the species collected from South Brazil. The anatomy and histochemistry of the leaves and stems were investigated by light and scanning electron microscopy, and the leaf and stem volatile oils were analyzed by GC–MS. Amphistomatic leaves, anomocytic stomata, presence of papillae and epicuticular waxes, slightly biconvex midrib with a bicollateral vascular bundle in open arc and two dorsal traces, secretory cavities, calcium oxalate druses and prismatic crystals, rounded petiole with a bicollateral vascular bundle in open arc with invaginated ends and rounded stem with sclerenchyma abutting the internal and external phloem are observed in this species. The main components of the volatile oil were *p*-cymene (28.90%) and cryptone (17.92%). These characteristics can help in the identification and quality control of *E. saligna*.

Introduction

Eucalyptus L'Hér. is one of the principal genera of Myrtaceae. The genus comprises more than 800 species, most are native to Australia (Flores et al., 2016) and many of them are cultivated elsewhere. Species of *Eucalyptus* are used in the production of paper, timber, honey, and volatile oils, which have extensive use in pharmaceutical and perfumery industries (Brooker and Kleinig, 2006; Flores et al., 2016; Barbosa et al., 2016).

Eucalyptus saligna Sm., Myrtaceae, commonly known as "Sydney blue gum" (Ritter, 2014), is a large tree, with a rough and persistent bark. The leaves are petiolate, about 9–19 cm long and 1.8–3.5 cm wide. They are lanceolate or falcate in shape, with acuminate apex, acute to attenuate base (commonly asymmetric), entire margin and prominent reticulate veins (Flores et al., 2016). Several species of *Eucalyptus* are morphologically similar and called by the same common name "eucalipto" in Brazil, causing confusions in the identification. For example, *E. saligna* is often confused with *E. deanei* Maiden, *E. dunnii* Maiden, *E. grandis* W.Hill or *E. botryoides* Sm. (Flores et al., 2016).

Several bioactivities of the volatile oils have been reported for *E. saligna*, such as antimicrobial (Sartorelli et al., 2007; Barbosa et al., 2016), insecticidal (Brooker and Kleinig, 2006; Barbosa et al., 2016), nematicidal (Salgado et al., 2003), repellent (Tapondjou et al., 2005; Ceferino et al., 2006) and cytotoxicity activities (Bhuyan et al., 2017). The biological activities of the species are mainly due to chemical compounds present in the volatile oil (Barbosa et al., 2016). Various studies have shown qualitative and quantitative differences in the volatile oil compositions in *E. saligna* collected from different geographical regions (Barbosa et al., 2016).

Considering the morphological similarities between different species of *Eucalyptus*, and the fact that *E. saligna* shows differences in the chemical composition of volatile oils sourced from different locations, the aims of this study were to illustrate the

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anatomical features of the leaves and stems that can facilitate correct identification of the species and to characterize the volatile oil composition of *E. saligna* collected in Paraná, South Brazil.

Materials and methods

Plant material

Samples of leaves and stems of *Eucalyptus saligna* Sm., Myrtaceae, were collected from plants growing in the campus of the State University of Ponta Grossa, Ponta Grossa, Paraná, Brazil (latitude 25°09′36″ S and longitude 50°10′18″ W) in March 2016. At least six samples of mature leaves (cut from median, intercostal and margin regions) were obtained from the sixth node and below, as well as stem fragments 5–15 cm from the shoot were collected. The plant material was identified using floras (Chippendale, 1988; Boland et al., 2006; Flores et al., 2016) and a voucher specimen was stored the under the number 21836 HUPG in the Herbarium of the State University of Ponta Grossa.

Sample preparation for light microscopy

Leaf and stem samples of *E. saligna* were cut into fragments and fixed in FAA solution consisting of a mixture of 70% ethanol (90%), formaldehyde (5%) and acetic acid (5%) (Johansen, 1940) and stored in 70% ethanol/water solution (Berlyn and Miksche, 1976). Semi-permanent mounts were prepared by free-hand sectioning from the third inferior portion of the midrib of the plant material using razor blades and mounting them on glass slides in a drop of glycerin. The sections were stained using astra blue/basic fuchsine (Roeser, 1962) or toluidine blue (O'Brien et al., 1964). Kraus and Arduin (1997) methods were used to analyze epidermal features. Epicuticular wax classification was based on Barthlott et al. (1998). The preparations were analyzed and photographed using an Olympus CX31 light microscope equipped with a C-7070 digital camera.

Sample preparation for field emission scanning electron microscopy

Fixed samples of leaves and stems were gradually dehydrated by passing through a series of ethanol/water solutions with increasing concentrations of ethanol (80%, 90% and 100%), then dried in a critical point dryer. The dried samples were mounted on aluminum stubs using glued carbon tabs and then sputter coated with gold using a Quorum SC7620 (Quorum Technologies, Laughton, UK) sputter coater. The samples were analyzed and photographed using a Mira3 (Tescan, Brno-Kohoutovice, Czech Republic) field emission scanning electron microscope (FESEM). Qualitative X-ray microanalyses were performed on crystals and in cells without crystals (control) using an EDS (*Energy-dispersive X-ray spectroscopy*) detector attached to the Mira3 SEM. This procedure as well as FESEM and light microscope studies were conducted at the multi-user laboratory (C-Labmu) of the State University of Ponta Grossa, Paraná, Brazil.

Histochemical analyses

Histochemical analyses were carried out using cross-sections of fresh leaves and stems obtained by the same method used in the anatomical assay. The following standard solutions were employed for histochemical tests: Sudan III for lipophilic substances (Foster, 1949), ferric chloride 2% (Johansen, 1940) and potassium dichromate 10% (Gabe, 1968) for phenolic components, phloroglucinol/HCl to test lignin (Sass, 1951) and iodine-iodide for starch (Berlyn and Miksche, 1976). Controls were made in parallel with the tests, and semi-permanent slides were prepared as described above.

Extraction of volatile oil and GC–MS analysis

Dried plant material (300 g) was subjected to hydrodistillation for 4 h, in triplicate, using a modified Clevenger-type apparatus for the extraction of volatile oils. The resultant oils were dried using anhydrous Na_2SO_4 and stored in glass vials with Teflon-sealed caps at 4 ± 0.5 °C with no light.

