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The anatomical and histochemical properties of endemic species *Leiotulus aureus* (Apiaceae)

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ABSTRACT:
Leiotulus aureus is an endemic umbellifer of the Balkan Peninsula and Turkey. It belongs to the rather complex tribe Tordylieae, whose genera are carpologically characterised in detail. However, information on the structure of its vegetative plant parts remains limited. Previous chemical research implies the presence of specialised structures related to the production of secondary metabolites. The aim of this study is the localisation of these anatomical features. The plant material was collected in Montenegro. The anatomy was studied using both paraffin sections and native sections stained with standard reagents for histochemical analysis, while fruit secretory products were characterised using Raman spectroscopy. Secretory structures were observed in all the plant parts of this monocarpic biennial or short-lived species, forming a network extending from the roots through the stem to the leaves, flowers and fruits. In the root, they are located exclusively in the cortical parenchyma, while in aboveground vegetative organs, they are found in the cortical parenchyma, in the ribs, aligned with vascular bundles. The fruits contain well-developed dorsal lateral and commissural vittae, while the dorsal median vittae and ducts associated with the vascular bundles are filiform and hardly recognisable. The presence of phenolic compound, flavonoids, fatty acids, and octyl-esters confirmed by histochemical staining, epifluorescence, Raman spectroscopy implies potential phytopharmaceutical properties and warrants further investigations of secondary metabolite pathways.

Keywords: *Malabaila aurea*, light microscopy, epifluorescence, micro-Raman, 532 nm, secretory structures

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INTRODUCTION

The genus *Leiotulus* Ehrenb., one of the genera of the *Apiaceae* family, has undergone significant nomenclatural changes throughout history (e.g. Hand 2011+; POWO 2024), which have not yet been harmonised. While Euro+Med (Hand 2011+) considers it as a synonym of *Malabaila* and implies that *Leiotulus, Malabaila, Pastinaca,* and *Trigonosciadium* might be grouped under a very broad interpretation of *Pastinaca*, POWO (2024) accepts it as a distinct genus. A comparable scenario applies to the species of our interest, *Leiotulus aureus* (Sm.) Pimenov & Ostr. (syn. *Heracleum aureum* Smith, *Malabaila aurea* (Smith) Boiss., *Pastinaca rectistyla* Cesat, *Malabaila rectistyla* (Cesat) Bois. et Sprun, *M. biradiata* Hausskn ex Nym., *M. burnatiana* Heldr.). Despite differences in nomenclatural treatment, both databases agree on the plant's geographical range and consider it an endemic species of Turkey and the Balkan Peninsula (Hand 2011+; POWO 2024).

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To date, research on *L. aureus* has comprised carpological characterisation (Pimenov & Ostroumova 1994), pollen morphology (Mačukanović-Jočić *et al.* 2023), and chemical characterisation (e.g. Tzakou *et al.* 2008; Vučković *et al.* 2014), with the anatomy of the vegetative plant parts remaining unexamined.

The aim of this study was to identify and locate those anatomical traits associated with the production of secondary metabolites, followed by the chemical characterisation of their secretory products through Raman spectroscopy. The synthesis and secretion of essential oils in plants are linked to specialised structures such as glandular trichomes, or cavities/ducts found in parenchyma tissue. The examination of histochemical localisation and spectroscopy analysis aims to provide a deeper understanding of the characteristics of the species relevant to both chemotaxonomy and phyto-pharmacy.

MATERIALS AND METHODS

Plant material. *Leiotulus aureus* is a viscid biennial or short-lived plant, up to 50 cm in height, with a pubescent, hollow and striate stem. The leaves are pinnate, with the lower ones exhibiting 3 to 4 ovate segments found in pairs, while the upper ones have 1 to 2 pairs of narrow to linear-lanceolate segments, deeply serrated or sometimes lobed. The umbels are compound, yellow, with 3–9 rays, reduced bracts, a few bracteoles, linear-lanceolate, deciduous. The fruit measures 8–10 mm, is winged, suborbicular, with the wings strongly thickened at the margin, conspicuous vittae near the margin and persistent styles (Fig. 1; FloraVeg.EU 2022–2024).

The plant material was collected from the site named Gorica in Podgorica (N 42.44916667°, E 19.26722222°), during the flowering and fruiting phase of the species: May–June 2017, and 2023. The voucher specimen of the investigated population was deposited in the herbarium collection at the Faculty of Natural Sciences and Mathematics, University of Montenegro (TGU no. 1500402).

Microscopic analysis. The general anatomy was studied on ten individuals fixed in FAA (formaldehyde: alcohol – 50% ethanol: acetic acid 5:90:5 v/v) for 24 hours and stored in 50% ethanol, while the histochemistry was studied on fresh material.

Permanent microslides for light microscopy were prepared according to standard paraffin/wax procedure (Ruzin 1999). Fixed plant samples were chemically dehydrated in a graded ethanol series, followed by clearing in xylol and impregnation in Histowax (56–58°C). After embedding the samples and solidifying the paraffin blocks on a cold plate, histological sections of about 5–7 µm were cut using a sliding microtome (Leica SM 2000 R) and placed on microslides. Tissue sections were deparaffinised, hydrated in a graded ethanol series to water, and double-stained in Safranin O (1%, w/v, 50% ethanol) and Alcian blue (1% w/v, aqueous), dehydrated in an ethanol series and mounted using Canada balsam. Several sections of the root, leaf blade, leaf petiole, stem, and fruits were sectioned from each individual plant. The permanent microslides were prepared and stored at the Department of Agrobotany, University of Belgrade, Faculty of Agriculture. Leica IM1000 imaging software was used to capture and measure the microslides. For each parameter, several sections of all the ten studied individuals were analysed.

