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Structural characterization of vegetative organs of the endangered Brazilian native species *Hesperozygis ringens* (Benth.) Epling

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ABSTRACT

The aim of this study was to describe the structural characterization of *Hesperozygis ringens* (Benth.) Epling vegetative organs. For this purpose, leaves, stems and roots of the endangered Lamiaceae were collected from a population located in Santo Antão, Santa Maria municipality, Rio Grande do Sul, Brazil. Results demonstrated that the *H. ringens* leaf blade presents glandular and non-glandular trichomes as well as two morphs of diallelocytic stomata, which are usually found above the epidermis level. The petiole is concave on ventral face and convex on its dorsal face, containing glandular and non-glandular trichomes as well as stomata in the epidermis. These types of trichomes were also detected in the stem. In addition, the presence of intercellular spaces within the organ is highlighted. Stomata above epidermis level also occurred in the stem. Phenolic idioblasts were found in the cortical region of plant root and deposit of lipophilic substance was observed in phloem cells. Great amount of apparently two different crystals were detected in all organs. Information obtained in this study provides knowledge about the characterization of *H. ringens*, which may be used to distinguish characters of taxa and can help understand the species survival in its occurrence sites.

Key words: Lamiaceae, leaf blade, petiole, root, stem.

INTRODUCTION

Hesperozygis ringens (Benth.) Epling is a woody herb native from Brazilian Pampa, which is currently on the list of endangered species (Governo do Estado do Rio Grande do Sul 2014). Initially called *Glechon ringens* Benth. and commonly known as *espanta-pulga*, the species occurs sparsely in rocky fields of southeastern region of Rio Grande do Sul, Brazil (Von Poser et al. 1996, Tropicos 2017). *Hesperozygis* Epling. is formed by shrubs or sub shrubs, seven species occurring in southern Brazil and one species in Mexico (Pereira and Pereira 1973, Harley et al. 2004, Bräuchler et al. 2010). The genus belongs to Lamiaceae Martynov, 1820 and within this family, it is placed

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in the Nepetoideae subfamily, Mentheae tribe, and Menthinae subtribe (Harley et al. 2004). A study on the molecular phylogeny of this subtribe classified *Hesperozygis* as a member of the monophyletic group *The New World*, along with other 21 genera (Bräuchler et al. 2010).

Lamiaceae is composed of 236 genera and about 7173 species, which are annual, biennial and perennial herbs, shrubs, sub shrubs or trees (Harley et al. 2004). Its species may be used in different areas such as medicinal, culinary and perfumery (Naghibi et al. 2005). The aromatic species classified in this family are characterized by essential oil production in glandular trichomes located on aerial organs (Werker 1993). *H. ringens* has a great potential for essential oil production (Pinheiro et al. 2016), it stands out among Lamiaceae species. According to Werker (1993), these extractives may present beneficial functions for producer plants as protection against herbivores and pathogens or as attraction of pollinator agents. This way, they often exhibit significant biological effects on different living organisms (Badawy and Abdelgaleil 2014, Pinheiro et al. 2017). Confirming this premise, a series of biological activities have already been described for *H. ringens* essential oil. In addition, the oxygenated monoterpenoid pulegone has been reported as its major compound and different authors attributed the activities detected for this extractive especially to this molecule (Von Poser et al. 1996, Ribeiro et al. 2010, Silva et al. 2014, Toni et al. 2014, Pinheiro et al. 2016, 2017). Studies regarding this species as well as other *Hesperozygis* representatives usually focus on essential oil production, describing their chemical characteristics and possible activities for the plant extractives (Von Poser et al. 1996, González-Chavez et al. 2011, Martini et al. 2011, Castilho et al. 2016). Despite the chemical importance of the genus, little information is known on the structural characterization of its species.

Although *H. ringens* essential oil has been studied under different aspects in the past years, there is a lack of information about the structural characterization of vegetative organs of this species in literature. Additionally, there are no reports on the production and storage structures of the essential oil in *Hesperozygis* representatives. Since such information could contribute to provide knowledge to be used to distinguish taxa characters and help understand the survival of *H. ringens* in its occurrence sites, the purpose of this study was to perform structural characterization of its leaf, stem and root.

MATERIALS AND METHODS

PLANT MATERIAL

Hesperozygis ringens has been threatened with extinction (Governo do Estado do Rio Grande do Sul 2014), thus a legal authorization for scientific activities was obtained through Sistema de Autorização e Informação em Biodiversidade (SISBIO, number 44197-2). *Hesperozygis ringens* leaf blade, petioles, stems and roots were randomly gathered from a single population located in Santo Antão (S 29° 37'; W 53° 52'), Santa Maria municipality, Rio Grande do Sul, Brazil, in April 2016. Leaf blades, petioles and stem samples were gathered from 10 individuals, while root samples were gathered from 3 individuals. A voucher specimen was deposited at the Herbarium of the Forest Science Department (HDCF 6720), UFSM, Brazil. Plant material was analyzed in the Structural Botany Laboratory (Federal University of Santa Maria).

PROCEDURES FOR HISTOLOGY AND HISTOCHEMISTRY

Hesperozygis ringens vegetative organs were analyzed and dissected under stereomicroscope. Then, the materials were fixed in 1% glutaraldehyde and 4% formaldehyde in sodium phosphate 0.1M

pH 7.2 buffer (Gabriel 1982, McDowell and Trump 1976). Thereafter, fixed materials were submitted to vacuum, followed by the procedure of washing in sodium phosphate buffer 0.1M pH 7.2 for 15 min, according to methodology adapted from Gabriel (1982). Afterwards, materials were washed in distilled water for 15 min, immersed in Tween 20 2 mL/L (adapted from Freudenstein et al. 2002), and submitted to rotation for 15 days to remove epicuticular waxes. In the next procedure, the dehydration of plant materials was performed in ascending ethylic series (10, 30, 50, 70, 90, 100%) for 15 min in each concentration, followed by pre-infiltration into (2-hydroxyethyl) methacrylate (HEMA) and absolute ethanol, finalizing by a process of infiltration in HEMA. Materials were put in embedding moulds filled with HEMA until polymerization, according to Gerrits and Smid (1983). The procedures for histology occurred at least 3 times for each plant material.