Volatile oils of *E. saligna* were analyzed on a Shimadzu 2010 Plus gas chromatograph coupled with a Shimadzu TQ8040 mass selective detector and equipped with a Rtx-5MS capillary column (30 m × 0.25 mm × 0.25 μm), operated under programmed temperature from 60 to 250 °C at 3 °C/min and an injector temperature of 250 °C, with an injection volume of 1 μl of the sample (1% (w/v) in hexane), in split mode (ratio 1:40). The interface ion source was at 300 °C, mass range of *m/z* 40–400, using helium as a carrier gas, with a flow of 1.0 ml/min, with the ionization mode: electron impact 70 eV. Quantitative analysis was performed using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector under the same conditions previously described. The relative areas were the average of triplicate analysis.

Experimental retention indices (RI) were calculated by injection of *n*-alkane series standard from nine to twenty carbon atoms and volatile oil samples under the same conditions. The identification of the components was based on the comparison of the RI, and mass spectra of each substance with spectra from the NIST02 library and with literature data (Adams, 2007). The identification of the main compound of volatile oil was confirmed using the standard of *p*-cymene. This analysis was carried out at the Federal University of Paraná. The results are shown in Table 1.

Results and discussion

Anatomical studies

In *E. saligna*, leaf (Fig. 1a) the adaxial and abaxial epidermises show straight anticlinal walls (Fig. 1b and c) and are covered externally by smooth cuticle (Fig. 1g). Anomocytic stomata are present on both adaxial (Fig. 1b) and abaxial (Fig. 1c) epidermises, characterizing the leaf as amphistomatic. The average size of stomata

Table 1
Chemical compounds in the volatile oil of *Eucalyptus saligna*.

Compound	RI cal.	RI lit.	Peak area (%)	Identification
α-Thujene	927	924	0.94	RI, MS
α-Pinene	933	932	1.04	RI, MS
α-Phellandrene	1006	1002	1.04	RI, MS
α-Terpinene	1017	1014	0.57	RI, MS
p-Cymene	1025	1020	28.90	RI, MS
Sylvestrene	1029	1025	4.89	RI, MS
1,8-Cineole	1032	1026	2.15	RI, MS
p-Cymenene	1091	1089	0.80	RI, MS
Sabina ketone	1152	1154	2.25	RI, MS
Terpinen-4-ol	1179	1174	5.33	RI, MS
Cryptone	1188	1183	17.22	RI, MS
α-Terpineol	1193	1186	0.73	RI, MS
Cuminaldehyde	1242	1238	5.25	RI, MS
trans-Ascaridol glycol	1277	1266	7.32	RI, MS
Thymol	1305	1289	0.89	RI, MS
Spathulenol	1582	1577	4.84	RI, MS
Compounds identified			84.16	RI, MS
Monoterpene hydrocarbons			38.18	RI, MS
Oxigenated monoterpenes			41.14	RI, MS
Sesquiterpenes			4.84	RI, MS

RI lit., retention index literature from Adams (2007); RI calc, calculated retention index; MS, mass spectra from NIST02 library.