Histochemical analysis was conducted to determine and localise certain chemical compounds using temporary microslides. For this purpose, the fresh plant material was sectioned by hand using commercial razorblades and stained with reagents listed in Supplementary Table 1. In addition, the maceration of stem and root parts was performed to obtain more detailed features of

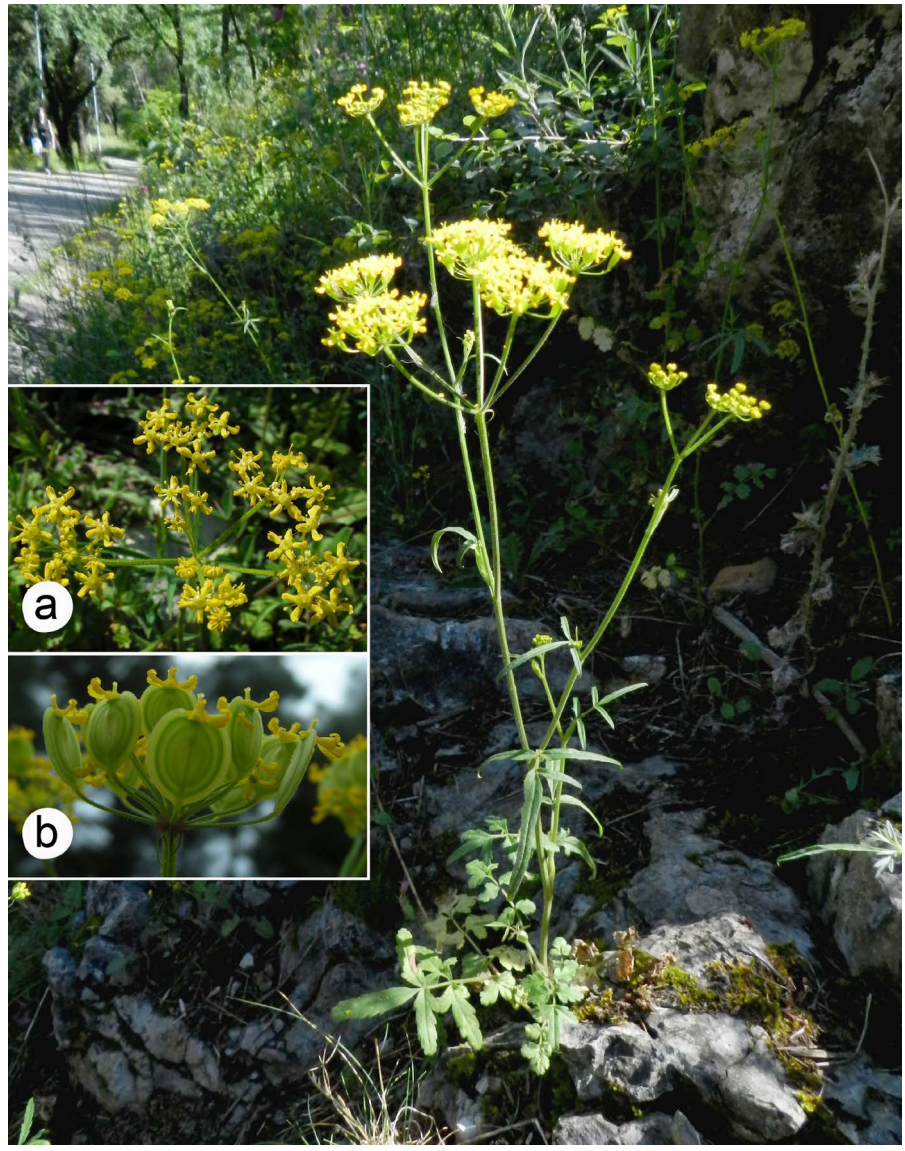

Fig. 1 The above-ground part of *Leiotulus aureus* from its natural habitat – Gorica in Podgorica, a – inflorescence, b – fruits (photo: Danijela Stešević)

Table 1 Measurements of the leaf anatomical features in micrometres (given in μm as mean ± stdev): the inner diameter of the secretory duct (isd), the size of the epithelial cells (ec), the upper epidermis (ue), palisade tissue (pt), spongy tissue (st), the lower epidermis (le), leaf thickness (lt).

	isd	ec	ue	pt	st	le	lt
Lower leaf	26.9±4.4	9.4±2.5	24.2±6.5	55.8±9.8	60.6±16.1	11.7±3.6	152.3±27.0
Upper leaf	35.6±5.2	9.7±1.7	18.7±4.8	61.8±12.0	78.3±18.9	12.4±3.1	171.2±29.8

the xylem elements (such as vessel element length), using Jeffrey's macerating fluid (equal volumes of 10% nitric acid and 10% chromic acid, for 24 hours at room temperature). The microslides were observed using a bright-field light microscope (Leica DM2000 equipped with a DFC320 digital camera) or a Leica DMLS epifluorescence microscope equipped with an HBO 50 W mercury vapor lamp and filter cubes I3 (band-pass filter wavelength 450–490 nm) and N2.1 (band-pass filter BP wavelength 515–580 nm).