Crystal characterization was performed by tests in fresh and included material. Samples were submitted to alcoholic phloroglucin for two minutes followed by mounting in conc. HCl for inulin identification, based on the formation of stained precipitate (Gahan 1984). Inulin was also identified by fresh material dehydration in ethanol and reaction of phenol crystals and H_2SO_4 (Zarlavsky 2014). HCl at 7 and 25% was used for crystal solubilization and eventual gas release, allowing to identify calcium oxalate or calcium carbonate (Zarlavsky 2014).

MICROSCOPIC ANALYSIS AND PHOTOGRAPHIC RECORD

From the embedded leaves, petioles, stems and roots, 4 μm thick sections were obtained by a Leica RM2245 rotary microtome. The sections were stained by Toluidine Blue O (Feder and O'Brien 1968) at 0.05% pH 4.4 in sodium benzoate buffer (Sidman et al. 1961). Sections of leaf blade containing glandular trichomes were stained by Sudan Red 7B (Brundrett et al. 1991). Sudan Black B was also applied on stem sections for detecting lipophilic substances in the endoderm (Pearse 1972). Iodine dissolved in potassium iodide (lugol's reagent) was applied for starch (Johansen 1940). Slide analyses were performed under a Leica DM2000 light microscope in bright field and polarized light for crystals. The plant material dissection and photographic records were performed by a Leica DM80 stereomicroscope. Photographic records for this study were obtained using a Leica DFC 295 digital capture system and software LAS (Leica™), as well as Zeiss Axio Imager A2 microscope, Zeiss MCr digital capture system and ZEN (Zeiss™) software.

RESULTS

LEAF BLADE

Leaf epidermis on abaxial and adaxial surfaces presents one cell layer which is amphistomatic (Figure 1a) with two different types of diallelocytic stomata (Figure 1b, c). The guard cells are commonly observed above the level of the epidermis, a structure that is promoted by the growth of the palisade cells surrounding the substomatic chamber (Figure 1a). The vascular system consists of collateral vascular bundles (Figure 1d). Non-glandular and glandular trichomes occur in the organ (Figure 1e-i). The set of trichomes does not form a dense indument (Figure 1e). Secretory cells of the glandular trichomes vary in a natural orange coloration (Figure 1f). Usually secretion accumulates between the cell wall and the cuticule of the glandular trichome, accompanied by cuticule distention (Figure 1f, g). The glandular ones are characterized by the presence of a single basal cell, a single stalk cell and eight secretory head cells arranged in circular form (Figure 1g, h). Cutinized outer portion of epidermal cells can also be observed (Figure 1g). Glandular trichomes occur below the epidermis level since the cells of the underlying palisade parenchyma do not