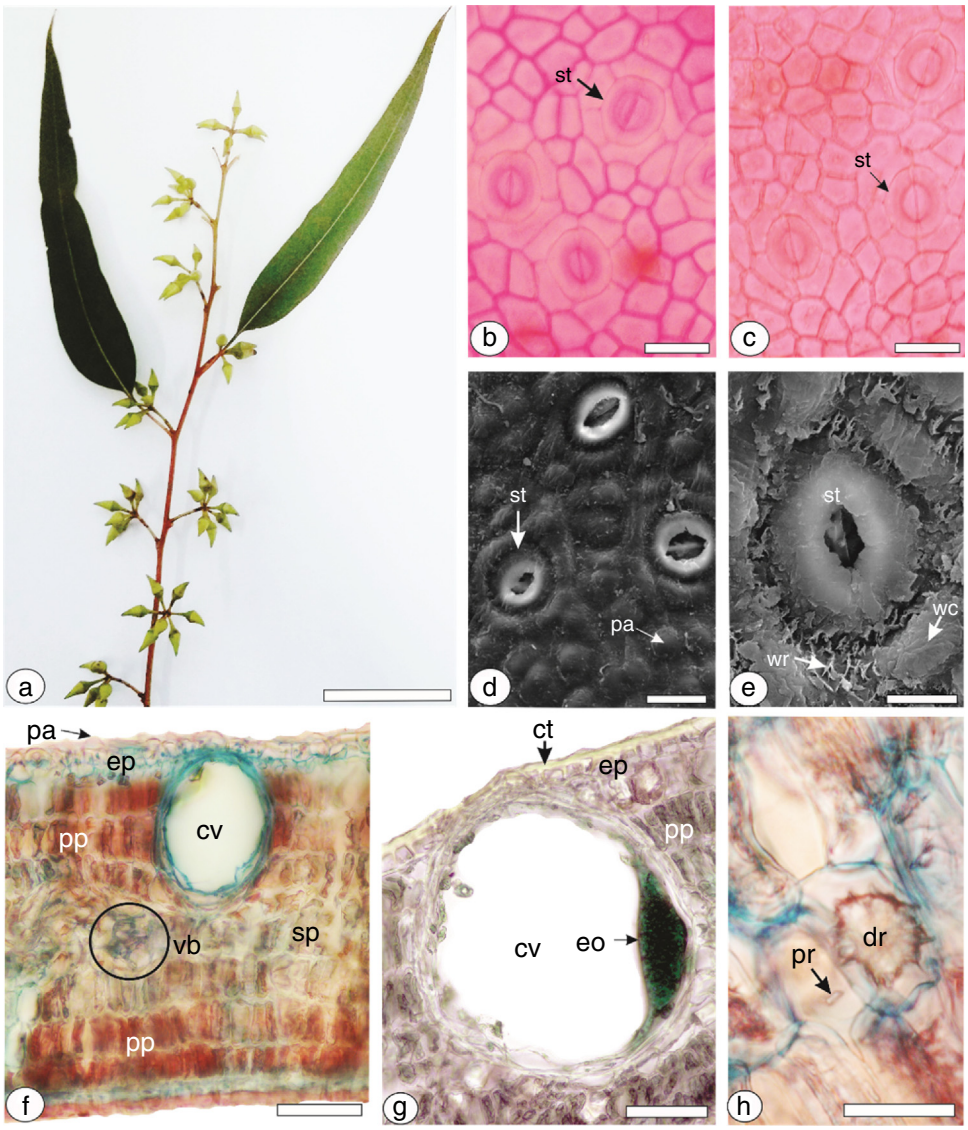

Fig. 1. Morpho-anatomy of leaves of *Eucalyptus saligna* [b, c, f, g, h: light microscopy; d, e: FESEM]. (a) A twig with leaves and flower buds. (b–e) Leaf epidermis in surface view (b, d: adaxial side; c, e: abaxial). (f–h) Transverse section of leaves showing secretory cavity (cv), prismatic crystal (pr) and druse (dr) [cv, secretory cavity; dr, druse; eo, volatile oil; ep, epidermis; pa, papillae; pp, palisade parenchyma; pr, prismatic crystal; sp, spongy parenchyma; st, stomata; vb, vascular bundle; wc, waxes in crusts; wr, waxes in rosettes]. Scale bars: a = 5 cm, f, g and h = 50 μm, b, c, d = 25 μm, e = 10 μm.

is 31 μm × 27 μm. The average stomatal index calculated for the adaxial side is 6 and 8 for the abaxial side. Different stomatal index was found in *E. saligna* analyzed with 120 days old, 0.35 and 15.55 for adaxial and abaxial, respectively (Santos et al., 2008).

In *Eucalyptus*, anomocytic (Santos et al., 2008; Döll-Boscardin et al., 2010) and anisocytic stomata (Tantawy, 2004) can be found, the former type being more common. Several species of *Eucalyptus* have amphistomatic leaves (Santos et al., 2008; Döll-Boscardin et al., 2010). However, hypostomatic leaves may also be observed (Malinowshi et al., 2009).

The cuticle can have a flat surface, ridges or papillate and wax can be deposited on top of and in the cuticle, showing great micromorphological diversity (Barthlott et al., 1998; Upton et al., 2011). *E. saligna* shows papillae on the adaxial side (Fig. 1d and f). Several species of this genus show papillae, such as *E. grandis*, *E. pellita* F.Muell. and *E. pilularis* Sm. (Santos et al., 2008; Guzmán et al., 2014).

Epicuticular waxes occur in crystalloid (rosettes) and crust-like forms (Fig. 1e) on the abaxial side. The leaves of *E. saligna* are clearer on the abaxial side (Flores et al., 2016), probably due to the presence of waxes. There is no previous information about the shape of the epicuticular waxes in *E. saligna*. The waxes are found as parallel-stacked platelets in *E. yalatensis* Boomsma (Knight et al., 2004) and filamentous crystalloid type in *E. globulus* var. *bicostata* (Maiden, Blakely & Simmonds) Ewart (Malinowshi et al., 2009). The presence and the type of the epicuticular waxes can help in species of *Eucalyptus* identification.

The leaf of *E. saligna*, in cross-section, has a unilayered epidermis with cells varying from tabular to round shapes and covered with a thick cuticle (Fig. 1f and g). Mesophyll is isobilateral, consisting of 1–3 layers of palisade parenchyma on either side and about two layers of spongy parenchyma in the median region. Minor collateral vascular bundles traverse the spongy tissue and are surrounded by a parenchymatous sheath (Fig. 1f). Minute prismatic crystals and druses are found in the mesophyll (Fig. 1h).

Isobilateral mesophyll is common in *Eucalyptus* (Santos et al., 2008; Iftikhar et al., 2009; Malinowshi et al., 2009; Döll-Boscardin et al., 2010). However, dorsiventral type can also be observed, as in *E. globulus* subsp. *bicostata* (Malinowshi et al., 2009), *E. grandis* and *E. urophylla* S.T.Blake (Santos et al., 2008).

The type of mesophyll and the number of layers of palisade and spongy parenchyma aid in the differentiation of *Eucalyptus* species (Santos et al., 2008). However, leaf anatomy can be altered by edaphic factors. For example, *E. camaldulensis* Dehnh. develops thicker leaves when it grows in arid environments (James and Bell, 1995). In *E. globulus* Labill., Johnson (1926) has observed dorsiventral mesophyll in some young leaves and isobilateral mesophyll in mature leaves.

Volatile oils can be obtained from different parts of plants depending upon the producing species. The volatile oils are stored in various types of secretory structures, such as secretory cells, glandular trichomes, secretory ducts, and secretory cavities (Barbosa et al., 2016). Species of *Eucalyptus* are aromatic due to the presence of volatile oil produced and stored in secretory cavities in the mesophyll, midrib (Santos et al., 2008) and in the cortex (Brisola and Demarco, 2011). In *E. saligna*, the secretory cavities are found in the mesophyll, especially in the sub-epidermal region, on both sides of the leaves. They are circular or oval in shape and measure 40–140 μm in diameter (Fig. 1f and g).

In cross-section, the midrib is slightly convex on both sides (Fig. 2a). This shape has also been observed in *E. pyrocarpa* L.A.S. Johnson & Blaxell by Santos et al. (2008). However, flat-convex shape has been reported for several species of *Eucalyptus*, such as *E. grandis*, *E. resinifera* Sm. and *E. urophylla* (Santos et al., 2008). The single-layered epidermis is covered by a thick and smooth cuticle (Fig. 2a and c). The cells are rounded in shape on both sides (Fig. 2a and c). Underlying the epidermis is angular collenchyma region, composed of about four layers on both faces. Idioblasts containing druses and prismatic crystals are found in this region (Fig. 2c and d). Phenolic compounds are observed in the phloem as well as in the cells adjoining the sclerenchyma sheath (Fig. 2a–c).

The vascular system is bicollateral, formed by a central slightly flat arc and two dorsal bundles (Fig. 2a). An incomplete sheath of sclerenchyma composed of up to 5 layers of fibers with different stages of lignification, surrounds the vascular system (Fig. 2a–c). Different organizations of midrib vascular systems are found in the genus *Eucalyptus*, such as, flat arc in *E. pellita* and *E. grandis*, flat arc with invaginated ends and dorsal trace in *E. resinifera*, and siphonostele type in *E. pyrocarpa* (Santos et al., 2008). These aforementioned authors found different vascular organization to *E. saligna* as follow, pattern arc with invaginated ends and dorsal trace type; however, they analyzed the leaves from the median portion of the midrib. In the present study, leaves were analyzed in the third inferior part of the midrib. Anyway, the midrib shape and the vascularization pattern can be anatomical markers, supporting the differentiation of species.