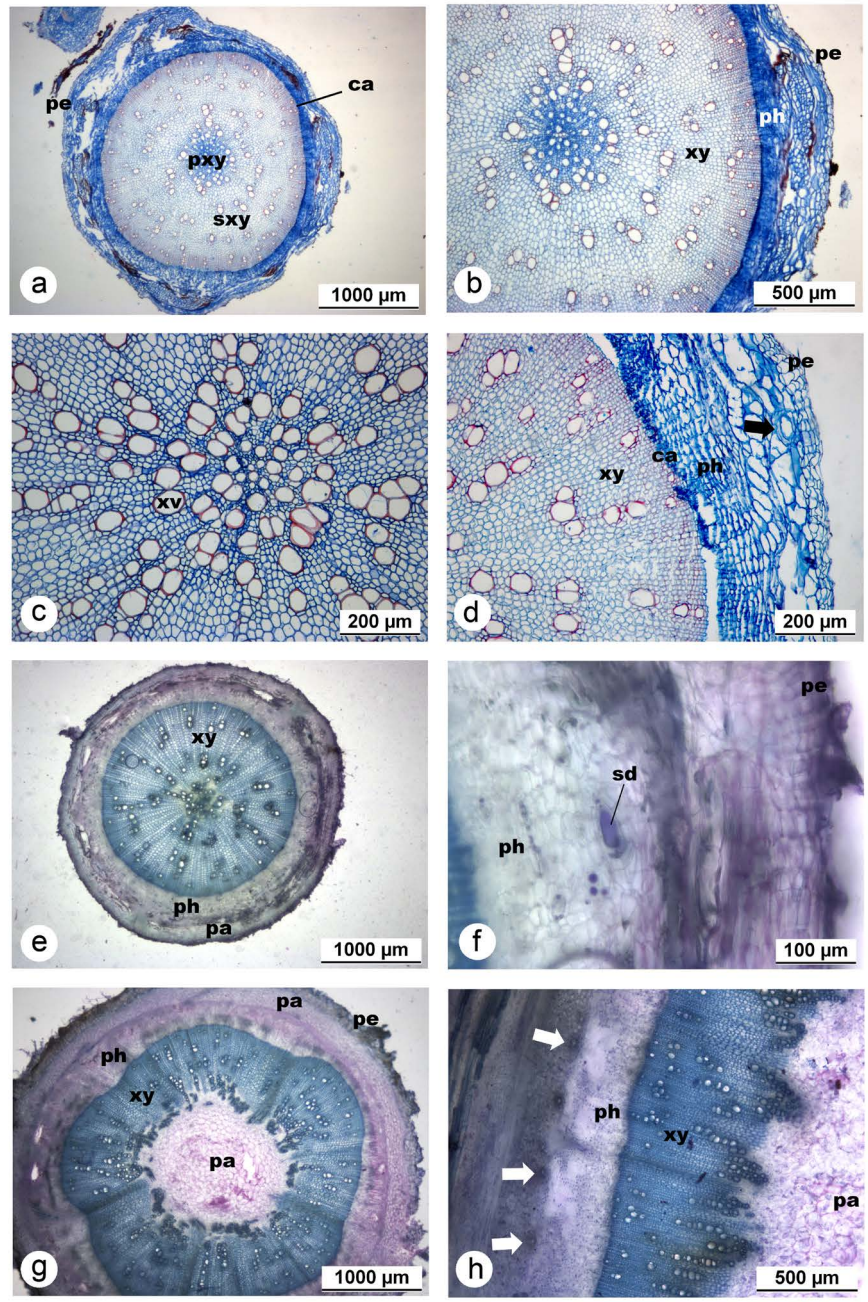

Fig. 2 Transverse section of the upper part of a taproot (originating from the hypocotyl part of the stem) of *Leiotulus aureus*, parafine cross sections (a–d), and Toluidine blue staining (e–h). Abbreviations: ca – cambium; pa – parenchyma; pe - periderm; ph – phloem; pxy – primary xylem; sd – secretory duct; xv – xylem vessel; sxy – secondary xylem, xy – xylem. The secretory ducts are indicated by arrows (d, h).

Raman microspectroscopy. Raman microspectroscopy of the cross-sectioned fruit samples was recorded using a Horiba Jobin Yvon XploRA Raman spectrometer equipped with an Olympus BX 41 microscope. Raman spectra were taken directly from the oil duct content of individual *L. aureus* fruits. The fruits were transversely sectioned prior to analysis using a razor blade. Raman scattering was excited by a laser with a wavelength of 532 nm focused on the sample through a 50 LWD objective (Olympus, Tokyo, Japan). Raman scattering was performed with a 1200 lines/mm grating, resulting in spectra in the range of 200–1800 cm^{-1}. The spectral resolution was about 3 cm^{-1} and the calibration was checked using a 520.47 cm^{-1} line of silicon. The spectra were recorded with an exposure time of 10 seconds and the samples were scanned five times. Data acquisition and instrument control were carried out using