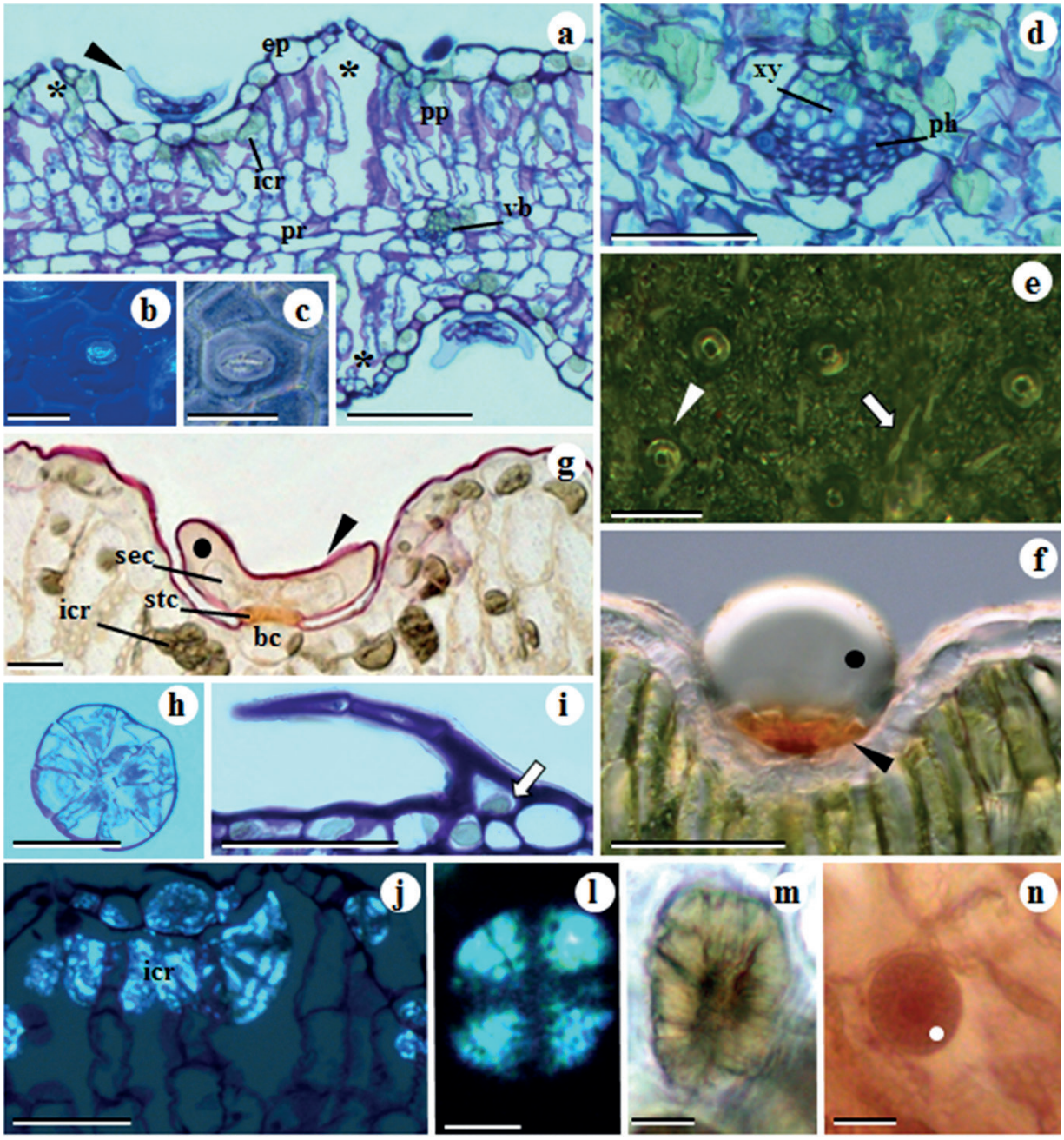

Figure 1 - Structural characterization of *Hesperozygis ringens* leaf blade. Figures 1a, d, f-g, i-n in transversal sections. Figures 1b-c and e in frontal view. Figure 1h in paradermal section. **(a)** Epidermis (ep), inulin crystal (icr), palisade parenchyma (pp), perpendicular parenchyma (pr), vascular bundle (vb), glandular trichome (arrow head), substomatic chambers (asterisks). **(b)** Detail of diallelocytic stomata. **(c)** Detail of diacytic stomata. **(d)** Detail of the vascular bundle, highlighting xylem (xy) and phloem (ph). **(e)** Leaf blade indumentum presenting glandular trichome (arrow head) and non-glandular trichome (arrow). **(f)** Detail of glandular trichome with a natural orange coloration (arrow head) and distended cuticle (black circle). **(g)** Detail of glandular trichome (arrow head) stained by Sudan Red 7B, highlighting inulin crystal (icr), basal cell (bc), stalk cell (stc), secretory cell (sec) and distended cuticle (black circle). **(h)** Detail of glandular trichome presenting eight secretory cells. **(i)** Detail of non-glandular trichome and inulin crystal (arrow).**(j)** Inulin crystal (icr) under polarization. **(l)** Detail of inulin crystal under polarization presenting a shape of maltese cross. **(m)** Detail of inulin crystal presenting streaks after dehydration period. **(n)** Detail of reddish precipitate (circle) after floroglucine reaction. Scale bars: 20 µm **(l, m** and **n)**; 50 µm **(b, c, d, h, i** and **j)**; 100 µm **(a, f** and **g)**; 500 µm **(e)**.

grow, unlike the surrounding cells. This generates a depression where the glandular trichomes are lodged (Figure 1f, g). Non-glandular trichomes have three cells in a single cell series (Figure 1i).

Mesophyll is isobilateral (Figure 1a). The palisade parenchyma on both faces presents two to three thick cell layers. It also has wide intercellular spaces which are well developed in the substomatic chambers (Figure 1a). In the central portion, the parenchyma tends to be compact with isodiametric or prismatic cells, but it is perpendicular to the palisade cells (Figure 1a).

Crystals are widely distributed in epidermis and mesophyll (Figure 1a, g, i and j). They may also be found in extracellular spaces as substomatic chambers, where they usually obliterate the space (Figure 1j), and in non-glandular trichomes (Figure 1i). These crystals, under polarization, show the typical maltese cross (Figure 1l) in fresh and included material, and streaks after a long dehydration period (Figure 1m). In dried material, precipitate was not detected in crystal areas. In fresh material, the crystals appear structured in a spherical contour, except for those found in intercellular spaces where they are molded according to the space (Figure 1j). The phloroglucin reaction generates reddish precipitate in this organ (Figure 1n). After the leaf imbibition in HCl, no crystal dissolution or gas release was detected. The set of results indicate the presence of inulin crystals in *H. ringens* tissues.

PETIOLE

The petiole is concave on its ventral face and convex on its dorsal one (Figure 2a). Epidermis is composed of a single cell layer with glandular and non-glandular trichomes and stomata (Figures 2a, b). The fundamental tissue is formed by superficial collenchyma and more internal parenchyma; intercellular spaces are developed in this region (Figure 2a). Although collenchyma is formed in both faces, it presents greater number of cell layers in ventral face, showing to be intermediate between angular and lamellar (Figure 2a, b). Collenchyma presents typical structure with elongated cells and irregular parietal thickenings (Figure 2c). Crystals occur in intercellular spaces and intracellularly including trichomes, mainly the non-glandular ones (Figure 2b). The endodermis appears as amiliferous sheath (Figure 2d). The vascular tissue occurs as a collateral bundle with a shallow arc contour, presenting secondary growth.

STEM

Primary stems are quadrangular in section, keeping this form during the beginning of the secondary growth (Figure 3a). The epidermis is composed of a single cell layer, which presents stomata, uniseriate non glandular trichomes (Figure 3a), and glandular trichomes similar to those found in leaves (Figure 3b). The stomata occur above epidermis level with guard cells elevated by the subsidiary cells (Figure 3c). The non-glandular trichomes present a series of 3 to 4 cells (Figure 3a). Crystals occur in epidermal cells and in the non-glandular trichomes basal cells, in addition to the intercellular spaces (Figure 3a). The phloroglucin and HCl test generates reddish and brownish precipitate in the stem (Figure 3e-g). After the organ imbibition in HCl, no crystal dissolution or gas release was observed. As occurred in leaf blade, the set of results indicate the presence of inulin crystals in stem tissues.