The petiole of *E. saligna*, sectioned transversely in the medial portion, showed rounded shape (Fig. 3a). The epidermal cells are anticlinally slightly elongated and covered externally by thick cuticle (Fig. 3d). Beneath the epidermis, up to seven layers of angular collenchyma are present (Fig. 3c and d). Several secretory cavities, similar to those found in the leaf blade, are observed in the ground parenchyma (Fig. 3a and c). Prismatic crystals are frequently observed in the collenchyma (Fig. 3d and e). The vascular system is bicollateral and is represented by a flat arc with invaginated ends (Fig. 3a). According to Cronquist (1981), the presence of a bicollateral vascular system in the petioles is a feature of the family Myrtaceae.

In an incipient secondary structure, the stem of *E. saligna* is circular in shape (Fig. 4a). The epidermis is uniseriate and formed by rectangular and square-shaped cells, and covered by thick cuticle (Fig. 4e). The genus *Eucalyptus* has variable stem shapes, such as circular in *E. urophylla* and rectangular in *E. grandis* (Brisola and Demarco, 2011). According to Bryant and Trueman (2015), the stems of *E. grandis* are stellate in cross-section near the shoot apex but they develop a more rectangular shape as the vascular tissues develop and the stems enlarge radially.

The cortex is made up of up to twelve layers of parenchyma cells with rounded shape (Fig. 4b), measuring about 5–6 μm in diameter; most of the cells are filled with phenolic compounds (Fig. 4b and c). Brisola and Demarco (2011) have observed 10–15 layers of parenchyma cells in the cortex in *E. grandis* and 6–10 layers in *E. urophylla*. These authors have observed a few idioblasts containing tannins in *E. grandis* and a large amount of these in *E. urophylla* (Brisola and Demarco, 2011).

Several prismatic crystals (Fig. 4b, c and e–g) of varying sizes and shapes are found scattered in all tissues of the stem. Secretory cavities (Fig. 4a, c and e), measuring 40–110 μm in diameter, are mainly distributed in the cortex and sometimes occupy the entire cortex region as observed in Fig. 4e. Secretory cavities in the stems of *E. grandis* and *E. urophylla* measure 78 μm and 45 μm in diameter, respectively (Brisola and Demarco, 2011).

The vascular system presents a bicollateral arrangement. Both the external and internal phloem are composed of sieve elements, parenchyma cells and phenolic idioblasts. A discontinuous sclerenchymatous ring of sclereids of about 5 μm in diameter is found adjoining the external and internal phloem. The xylem is formed by vessel elements arranged in radial rows separated by lignified parenchyma cells (Fig. 4b, d and h). Pith occupies the central part of the stem and is composed of thin-walled parenchyma cells. Prismatic crystals and druses are commonly observed in the pith (Fig. 4d).

Formation of sclerenchyma cells along the periphery of the vascular system is apparent in the species of *Eucalyptus*, such as *E. tetrodonta* F.Muell., *E. pilularis* and *E. nitens* (H.Deane & Maiden) Maiden (Bryant and Trueman, 2015). Brisola and Demarco (2011) have observed important anatomical differences in the young stems of *E. grandis*, *E. urophylla* and the hybrid *E. grandis x urophylla*. These authors have stated that the stem shape, the presence of sclerenchyma and phenolic idioblasts are helpful in the differentiation of the three taxa.

In transverse section, the vascular tissue of *E. saligna* has a rectangular shape (Fig. 4a), as also described for *E. microcorys* F.Muell., *E. marginata* Donn ex Sm. and *E. grandis* by Bryant and Trueman (2015). These authors have also indicated that the shape of the vascular tissue, as observed near the fourth node, is variable in the species of *Eucalyptus* such as rectangular or slightly stellate in *E. camaldulensis*, *E. globulus* and *E. nitens*, and rectangular/circular in *E. tetrodonta* and *E. pilularis*.

The histochemical tests have aided in the localization of lipophilic, lignified and phenolic compounds and starch grains in *E. saligna*. Lipophilic compounds were detected in the cuticle (Fig. 5a) and in the secretory cavities (Fig. 5b), using Sudan III. Total lipids were also found in the lumen cavities and in cuticles in seven species of *Eucalyptus*, *E. grandis*, *E. pellita*, *E. pilularis*, *E. pyrocarpa*, *E. resinifera*, *E. saligna* and *E. urophylla* (Santos et al., 2008). Cutin is the main component of the cuticle and is a hydrophobic lipid polymer that help the plant to avoid losing water (Riederer and Schreiber, 2001).

In *Eucalyptus* species, volatile oils are stored in secretory cavities (Santos et al., 2008) and they are considered as signals of chemical communication with other plants and as chemical protection against animals (Gottlieb and Salatino, 1987). Besides that, they can be associated with the strategy of reducing excessive loss of water, acting as a thermal isolating agent (Craveiro and Machado, 1986).

Phenolic components were detected using ferric chloride (Fig. 5c, d and e) and potassium dichromate (Fig. 5f) solutions. They were found in the epidermis and mesophyll of the leaf blade, and in the epidermis, ground parenchyma and in the phloem of the midrib