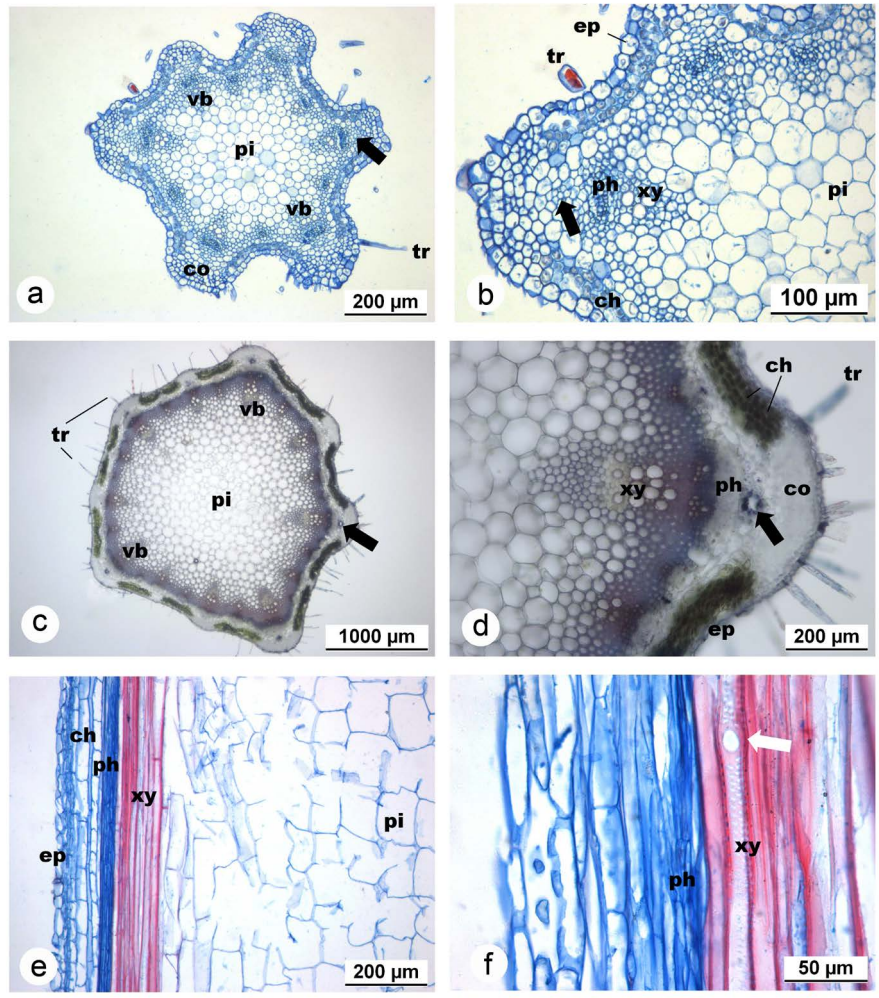

Fig. 3. Anatomical features of the upper (a, b) and lower (c–-f) part of the stem of *Leiotulus aureus*. Nadi staining indicated the presence of essential oils in the epithelial cells (violet-blue) (c, d). In the longitudinal stem sections (e, f), pitted vessels (alternate pitting) were visible in the sparsely developed secondary xylem. Abbreviations: ch – chlorenchyma; co – colenchyma; ep – epidermis; ph – phloem; pi – pith; tr – trichomes; vb – vascular bundle; xy – xylem. The black arrow shows the stem secretory ducts (a–d). The white arrow shows a simple perforation plate (f).

LabSpec 6 software (Horiba Scientific, France). The assignment of the main bands was based on literature data. The interpretation of the Raman spectra relied on the results of the chemical characterisation of volatile constituents of *L. aureus* fruits, collected from the same population/site – Gorica in Podgorica (Vučković *et al.* 2014).

RESULTS

Root anatomy. *Leiotulus aureus* had a taproot. The anatomical analysis showed that typical secondary growth occurred in the older roots (Fig. 2a, b, e, g). The surface was covered by a periderm, and the cortex consisted of parenchymatous tissue with large air spaces, followed by a compact layer of primary and secondary phloem positioned next to the cambium. On the inner side of the cambium, the secondary xylem was well developed (Fig. 2a, d), and in the central part of the root vessels originated from diarch primary xylem (Fig. 2c). All the results are presented as mean ± standard deviation.

The inner tangential diameter of the secondary xylem vessels was 40.0 ± 12.5 µm (min–max 12.4–63.0 µm), and the thickness of their cell walls was about 2.7 ± 0.5 µm. The length of the vessel elements in the root was about 156.6 ± 48.0 µm (min-max 99.0–253.0 µm). Small secretory ducts were distributed through the cortex parenchyma. The average inner diameter of the ducts was 13.3 ± 9.9 µm, and the size of the epithelial cells averaged 10.8 ±

4.3 µm. The upper part of the taproot, which is actually the lower part of the stem originating from the hypocotyl part of the seedling, contained numerous secretory ducts, mainly located at the interface of the primary cortex and phloem (Fig. 2d, f, h).

Stem anatomy. The stem was erect and sulcate, with a unicellular layer of epidermal cells on the surface, and a relatively thin cortex below the epidermis made up of chlorenchyma and collenchyma, followed by a few layers of cortical parenchyma cells (Fig. 3a–d). Collateral vascular bundles were arranged in a ring at the periphery of the central cylinder, separated by wide parenchyma rays. The majority of the stem, including the pith, consisted of parenchyma tissue (Fig. 3a–c).

The stem cross-section in the upper part of the plant, just below the inflorescence, was characterised by an angular shape with 5–7 ribs, which consisted of collenchyma (Fig. 3a, b). The central cylinder contained 10–11 collateral vascular bundles separated from one another by parenchyma tissue. The first-formed protoxylem elements exhibited spiral thickening, while in the mature metaxylem they were reticulate. The average vessel diameter of in the primary xylem was 40.5 ± 12.4 µm, with a cell wall thickness of 2.7 ± 0.5 µm (min-max 1.6–3.6 µm). The length of these vessel elements was about 247.3 ± 86.1 µm (min-max 51.4–411.4 µm). The stem section of the lower part of the plant (Fig 3c, d), just above the ground, had a rounder shape, the stem ribs were weakly pronounced, and compared to the upper part of the stem, possessed a larger number of vascular bundles (20–30). Secondary growth was only initiated and observed in the basal parts of the stem (Fig. 3c, d). Thus, the vascular cambium was characterised as being weakly active. The vessels of the secondary xylem were pitted (pits mostly circular and rounded), with simple perforation plates (Fig. 3 e, f) and narrower than the vessels in the primary xylem, averaging 23.6 ± 10.3 µm, but of a similar length (241.1 ± 70.3 µm). Vessels were not confined exclusively to the fascicular regions, but the interfascicular regions were predominantly occupied by fibres. The diameter of the fibres in the fascicular region was almost the same as in the interfascicular region, averaging 13.8 ± 3.9 µm and 14.1 ± 4.0 µm respectively, while the length of the fibres was 406.5 ± 163.4 µm. Mechanical tissues were also present, developing by lignification and wall thickening of the ray parenchyma cells surrounding the protoxylem part of each bundle (Fig 3c, d). The largest parenchyma cells occurred in the pith. In the middle of the cross-section, a variably sized cavity was observed, tending to be particularly wide in the lower parts of the stem.

Secretory ducts were present in the stem cortical parenchyma, in the ribs, aligned with the vascular bundles (Fig. 3b, c, d). In the lower part of the stem the secretory ducts were wider, averaging 24.0 ± 7.1 µm, with the epithelial cells measuring 10.4 ± 2.5 µm, while in the upper part, the secretory ducts were narrower, measuring 18.9 ± 3.8 µm and the epithelial cells smaller, averaging 6.0 ± 1.7 µm.