Cortical region has lacunar subepidermal collenchyma in the angles, composed of 1 to 3 layers of tipical elongated cells, and internal fiber bundles (Figure 3a, b). In general, the fundamental tissue in this region presents large intercellular spaces (Figure 3a). In the stem, the superficial layers are chlorenchymatics (Figure 3a). Parenchymal tissue occurs internally, until the endodermis (Figure 3a). The endodermis is formed by a single layer of juxtaposed cells showing plasts with starch and Casparian strips, as well as alternating cells with

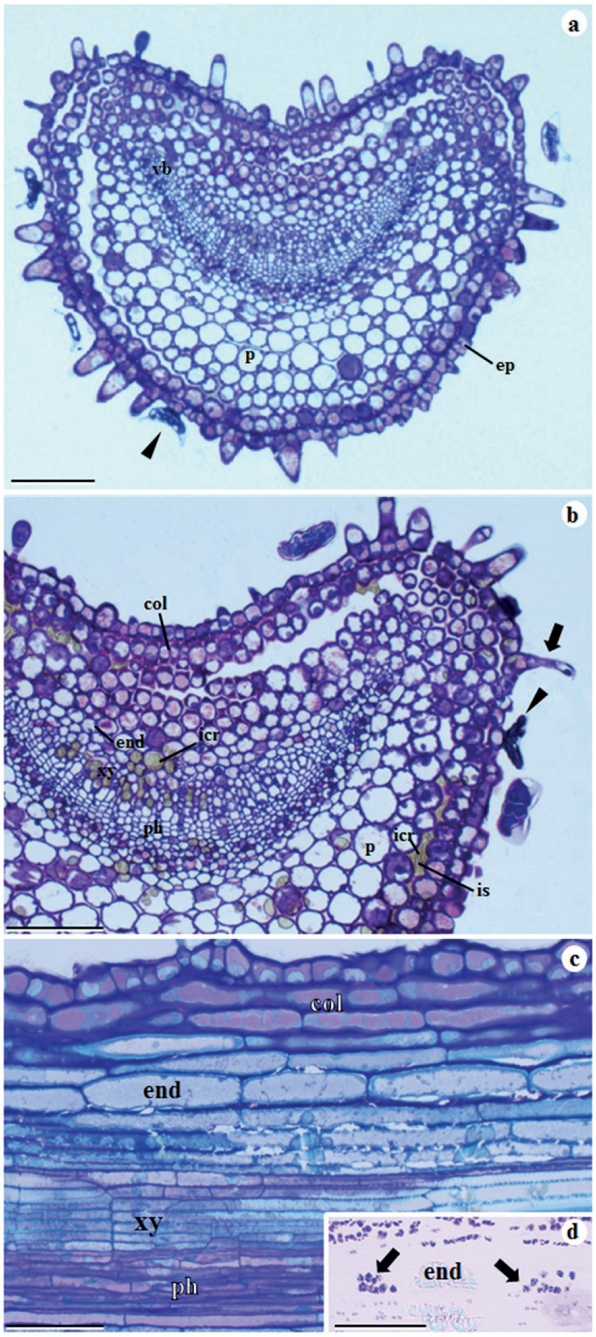

Figure 2 - Structural characterization of *Hesperozygis ringens* leaf petiole. Figures 2a-b in transversal sections. Figures 2c-d in longitudinal section. **(a)** Epidermis (ep), parenchyma (p) and vascular bundle (vb). **(b)** Non-glandular trichome (arrow), glandular trichome (arrow head), collenchyma (col), endodermis (end), inulin crystals (icr), xylem (xy), phloem (ph), parenchyma (p) and intercellular space (is). **(c)** Detail of collenchyma (col), endodermis (end), xylem (xy) and phloem (ph). **(d)** Detail of starch (black arrow) in endodermis (end) after reaction with lugol. Scale bars: 50 µm **(d)**; 100 µm **(a, b** and **c)**.

lipophilic substance in the cell wall composition (Figure 3d). Such cells are relatively bulkier and demonstrate thickened walls.

The primary phloem shows conductive cells, parenchyma, and rare isolated fibers (Figure 3a). The formed secondary phloem also demonstrated conductive elements and parenchyma, in addition to companion cells soon after the beginning of the cambial activity (Figure 3a). The primary and secondary xylems show vessel elements, fibers and parenchyma. The vascular cambium differentiation is observed in very young stems, in addition to continuous xylem and phloem (Figure 3a). Crystals are observed in both primary and secondary xylem and phloem (Figure 3a).

The pith has a peripheral region composed of sclerenchymatous tissue, which is derived from typical parenchyma when younger internodes are analyzed (Figure 3a). The central portion presents parenchymatous tissue with bulky cells and disaggregation from the middle lamella, generating large intercellular spaces (Figure 3a). Crystals are commonly found in this region, especially intracellularly.

SECONDARY STEM

The secondary xylem frequently presents isolated vessel elements in pairs and rarely in trios (Figure 3h). Interfascicular cambium produces only ray cells for both sides (Figure 3h). Crystals are found on the side of the newly formed xylem and phloem (Figure 3h). A great number of cells with phenolic compounds occur in the secondary phloem and xylem (Figure 3h). The pith is partially sclerified and fistulous (Figure 3h). In mature stems, the cells of the xylem ray are not sclerified, generating a contrast between the rays and the axial elements of the xylem tissue (Figure 3h). There are rays with different widths (Figure 3h).