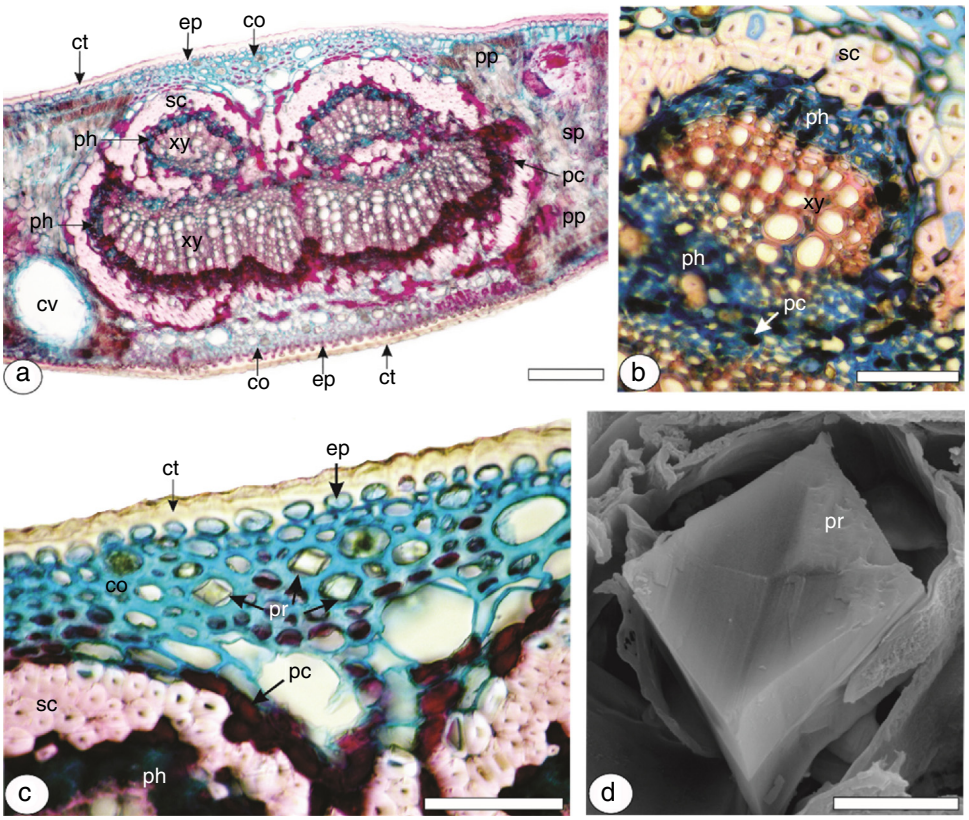

Fig. 2. Midrib anatomy of *Eucalyptus saligna* [a, b, c: light microscopy; d: FESEM]. (a–c) Transverse sections. (d) View of a prismatic crystal [co, collenchyma; ct, cuticle; cv, secretory cavity; ep, epidermis; sp, spongy parenchyma; pc, phenolic compounds; ph, phloem; pp, palisade parenchyma; pr, prismatic crystal; sc, sclerenchyma; xy, xylem]. Scale bars: a and b = 100 μm, c = 50 μm, d = 5 μm.

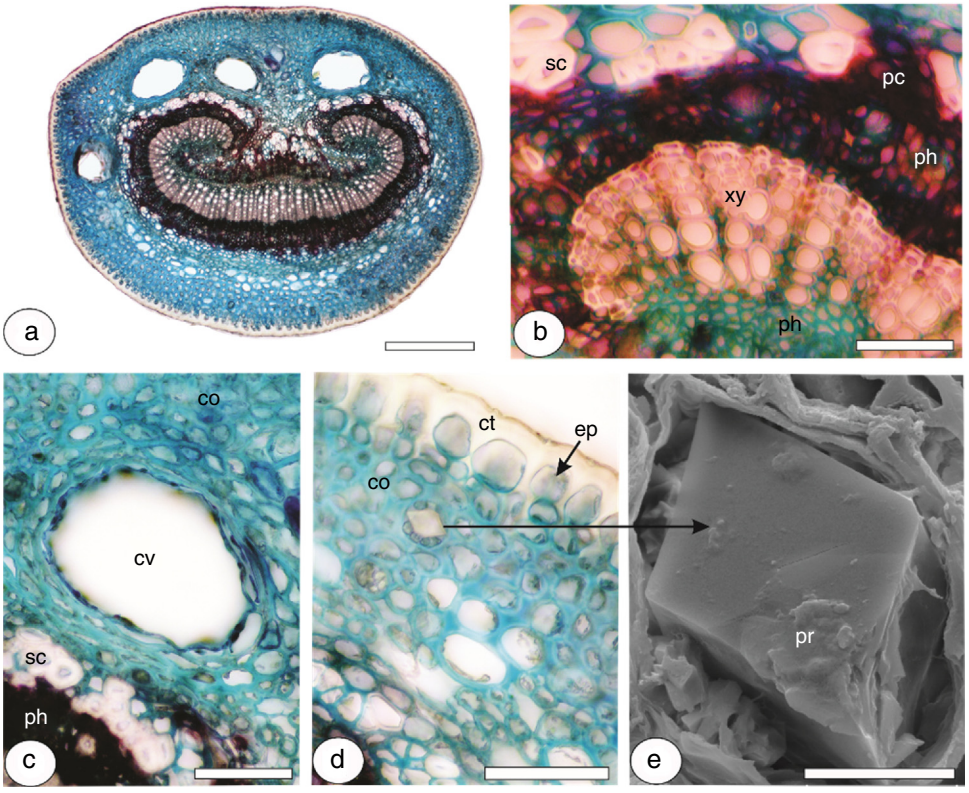

Fig. 3. Petiole anatomy of *Eucalyptus saligna* [a–d: light microscopy; e: SEM]. (a–d) Transverse sections; (e) view of a prismatic crystal [co, collenchyma; ct, cuticle; cv, secretory cavity; ep, epidermis; ph, phloem; pr, prismatic crystal; sc, sclerenchyma; xy, xylem]. Scale bars: a = 200 μm, b–d = 50 μm, e = 10 μm.