Leaf anatomy. All the leaves were 1-pinnate. The basal leaves possess a petiole, while the upper leaves were more or less sessile (Fig. 1). The leaf blade cross-section exhibited a linear shape and bilateral symmetry, with a palisade and spongy parenchyma structure (Fig. 4a, b). A single-layered epidermis was observed on both the leaf sides. The epidermal cells were irregularly polygonal in shape and larger on the upper leaf side. The leaf blade was amphistomatous and non-glandular trichomes were present on the surface of both sides of the leaf. The leaves in the upper part of the stem were slightly thicker than the lower ones, exhibiting a thicker palisade and spongy tissue, while the thickness of the epidermis in both types of leaves was similar (Table 1). Secretory ducts were observed within the leaf (Fig. 4c). Ducts larger in diameter were observed

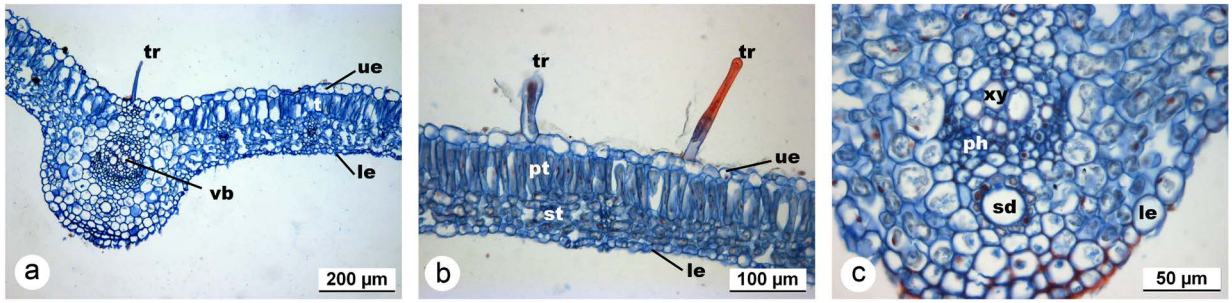

Fig. 4 Anatomical features of *Leiotulus aureus* leaf. a – midvein, b – trichomes on the upper leaf surface, c – secretory ducts. Abbreviations: ep – epidermis; ph – phloem; pt – palisade parenchyma; st – spongy parenchyma; sd – secretory duct; tr - trichomes; vb – vascular bundle; xy – xylem.

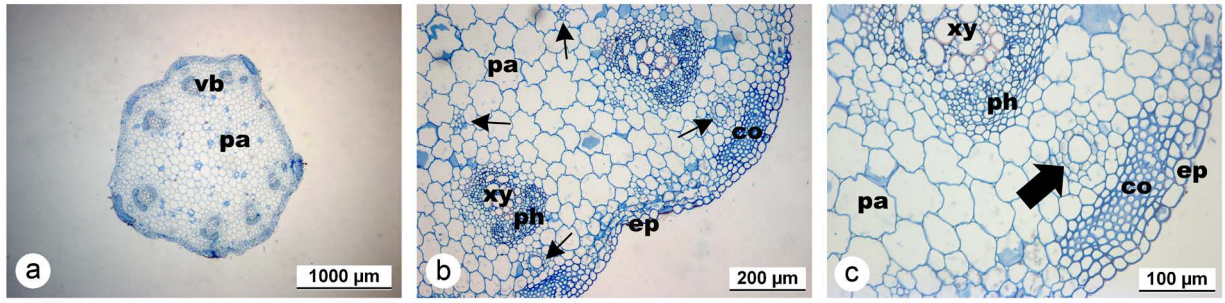

Fig. 5 Anatomical features of the leaf petiole of *Leiotulus aureus*. a – shape of the leaf petiole, b – cross section through the epidermis, primary cortex and outer parts of the central cylinder, c – the secretory ducts in the primary cortex. Abbreviations: ep – epidermis; co – colenchyma; pa – parenchyma; ph – phloem; vb – vascular bundle; xy – xylem. The arrow points to the secretory ducts.

in the parenchyma surrounding the vascular bundles in the midvein, next to the phloem, while smaller ones were observed next to the xylem. The secretory ducts present in the leaves were larger than those in the stem, particularly in the upper leaves.

Leaf petiole anatomy. The petiole had a polygonal shape, with small ribs and slightly concave areas between the ribs (Fig. 5a). The surface layer consisted of a single-layered epidermis, with alternately arranged collenchyma and chlorenchyma below (Fig. 5b, c). The central part of the leaf petiole was made up of 7–9 vascular bundles surrounded by parenchyma, with the largest parenchyma cells located in the middle part of the petiole. The vascular bundles were aligned with the ribs. In the ribs, in the parenchyma just below the collenchyma and next to the phloem of each vascular bundle, large intercellular secretory ducts were visible (Fig. 5b, c), with an inner diameter averaging 35.8 ± 8.0 µm. Each intercellular duct was surrounded by small epithelial cells (Fig. 5c), with a single cell size averaging 10.8 ± 3.7 µm. Moreover, smaller secretory ducts were positioned on the other side of the vascular bundles, in the parenchyma tissue next to the xylem of the vascular bundles (Fig 5b).

Fruit anatomy. The fruit was a dorsally compressed schizocarp composed of two single-seeded mericarps cordate-obovate in outline (Fig. 1), 2.54 ± 0.31 mm in width and a thickness of 0.64 ± 0.08 mm. Numerous non-glandular trichomes were present on the dorsal face of the mericarp, while the commis-