ROOT

In young root the epidermis has isodiametric cells in transverse section (Figure 4a). The region

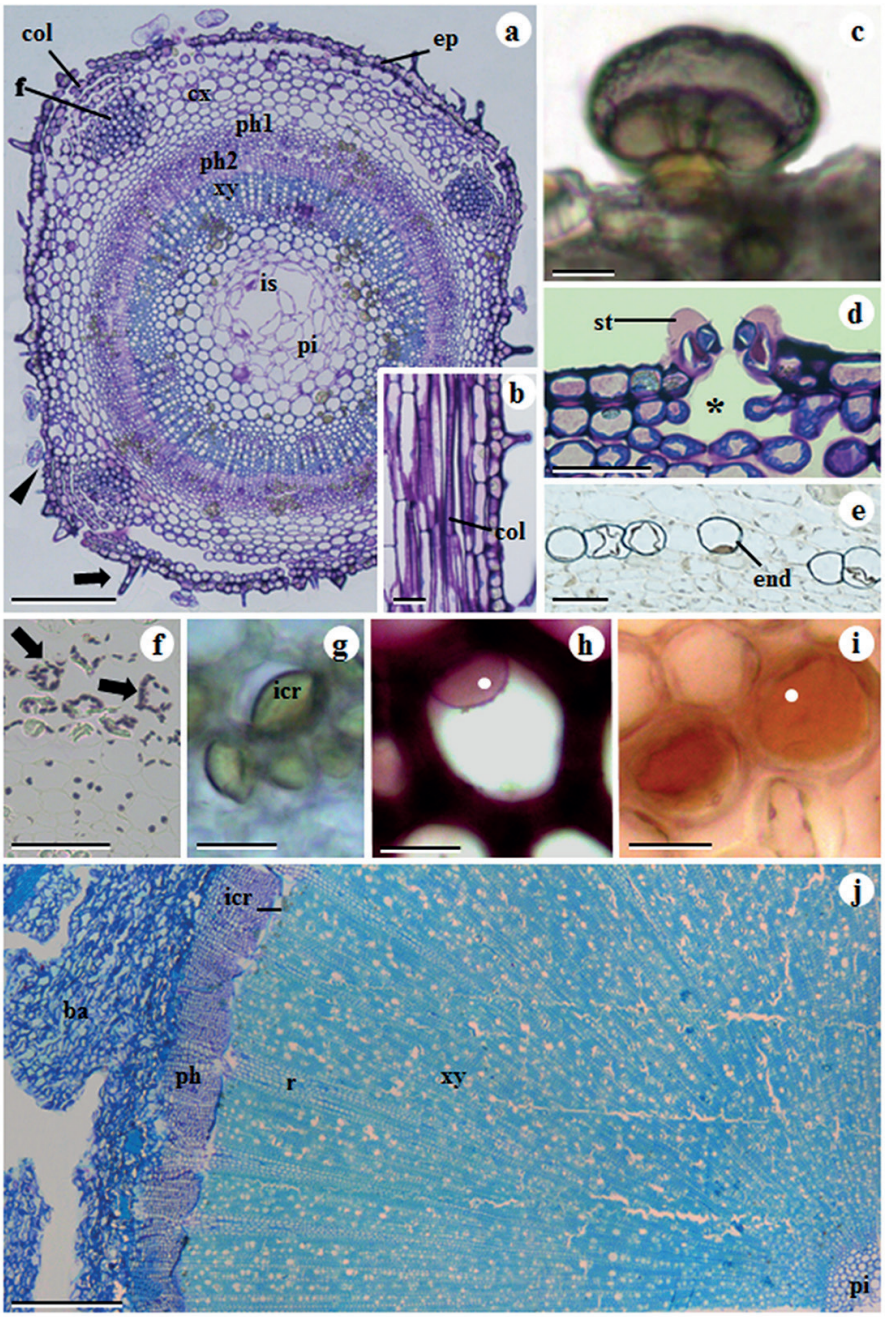

Figure 3 - Structural characterization of *Hesperozygis ringens* stem. Figures 1a, c-j in transversal sections. Figure 1b in longitudinal section. **(a)** Glandular trichome (arrow head), non-glandular trichome (arrow), epidermis (ep), collenchyma (col), fiber (f), cortex (cx), primary phloem (ph1), secondary phloem (ph2), xylem (xy), intercellular space (is), pith (pi). **(b)** Detail of collenchyma (col). **(c)** Detail of glandular trichome. **(d)** Detail of stomata (st) and substomatic chamber (asterisk). **(e)** Detail of endodermis (end) stained by Sudan Black B. **(f)** Detail of starch (black arrow) in endodermis after reaction with lugol. **(g)** Detail of inulin crystals (icr). **(h)** Detail of reddish precipitate (circle) after phloroglucin reaction. **(i)** Detail of orange precipitate (circle) after phloroglucin reaction. **(j)** Bark (ba), inulin crystal (icr), phloem (ph), xylem (xy), rays (r) and pith (pi). Scale bars: 20 μm **(c, g, h, i** and **j)**; 50 μm **(b, d** and **f)**; 100 μm **(a)**; 500 μm **(e)**.

demonstrates more thickened radial and external tangential walls as well as deposit of lipophilic substances (Figure 4b). In mature root, outer and inner portions of the bark accumulate a great quantity of secondary metabolites (Figure 4b). The cells of xylematic ray present a lignification gradient where the most recent ones are non lignified. The presence and absence of lignin in sclerified radial tissues generate a contrast between non lignified and lignified portions (Figure 4b). Secondary phloem is not associated to sclerified tissue. Within the vessel elements, crystals occur in great quantity, occasionally obliterating the vessel elements. Such crystals also occur in newly formed vessel elements and sclerenchyma. The crystals of xylem differ from those found in other tissues. These crystals are acicular and present arrangement in rosette usually connected to the walls of the vessel elements (Figure 4c). Phloroglucin and HCl test produced no reaction in the crystals, but the structures were dissolved by H_2SO_4 and phenol (Figure 4d), indicating the presence of hesperidin crystals.