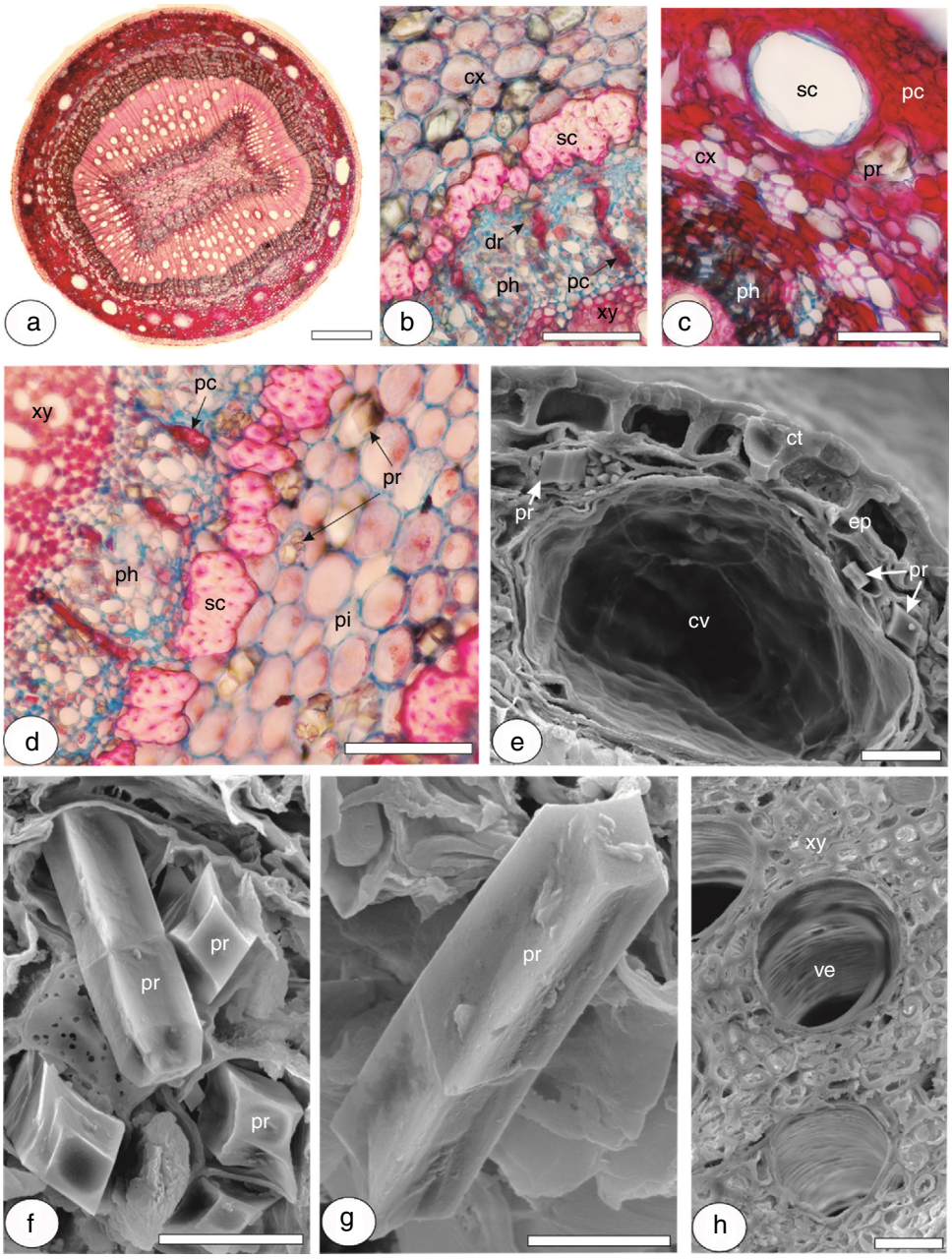

Fig. 4. Stem anatomy of *Eucalyptus saligna* [a, b, c, d: light microscopy; e, f, g, h: FESEM]. (a–e, h) Transverse sections; (f and g) view of prismatic crystal [ct: cuticle; cv: secretory cavities [cx, cortex; dr, druse; ep, epidermis; pc, phenolic compounds; ph, phloem; pi, pith; pr, prismatic crystal; sc, sclerenchyma; ve, vessel element; xy, xylem]. Scale bar: a = 200 μm, b–d = 50 μm, e, h = 20 μm, f = 10 μm, g = 5 μm.

(Fig. 5c). In the petiole, phenolic compounds were also observed in the phloem and near the sclerenchyma (Fig. 5d and e).

In the same way, Santos et al. (2008), using potassium dichromate solution, evidenced phenolic compounds in the mesophyll and in the epidermis of leaves of *Eucalyptus* species. The aforementioned authors also used vanillin-hydrochloric acid in the histochemical test and verified the presence of tannins in the mesophyll of *E. pilularis*, *E. pyrocarpa* and *E. saligna* and in the epidermis of *E. pilularis*. Phenolic compounds are responsible for resistance to hostile environmental factors and in the defense of plants against insect herbivores and fungal pathogens (Lattanzio et al., 2006).

Lignified elements, which reacted with phloroglucinol/HCl, are found in the discontinuous sclerenchymatous ring adjoining the phloem, in addition to xylem in the leaves and stems (Fig. 5g). Disagreeing from this, Santos and co-workers (2008) obtained negative results when used phloroglucinol to test leaves of *E. saligna* and other species of *Eucalyptus*. However, these species were analyzed with 120 days old. Lignin is a polymer high in phenolics, which confer structural integrity to the cell wall, stiffness and strength of the stem, waterproofs the cell wall, permitting transport of water and solutes through the vascular system, and protecting plants against pathogens (Boerjan et al., 2003).

Starch grains react positively with iodine solution, are small and rounded, occur in aggregates of two or more granules and are found in the endodermis of the stem. Starch grains were detected in the mesophyll of seven species of *Eucalyptus* (Santos et al., 2008), feature that was not confirmed in this study. Starch is widely distributed throughout plant tissues, but is commonly found in highest concentrations in roots, stems, rhizomes, and fruits (Upton et al., 2011).