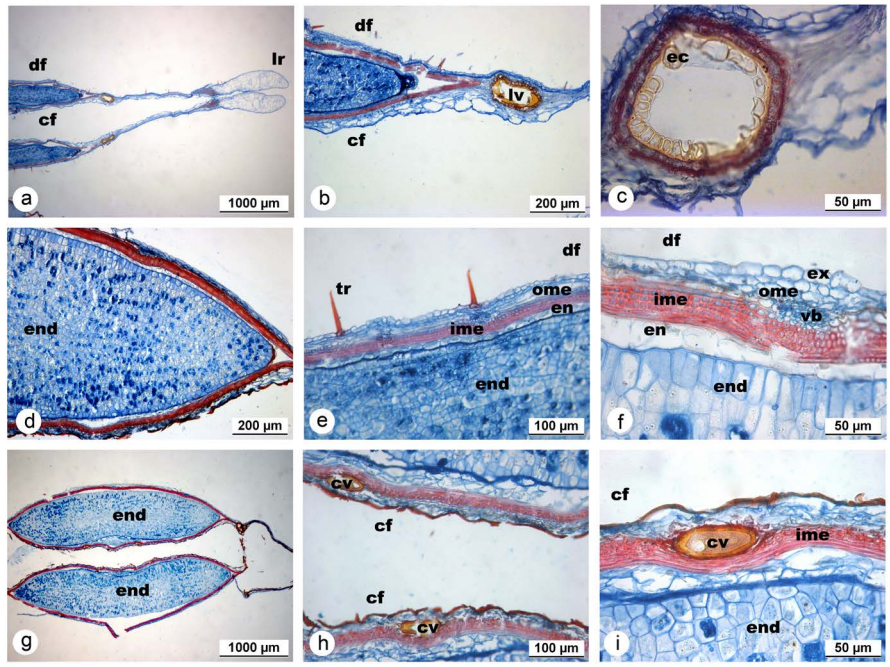

Fig. 6 Transverse section of the *Leiotulus aureus* fruit: a – lateral ribs, b, c – lateral vittae, d – endosperm, e, f – dorsal face, pericarp layers, g – the central part of the fruit, h, i – the commissural face and commissural vittae. Abbreviations: cf – commissural face; cv – commissural vittae; df – dorsal face; ec – epithelial cells; en – endocarp; end – endosperm; ex – exocarp; ime – inner mesocarp; lr – lateral ribs; lateral vittae; ome – outer mesocarp; tr – trichomes; vb – vascular bundle.

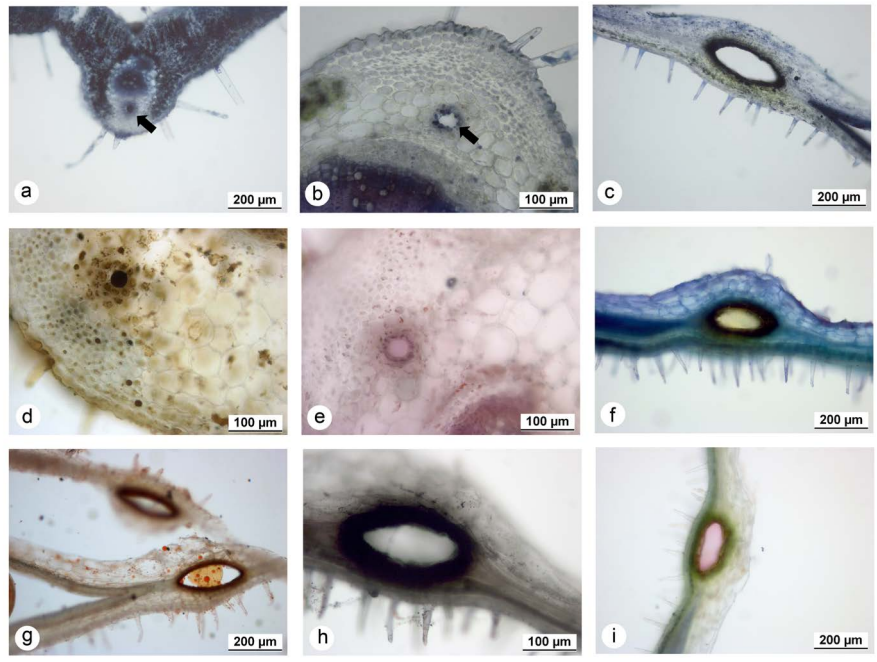

Fig. 7 Transverse sections of different plant parts of *Leiotulus aureus* with positive reactions to histochemical tests: a – leaf, essential oils (Nadi blue, indicated with arrow), b – stem, essential oils (Nadi blue, indicated with arrow), c – fruit, essential oils (Nadi blue), d – leaf petiole, alkaloids (Ditmar's reagent), e – stem, proteins (Acid Fuchsin), f – fruit, proteins (Naphthol Blue Black), g – fruit, lipids (Sudan III and Sudan Black), i – fruit, pectic substances (Toluidine Blue).

sural face ranged from sparsely hairy to glabrous and waxy (Fig. 6b, e, h, i). There were three filiform dorsal ribs, while the lateral ribs were winged, elongated in the neck and inflated in the distal part (Fig. 6a). The width of the proximal part measured 1.09 ± 0.24 mm, the width of the distal inflated part 1.04 ± 0.97 mm, and the thickness of the distal inflated part 0.29 ± 0.05 mm. The exocarp consisted of a single layer, which covered the dorsal side, the edges of the lateral ribs, and one-quarter to half of the commissural face on the inflated part of the

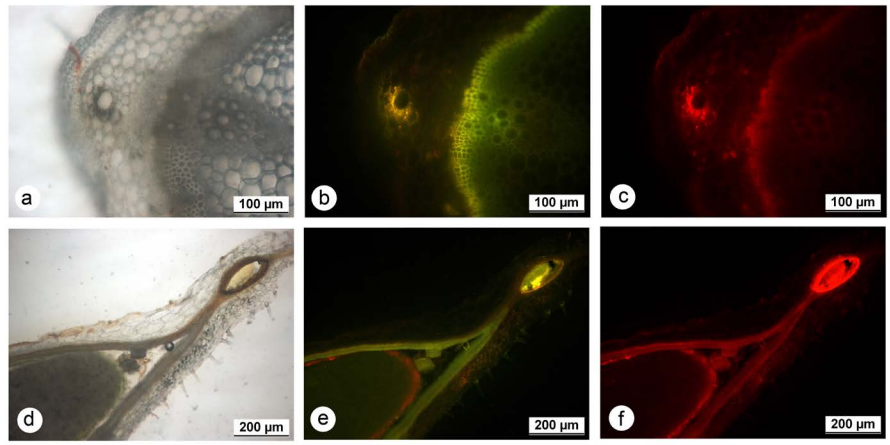

Fig. 8 Transverse sections of the stem (a–c) and fruit (d–f) of *Leiotulus aureus* observed by epifluorescence microscope in filter I3 showing fluorescence in the secretory ducts in the stem and in the large vitae in the mericarp wing, indicating the presence of phenolic compounds.