In xylem, the vessel elements are predominantly isolated; they are frequently found in pairs and rarely in trios (Figure 4b). Phellogen appears internally (Figure 4a). The cortical region has phenolic idioblasts and phloem cells presenting deposit of lipophilic substances (Figure 4a, b). The occurrence of fungal hyphae in this region is high. Some cells in cortex and phloem are compressed and others grow very large (Figure 4a).

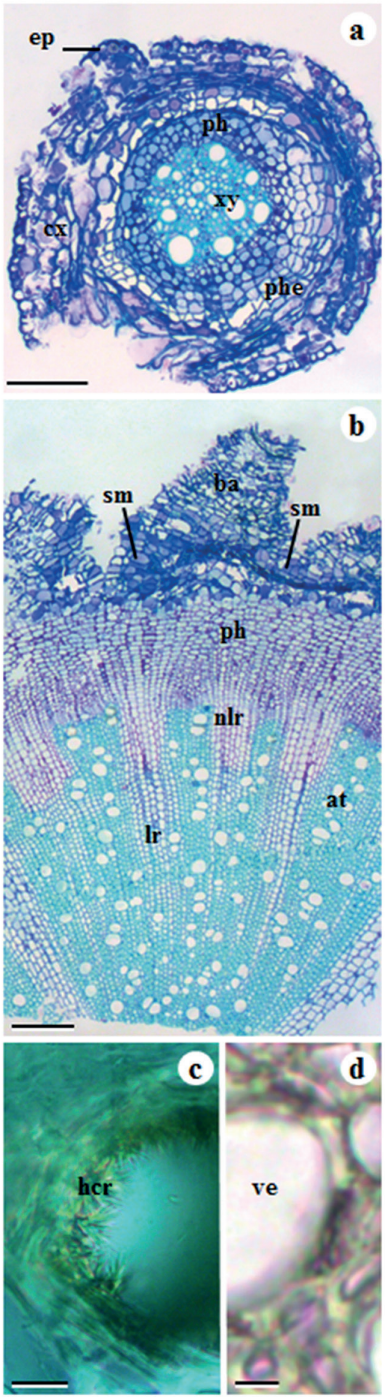

Figure 4 - Structural characterization of *Hesperozygis ringens* root in transversal sections. **(a)** Epidermis (ep), cortex (cx), phellogen (phe), phloem (ph), xylem (xy). **(b)** Bark (ba), secondary metabolites (sm), phloem (ph), non lignified rays (nlr), lignified rays (lr), axial tissue (at). **(c)** Detail of vessel element highlighting hesperidin crystals (hcr). **(d)** Detail of vessel element (ve). Scale bars: 10 µm **(c** and **d)**, 200 µm **(a** and **b)**.

DISCUSSION

LEAF BLADE

Leaves of species classified in the subtribe Menthinae present indumentum characterized by the presence of glandular and non-glandular trichomes in both faces (Satil et al. 2002, Toledo et al. 2004, Novoa et al. 2005). In *H. ringens* diallelocytic, stomata were detected in both leaf

faces in the blade and petiole. Amphistomatic leaves and stomata, predominantly diacitic, are commonly found in species of the same subtribe, as occurred in *Cunila microcephala* Benth., *Hedeoma multiflora* Benth. and *Satureja* L. genus. In the latter, stomata occur above epidermis level (Satil et al. 2002, Toledo et al. 2004, Novoa et al. 2005, Satil and Kaya 2007). Amphistomatic leaves are also found in *Salvia nutans* L. (Gürcan et al. 2016). Anisocytic stomata have also been detected in Lamiaceae, as occurred in *Stachys iberica* Bieb. subsp. *iberica* var. *densipilosa* Bhattacharjee (Erkara et al. 2010). Diallelocytic stomata are characterized by the presence of subsidiary cells positioned perpendicularly to the guard cells and with common walls obliquely positioned described as C-shape (Cantino 1990). Those found in *H. ringens* demonstrate two and three subsidiary cells. Diallelocytic stomata have already been identified in Nepetoideae representatives, including some of *The New World* genus such as *Cunila* D. Royen ex L., *Rhododon* Epling, *Pogogyne* Benth. and *Monardella* Benth. They were also described in other Menthinae genus, such as *Mentha* L., *Hedeoma* Pers., *Micromeria* Benth., *Satureja* L. and *Thymus* L. (Cantino 1990). This type of stomata was also detected in *Wenchengia* C. Y. Wu & S. Chow, another genus belonging to Lamiaceae (Cantino and Abu-Asab 1993). Attention should be given to the palisade parenchyma and its intercellular spaces. It apparently assumes the role of the spongy parenchyma that is poorly developed and tends to be compact and reduced to a few cells beyond the endodermis. Although the central tissue of the mesophyll is not typically spongy, it presents a distinct basic structure when compared to the palisade tissue. This allows to interpret the mesophyll as isobilateral and heterogeneous.