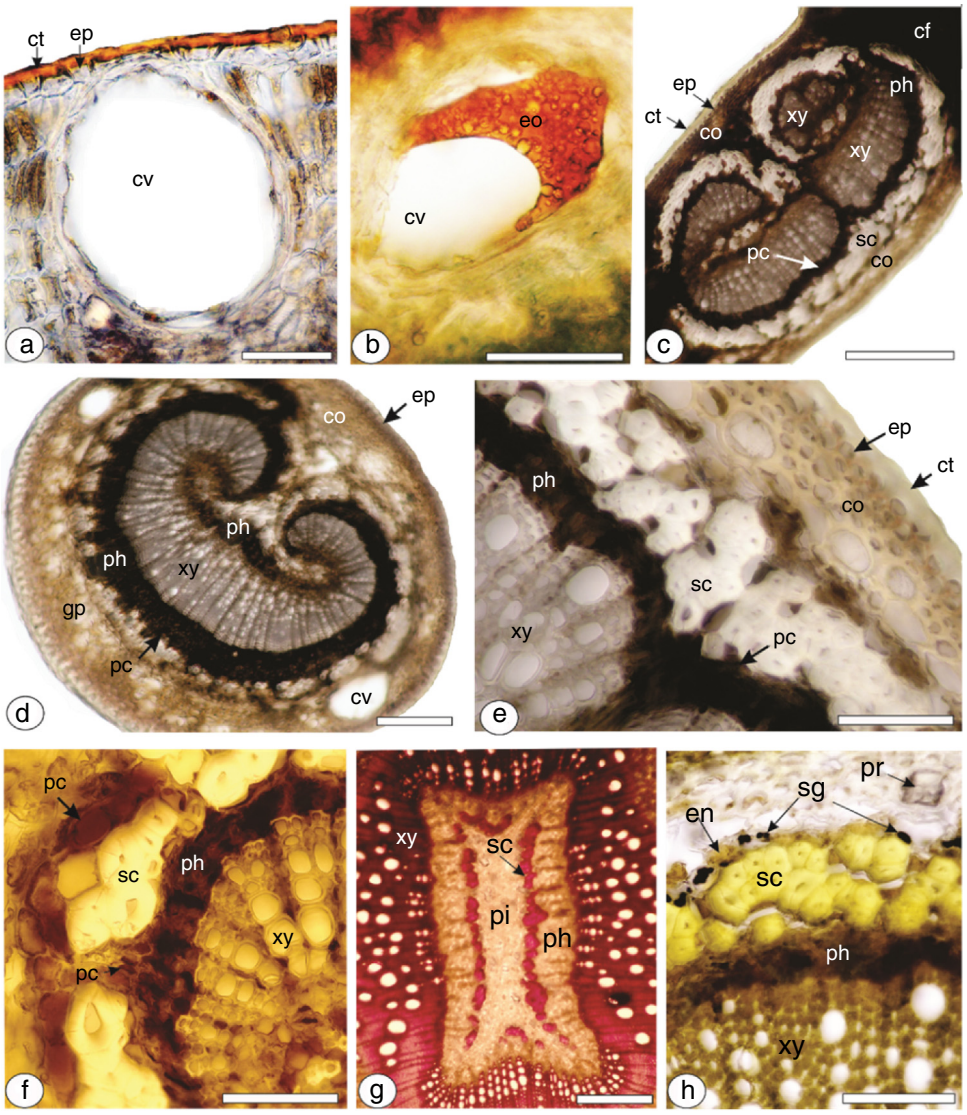

Fig. 5. Histochemistry of *Eucalyptus saligna* [a, b: Sudan III; c, d, e: ferric chloride; f: potassium dichromate solution (10%); g: phloroglucinol/HCl; h: 1% iodine solution]. Transverse sections a–c: leaf; d, e: petiole; f, g, h: stem [co, collenchyma; ct, cuticle; cv, secretory cavities; en, endodermis; eo, volatile oil; ep, epidermis; gp, ground parenchyma; pc, phenolic compounds; ph, phloem; pi, pith; pr, prismatic crystal; sg, starch grains; sc, sclerenchyma; xy, xylem]. Scale bar: a = 200 μm, b–d = 50 μm, e = 10 μm.

Two crystalline morphotypes are found in *E. saligna*, namely, prismatic crystals and druses. These are present in the mesophyll (Fig. 1h), midrib (Fig. 2c and d), petiole (Fig. 3c, d and e) and stem (Figs. 4b and d–h). The presence of crystals is common in *Eucalyptus* (Santos et al., 2008; Malinowshi et al., 2009; Döll-Boscardin et al., 2010; Brisola and Demarco, 2011).

Even though the size and quantity of crystals vary among different taxa, the shape and location of the crystals within a taxon are frequently very specific and may be considered as a taxonomic feature. The plant may produce a single type or multiple types of crystals throughout the plant, multiple types in different organs or multiple types in the same organ but in different tissues. Additionally, there are several functions of crystals in plants, such as tissue stiffness, ion balance, plant protection and detoxification (Franceschi and Nakata, 2005).

The crystals were analyzed for their elemental chemical composition using an EDS. The EDS spectrum of a prismatic crystal (Fig. 6a) shows prominent peaks of calcium (37.18%), carbon (13.09%) and oxygen (49.73%). Whereas, the EDS spectrum of a druse (Fig. 6b) shows major peaks of calcium (42.23%), carbon (11.17%) and oxygen (46.60%). The chemical composition confirms that these crystals are of calcium oxalate. In general, calcium salts may precipitate in the form of oxalate, phosphate, malate, sulfate, silicate, carbonate or citrate (Weiner and Dove, 2003); however, calcium oxalate seems to be more common in vegetable species (Santos et al., 2008; Malinowshi et al., 2009; Döll-Boscardin et al., 2010; Andrade et al., 2017; Santos et al., 2018). The major unlabeled peaks represent gold element used to spraying the samples.

Yield and chemical composition of volatile oil

Volatile oil extracted by hydrodistillation of the leaves and stems of *E. saligna* was light yellow with strong and characteristic odor. Similar characteristics were observed in *E. benthamii* by Döll-Boscardin and co-workers (2010). Volatile oils of *Eucalyptus* are aromatic, spicy, and light yellow or colorless, yet brownish or greenish were also reported (Araújo et al., 2010).

In Brazil, *E. citriodora* Hook. (1.0–1.6%), *E. globulus* (1.7–5%) and *E. staigeriana* F.Muell. ex F.M.Bailey (1.2–1.5%) are the main species present the greatest yield in volatile oils from the leaves (Vitti and Brito, 2003). In the present study, the yield of volatile oil from *E. saligna* was 1.03% (v/w). But, Bett et al. (2016) reported only 0.38%

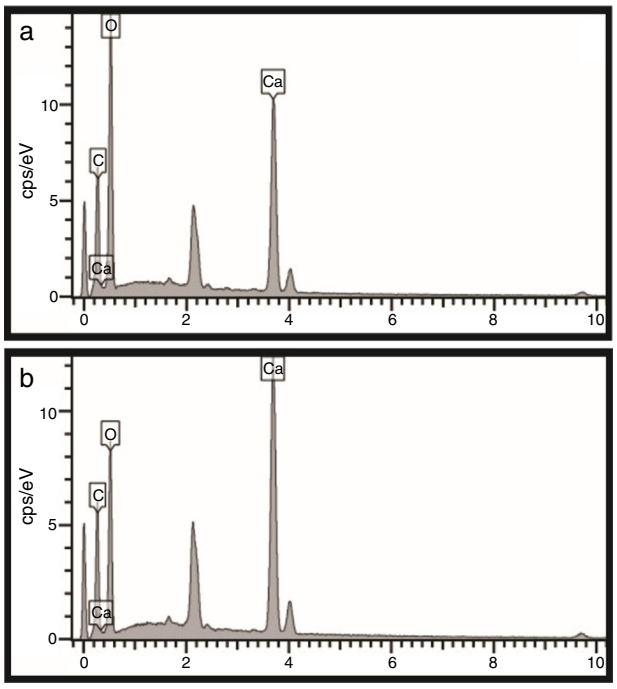

Fig. 6. EDS spectrum of prismatic (a) and druse (b) crystals of *Eucalyptus saligna*.