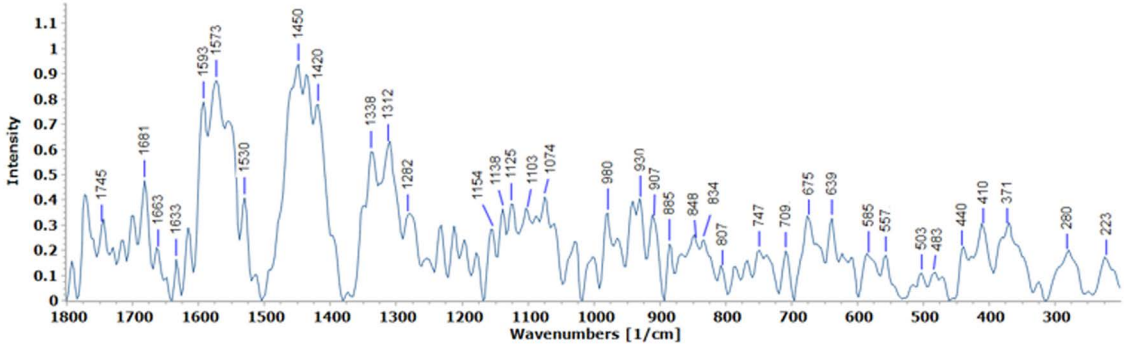

Fig. 9 Averages of normalized Raman spectra of *Leiotulus aureus* fruit samples, recorded within the spectral range from 200 to 1800 cm^{-1}

lateral ribs (Fig. 6d–f). The cells were rectangular with thickened outer walls. The outer mesocarp consisted of 4-5 layers of thin-walled parenchymatic cells, while the inner mesocarp (hypendocarp) was composed of 5–6 layers of lignified cells (fibres) with different orientations (Fig. 6e–f). The fibres in the outer part were vertical, while those in the inner part were horizontal. The endocarp consisted of a single layer of large thin-walled cells (Fig. 6f). The total pericarp was 53.2 ± 9.2 µm wide (min–max 31–76.6 µm), while the size of the seed cavity was almost identical to the seed size (Fig. 6g). Vascular bundles were situated in the outer mesocarp, under the ribs, just above the hypendocarp (Fig. 6f), as well as in the lateral ribs at in the base of the inflated part, next to the vertical lignified fibres. The inflated part of the marginal rib was mostly composed of large parenchymatic cells (Fig. 6a). The rib secretory ducts were filiform, and hardly recognisable. Four dorsal vittae were present: two lateral, 203.0 ± 32.9 µm wide (min–max 158.3–279.2 µm) and 114 ± 35.6 µm thick (min–max 59.6–165.7 µm), symmetrical and extending beyond half of the mericarp; two median 54.4 ± 14.3 µm wide (min–max 31.7–105.5 µm) and 28.7 ± 7.3 µm thick (min–max 16–49.7 µm), asymmetrical, and shorter that half of the mericarp. There were two commissural vittae, 61.8 ± 9.6 µm wide (min–max 46.1–78.4 µm), and 28.6 ± 6.2 µm thick (min–max 18.1–38.1 µm), symmetrical and extending to half of the mericarp. Both types of vittae were lined with epithelial cells (Fig 6c, h, i). In contrast to the thin-wall epithelial cells in the other plant parts, the walls of the epithelial cells in the fruit exhibited suberisation (Fig. 6c).

Histochemical analysis and fluorescence of the root, stem, leaf petiole, leaf and fruit of *Leiotulus aureus*. Histochemical tests using the Nadi reagent showed the presence of essential oils in the epithelial cells in the root, stem and in the secretory ducts in the leaves, leaf petioles and fruit (blue colour) (Fig 7a–c). Histochemical staining to detect alkaloids (with Ditmar's reagent) showed a slightly positive reaction in the secretory ducts in the fruits and leaf petiole (red-brown colour) (Fig 7d) and a negative one for the rest of the plant parts. Proteins were detected in the epithelial cells in the stem, leaf petiole and fruit (pink colour with Acid Fuchsin, and black colour with Naphthol Blue Black) (Fig. 7e, f), and lipids in the epithelial cells in the leaf petiole and fruits (red colour for positive staining by Sudan III Nile blue and black colour for positive staining with Sudan Black B) (Fig 7g, h).

Staining with Toluidine blue indicated the presence of pectic substances in the lumen of the secretory ducts in the fruit (pink colour) (Fig 7i). Histochemical staining to detect flavonoids was positive (with aluminium trichloride observed in the epifluorescence at 340–380 nm) in the peripheral tissue and the epithelial cells in the fruits. Histochemical staining for detecting steroids (with antimony trichloride) was slightly positive in the secretory ducts in the fruits and leaves (red colour). Autofluorescence of the non-stained sections (observed under epifluorescence at 340–380 nm) indicates the presence of phenolic compounds in the lumen of the secretory ducts, particularly in the stem and fruits (Fig 8).

Spectral fruit features. The average Raman spectrum of the *L. aureus* fruits exhibited the main bands which indicate the chemical composition of the oil ducts' content (Fig. 9). The higher intensity bands were positioned at 1573, 1450 and 1338 cm^{-1}, indicating the highest chemical contributions inside the ducts. Lower intensity bands positioned from 200 to 1200 cm^{-1} indicated the presence of less abundant components in the oil ducts. The predominant bands could be assigned to the octyl esters and octanol octyl esters (octyl butyrate or octyl isobutyrate).