PETIOLE

Similarly to *H. ringens,* petiole contour was also described for *Lamium* L. and *Salvia* L. species (Bagherpour et al. 2010, Bercu et al. 2011, Erbano et al. 2012, Celep et al. 2014, Atalay et al. 2016, Özdemir et al. 2016). Petiole contour is a mandatory state in morphological studies, being taxonomically useful in Lamiaceae, as observed for *Lamium* (Atalay et al. 2016) and *Salvia* (Özdemir et al. 2016). However, this feature was not considered a diagnostic character for *Hesperozygis* species (Pereira and Pereira 1973). Given the great similarities between species of *Hesperozygis* and *Glechon* and the obvious basis for support in taxonomic difficulties, it is noteworthy that the petiole form found in this study is different from that of *G. spathulata* (Banderó Filho et al. 2010). According to the authors, the plant presents mainly circular form in cross section analysis. The presence of glandular and non-glandular trichomes in Lamiaceae petioles was already described (Akçin et al. 2011). The report of these structures on the petiole could be of interest considering *Hesperozygis* representatives, since *Hesperozygis nitida* (Benth.) Epling is the only one presenting glabrous leaves on its adaxial surfaces (Pereira and Pereira 1973). The vascular structure is considered a very important feature of petiole (Metcalfe and Chalk 1972), mainly among species of the same genus. A great overlap of characteristics is found in Lamiaceae genera, as mentioned for *Salvia* and *Lamium* (Bagherpour et al. 2010, Bercu et al. 2011, Erbano et al. 2012, Celep et al. 2014, Atalay et al. 2016, Özdemir et al. 2016).

STEM

Other Menthinae representatives have shown quadrangular stems in cross section (Novoa et al. 2005, Ozcan and Eminagaoglu 2014), as detected in *H. ringens*. This is considered a usual Lamiaceae characteristic (Harley et al. 2004). The subtribe stem indumentums present glandular and non-glandular trichomes, as already described for *Hedeoma multiflora* Benth., *Origanum rotundifolium* Boiss. and *O. vulgare* L. ssp. *viride* (Boiss.) Hayek (Novoa et al. 2005, Ozcan and Eminagaoglu 2014). Stomata

were also found above epidermal level in stems of *Salvia nutans* L., a species belonging to another subtribe of the same family (Bercu et al. 2011). In *H. ringens* stem, the endodermis has remarkable features such as impregnation of some cell walls with lipophilic substances. This characteristic has not been described for other Lamiaceae species yet. The stem pith with parenchymatous tissue including intercellular spaces was found in *Lamium moschatum* Miller var. *rhodium* (Gand.) R. Mill; however, it presented roundish cells (Baran and Özdemir 2011). Apparently, the disaggregation described for *H. ringens* pith culminates in typical fistulous internodes of some Lamiaceae genus (Harley et al. 2004). The pith of the *Satureja parnassica* Heldr. et Sart. subsp. *sipylea* P.H. Davis (Menthinae) demonstrates parenchymatous cells as well as the central region frequently broken in pieces (Satil et al. 2002). *Salvia divinorum* Epling & Játiva stem presents degraded cells in pith centre forming a large cavity including internodes and being visible to the naked eye in adult organs (Kowalczuk et al. 2014). In some semi-aquatic plants, the description of large intercellular spaces was also reported (Harley et al. 2004). Their places of occurrence clearly differ from *H. ringens* habitat, which occurs in drained soil, both sandy and rocky ones. Thus, the formed spaces can be either considered constitutive or associated with some other functionality.

ROOT

In this study, fungal hyphae were detected in *H. ringens* roots. In general, the soil presents a wide diversity of microorganisms in the plant rhizosphere due to the nutrients secreted by the organ (Sala et al. 2007). On the other hand, secondary metabolites exuded by roots are often sources of chemotaxis, favoring symbiotic interactions of plant organs with microorganisms (Cheynier et al. 2013). Associations with mycorrhizal fungi may confer benefits to different plant species, which were also described for Lamiaceae representatives. According to Tarraf et al. (2015), arbuscular mycorrhizae have already provided increase in essential oil and biomass productions of aerial parts of *Salvia officinalis* L., *Origanum vulgare* L. and *Thymus vulgaris* L. In addition, *Satureja macrostema* (Benth.) Briq. was benefited by the mycorrhizae colonization, presenting increase in contents of the essential oil major compounds (Carreón-Abud et al. 2015).

GLANDULAR TRICHOMES

Lamiaceae species usually produce chemical substances in different types of glandular trichomes with distinct function, aiming at the survival and perpetuation of the producer plant (Werker 1993, 2000). In this study, glandular trichomes, classified as peltate containing eight secretory head cells, were found in *H. ringens* leaves and stems, a similar type described for *Cunila microcephala* (Toledo et al. 2004). Peltate trichomes with multicellular heads are commonly found in representatives of Menthinae subtribe, as observed in studies conducted with *Lamium*, *Thymus quinquecostatus* Celak, *Satureja horvatii* Silic and *Micromeria thymifolia* (Scop.) Fritsch (Baran and Özdemir 2009, 2011, Marin et al. 2012, 2013, Jing et al. 2014).

NON-GLANDULAR TRICHOMES

The function of non-glandular trichomes depends on the organ location, morphology and orientation (Werker 2000). Results indicate that these structures are distributed in abaxial and adaxial surfaces of leaf blade, petiole and stem in *H. ringens*. This distribution may be related with a defense mechanism, since these structures have the function of providing protection to glandular trichomes, when the indumentum is dense (Werker 2000). The municipality of Santa Maria presents

temperatures over 30 °C in the hottest months of its summer (Moreno 1961), thus we hypothesized that non-glandular trichomes may favor the *H. ringens* survival in months with less rain and higher temperatures. The referred structures may serve as a mechanical barrier against extreme temperatures, extensive light and water loss (Werker 2000).