(v/w) of yield for this species. The difference in the volatile oil yield within species may be due to environmental and edaphic factors, the methods applied for volatile oil extraction and storage conditions (Brooker and Kleinig, 2006; Lemos et al., 2012). However, the aforementioned authors (Bett et al., 2016) extracted the volatile oil only from the leaves of *E. saligna*. In the present study, mixtures of leaves and stems were used for extraction. The anatomical study showed several large secretory cavities in the cortex of the stem (Fig. 4a, c and e) which may have contributed to the increased yield.

Table 1 shows retention time (min), retention index, chemical identity and relative percentage (%) concentration of chemical constituents of *E. saligna*. The GC–MS analysis of the volatile oil revealed identification of sixteen compounds corresponding to 84.16% of the total number of compounds in the volatile oil. A comparison of the chemical groups in the volatile oil of *E. saligna* shows a high fraction of monoterpenes (79.32%), of which 41.14% are oxygenated monoterpenes. The main components of the volatile oil were *p*-cymene (28.90%) and cryptone (17.92%).

Differences in the volatile oil composition of *E. saligna* have been reported for the materials collected from Busia in Kenya, in which the major components of the volatile oil were 1,8-cineole (24.26%), *o*-cymene (9.92%) and α-terpineol (8.81%) (Bett et al., 2016). Volatile oil obtained from *E. saligna* growing in Cameroon contained 1,8-cineole (45.2%), *p*-cymene (34.4%) and α-pinene (12.8%) as the main components (Mossi et al., 2011). Volatile oil of *E. saligna* collected in Argentina evidenced a very high percentage of 1,8-cineole (93.2%) (Toloza et al., 2006).

There are also considerable differences in the chemical composition of volatile oil from the leaves of *E. saligna* collected in different locations within Brazil. For instance, the volatile oil of leaf materials collected from Goiás State is rich in *p*-cymene (25.6%), α-terpineol (9.3%), α-camphonellal (8.0%) and 1,8-cineole (6.2%) (Estanislau et al., 2001); volatile oil of the leaves obtained from Minas Gerais State has 92.3% α-pinene (Filomeno et al., 2008); materials from São Paulo State contain α-pinene (45.1%), *p*-cymene (22.5%), α-pinene oxide (11.3%) (Sartorelli et al., 2007); and α-pinene (25.9%), *p*-cymene (24.4%), γ-terpinene (24.6%) are the main compounds in the materials collected also from São Paulo State (Batista-Pereira et al., 2006).

Barbosa and co-workers (2016) have reported that *E. saligna* is widely cultivated in Brazil for cellulose pulp production and is composed of various chemotypes, some of them rich in 1,8-cineole. Volatile oil from the leaves of *E. saligna* presented higher concentration of α-pinene during blossoming and *p*-cymene during vegetative phase (Sartorelli et al., 2007). In the present study, volatile oils from leaves and stems of *E. saligna* evidenced lower 1,8-cineole concentration (2.18%).

In the present study, *p*-cymene (28.90%) has been found as the main compound in *E. saligna*. This compound is also found in *E. camaldulensis* (17.9%) (Lucia et al., 2008) and in *E. tereticornis* (22%) (Toloza et al., 2006). Some studies indicate a possible relationship of this compound with fungicidal activity (Lopez-Reyes et al., 2010; Camele et al., 2012; Elshafie et al., 2015). Santana et al. (2011) have observed in an *in vivo* study that *p*-cymene reduces orofacial nociceptive response, and may represent an important biomolecule in the treatment of pain in the orofacial region. Other studies have attributed potential anti-inflammatory activities to this compound (Chen et al., 2014; Zhong et al., 2013).

The second major component of the volatile oil of *E. saligna* was cryptone (17.92%). *Eucalyptus odorata* Behr presented 20.9% of cryptone (Elaissi et al., 2011) while *E. deglupta* Blume and *E. urophylla* showed 25 and 4%, respectively (Cimanga et al., 2002). Coffi and co-workers (2012) have reported cryptone in volatile oil from the leaves of *E. camaldulensis* collected in Argentina (5.71%,) and Australia (9.81%). This compound has potential antibacterial and fungicidal activities (Elaissi et al., 2011, 2012).

Conclusion

The anatomical features highlighted in this study include amphistomatic leaves, anomocytic stomata, presence of papillae, epicuticular waxes in crystalloid form (rosettes) and crust-like type, isobilateral mesophyll, slightly biconvex midrib with a bicollateral vascular bundle in open arc and two dorsal traces, secretory cavities with volatile oils, calcium oxalate druses and prismatic crystals, rounded petiole with a bicollateral vascular bundle in open arc with invaginated ends, rounded stem with sclerenchyma abutting the internal and external phloem, and the rectangular outline of the vascular system. These features can help in the identification and quality control of *E. saligna*.

Volatile oils of *E. saligna* were dominated by a high fraction of monoterpenes (79.32%), 41.14% of oxygenated monoterpenes and 38.18% of monoterpene hydrocarbons. The main components of the volatile oil are *p*-cymene (28.90%) and cryptone (17.92%). There is significant variability in the chemical composition of volatile oil described is this study as well as reported in the literature. New studies that address the factors influencing the chemical composition of volatile oils are needed for better understanding of the specific biology and purpose of volatile oils

Authors' contributions

CCS and AVGO collected the plant and carried out the laboratory work. TBF identified the plant material. BHLNSM and EKM performed GC–MS analysis. VR and IAK provided critical reading and insightful recommendations of the manuscript. JMB and PVF created the project. JMB supervised the laboratory work and wrote 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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