TAXONOMIC CONSIDERATIONS

Characters used to differentiate *Hesperozygis spathulata* Epling, *H. nitida* and *H. rhododon* Epling consider calyx tube length, leaf type, glabrous or hairy upper page, as well as sessile or pedunculated summits (Pereira and Pereira 1973). However, *H. ringens* characteristics are not described in the referred work. No information on the structural aspects of *H. ringens* such as trichome distribution and its classification or vegetative organ characteristics has been found in literature. For this reason, this study is necessary in order to provide information about the diagnostic characters of the species.

Studies have been conducted with other Lamiaceae representatives searching for description and classification of structural aspects of taxonomic importance. Characters such as glandular and non-glandular trichomes, stem form in cross-section, epidermal cell form, cortex pith, vascular bundles, leaf form in cross section and mesophyll characteristics have been shown to be useful for species identification (Satil and Kaya 2007, Kalicharan et al. 2015, Seyedi and Salmaki 2015, Khalik and Karakish 2016).

CRYSTAL INCLUSIONS

The anatomical characterization of 39 Lamiaceae representatives (Abu-Asab and Cantino 1987) and morphological review of the family (Harley et al. 2004) indicated that crystal inclusions are common. However, information on their chemical compositions were not found. In our study, the great number of crystals in *H. ringens* leaf, stem and root is highlighted. They can be found even in apoplast, including inside vessel elements. This morphological feature resembles the one described for stems and roots of other taxonomic groups of Lamiaceae (Romberger et al. 1993). Crystals presenting similar structures were found in the adaxial epidermis of *Teucrium sandrasicum* O. Schwarz (Dinç et al. 2008). Morphologically differing from the crystals visualized in the aerial organs, *H. ringens* roots demonstrate crystalline structures in vessel elements. Similar crystals composed of hesperidin were described inside vessel elements of *Citrus sinensis* (L.) Osbeck as a response to infections caused by *Xylella fastidiosa* (Alves et al. 2009) and by *Phytophthora citrophthora* (Del Rio et al. 2004). In *H. ringens* roots, crystals were dissolved by H_2SO_4. This diluted acid was already used as catalyst for hesperidin hydrolysis in different temperatures (Grohmann et al. 2000), suggesting the characterization of hesperidin crystals in the *H. ringens* roots. Hesperidin was already found in Lamiaceae representatives (Metcalfe and Chalke 1972), as described in *Clinopodium gracile* (Benth.) Matsum, where the substance was considered part of the defense response to the attack of *Aedes albopictus* Skuser mosquito (Chen et al. 2013).

Histochemical tests were performed for detection of inulin, calcium oxalate and calcium carbonate. Calcium oxalate and calcium carbonate were disregarded due to the absence of solubilization. Inulin is one of the possible crystals occurring in *H. ringens* leaf and stem based on the presence of maltese cross and streaks after the dehydration period. In this study, phloroglucin reaction promoted disappearance of crystals and formation of a red-brown precipitate, a similar reaction described by Gahan (1984), apart from crystals in vessel elements. However, some results do not corroborate with this hypothesis due to the absence of coloration after phenol and

H_2SO_4 test. Inulin crystals were already described in the Menthinae species *Cunila microcephala* (Toledo et al. 2004). The authors reported the presence of polysaccharide crystals, with similar morphology to those described in our study, demonstrating that a wide distribution of such structures may occur in leaves of *The New World* group representatives. Although *Hesperozygis* and *Glechon* were considered synonymous, in an anatomical study performed with *G. spathulata*, no crystals were detected (Banderó Filho et al. 2010). This suggests that the presence of these structures can be considered a differentiation characteristic between the genera. Inulin is considered a reserve of sugar, specially of fructose (Toledo et al. 2004), and is explored commercially and medicinally for different purposes (Kierstan 1978, Fuchs 1987). For plants in general, fructans originating from inulin generate cold and drought tolerances (Ritsema and Smeekens 2003), which are common environmental situations to *H. ringens* individuals at the occurrence site. In addition to hesperidin and inulin, the Lamiaceae representatives may also present other crystals with distinct features in relation to those found in *H. ringens,* such as calcium oxalate, as occurred in *Salvia divinorum* Epling & Játiva (Kowalczuk et al. 2014).

ECOLOGICAL CONSIDERATIONS

As detected for *H. ringens,* amphistomatic leaves, stomata above epidermis level and intercellular spaces are related to mesic environments. In a study of *Aegiphila sellowiana* Cham. juveniles submitted to flooding, amphistomatic leaves and the position of the stomata above epidermis level were reported (Medri et al. 2011). However, *H. ringens* shows small leaves and developed indumentum related to xeric environments. These structural aspects may mean part of the complexity of *H. ringens* adaptations, since Santa Maria may present rainy months along the year, rainless periods with high temperatures or cold and dry periods (Da Silva et al. 2007). These phenomena may even alternate in the same month. The referred structural features can also serve as a defense mechanism of the plant against possible biotic factors. The production of the two types of crystals may be associated with the conditions at which the species is exposed to in its habitat. No information regarding the concomitant production of these crystals in Lamiaceae representatives has been found in literature. For a better understanding of composition and functions of the crystals, further studies are suggested, considering the different plant organs.

CONCLUSIONS

Hesperozygis ringens presents remarkable characteristics such as the presence of glandular and non-glandular trichomes in leaf and stem, two morphs of diallelocytic stomata above epidermis, intercellular spaces in stem, presence of lipids in cell walls of stem endodermis, phenolic idioblasts in roots, and wide amount of crystals spread in all vegetative organs. Results found in this study may help understand the plant survival in its sites and encourage further studies aiming to preserve and reproduce this species.

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