DISCUSSION

Like all other Tordylinae, *Leiotulus aureus* exhibits a herbaceous habit, but unlike most of the representatives of Apioideae, its secondary growth is inconspicuous (Frankiewicz *et al.* 2022). However, it showed an ancestral/derived type of secondary growth because the cambial activity was not confined only to the vascular bundle, but was also present to some degree in the intervascular region (Fig. 3d, f). Considering the distinction between fascicular and interfascicular regions, *L. aureus* belongs to the group of apoids in which the vessels are mostly confined to the fascicular region, while the fibres did not differ among the regions (e.g. *Conium maculatum*). The perforation plates were simple, and intervessel pitting was alternate. According to Frankiewicz *et al.* (2021) intervessel pitting is associated with the type of background tissue; in stems with more fibres, alternate or transitional forms are dominant. Furthermore, the type of background tissue is correlated with reproductive strategy and it has been observed that species with monocarpic reproductive strategy and long internodes tend to develop fibrous wood, while polycarpy and shortening of internodes favour wood parenchymatisation (Frankiewicz *et al.* 2021, 2022). As a monocarpic biennial or short-lived species with long internodes, *L. aureus* deposits fibrous wood. The vessel outline observed in the transverse section ranged from rounded to slightly angular (Fig. 3d), which is considered a uninformative trait in apoids (Frankiewicz *et al.* 2022). Axial parenchyma was not distinguished, while the rays were of the medullary type. Both features are commonly present in herbaceous umbellifers (Frankiewicz *et al.* 2021).

In addition to the interdependence of background tissue and reproductive strategy, several studies of the stem anatomy of Apioideae have tested the hypothesis of a correlation among wood traits and plant size (Olson *et al.* 2014; Frankiewicz *et al.* 2022). Vessel elements and fibre length, vessel diameter and intervessel vertical pit diameter showed a positive correlation with the plant height, while vessel frequency was negatively correlated.

As implied in previous research on the chemical constituents of different plant parts of *L. aureus* and their biological activities, secretory structures were present in both the root (Vučković *et al.* 2014) and aboveground organs (Tzakou *et al.* 2008; Vučković *et al.* 2014). The current anatomical and histochemical investigation confirmed that the secretory ducts form a network extending from the roots through the stem to the leaves, flowers and fruits (Figs. 2–6), just as in the majority of representatives of the Apiaceae family (Svoboda & Svoboda 2000). In the root, they are located exclusively in the cortical parenchyma, although in some genera of *Tordylieae* (e.g. *Opopanax*) they also appear in the secondary xylem (Metcalfe & Chalk 1950). In the aboveground vegetative organs, they are situated in the cortical parenchyma, in the ribs, aligned with the vascular bundles, just like in other studied Tordylieae, e.g. *Trigonosciadium* (Eroğlu *et al.* 2017).

The fruit secretory structures of the Apiaceae family are very specific, because they are dimorphic and contain: i) the ducts associated with the vascular bundles, occurring in all of the aerial vegetative parts and extending to the fruits, and ii) the vittae, located exclusively in the fruits (Bradley & Fell 1966).

Traditional classification within the entire Apiales order relies on carpological characteristics (Liu *et al.* 2006). Thus, the fruits of *L. aureus* were studied in detail by Pimenov & Ostroumova (1994), in order to support the delimitation of similar genera within Tordylieae. The fruit margin played a significant role, and according to this feature, *Pastinaca* is clearly distinguished from *Malabaila* s.str.*, Leiotulus* and *Trigonosciadium* by the almost complete absence of parenchymatic tissue in the marginal wings. The carpological characteristics of *L. aureus* are considered intermediate between *Pastinaca* s.l. and *Zosima,* almost equally distant from both (Pimenov & Ostroumova 1994). Like the *Pastinaca* fruit type (including *Malabaila gravolens*), *L. aureus* is characterised by a markedly thickened distal part of the marginal mericarp ribs, while similar to *Zoosima,* it has rather narrow secretory ducts and a less developed distal part of the marginal mericarp ribs. These general observations were confirmed by our results (Fig. 6).

Interpretations of the origin of the lignified pericarp layer and type of endocarp differ among authors. Pimenov & Ostroumova (1994) consider lignified fibres as part of the mesocarp (hypendocarp), while the endocarp consists of thin-walled parenchyma cells, which are not visible in fully ripened fruit. The endocarp is lignified. In this study, the approach of Pimenov & Ostroumova (1994) was followed. The measurements of most mericarp sections of the Montenegrin population (e.g. mericarp width, lateral ribs, the width of the dorsal and commissular vitae, etc.) corroborate the results of previous carpological studies, which included Greek and Macedonian populations of *L. aureus* (Pimenov & Ostroumova 1994).

Histochemical staining of the fruits confirmed the presence of essential oils, free fatty acids, phospholipids, proteins, and phenolic compounds (Fig. 7). The most important and characteristic fatty acid methyl esters of the *L. aureus* fruits were identified as the octyl esters (octyl butyrate or octyl isobutyrate) and octanol (Vučković *et al.* 2014). They account for more than 95% of the seed lipids, with the CH_2 group belonging to the butyric acid residue. The predominant bands in the Raman spectrum of *L. aureus* oil ducts' content could be assigned to the octyl esters and octanol octyl esters (Fig. 9). Other signals probably originate from octanol, the second most abundant component in the fruits (Vučković *et al.* 2014).

The bands in the 1730–1750 cm^{-1} range were assigned to C=O stretching in the -CH_2-COOR ester-carbonyl stretching mode, which was present in all the Raman spectra of the oil samples and could be associated with the presence of fatty acid methyl ester (Farber *et al.* 2020). The signals in the 1630–1680 cm^{-1} region (Fig. 9) were typical of the olefinic ν(C=C) stretching vibration of unsaturated fatty acids (Popović-Djordjević *et al.* 2023). The intense and broad bands in the range of 1400–1500 cm^{-1} (e.g. bands at 1420, 1450 cm^{-1}) could be assigned to the CH_2 scissoring deformation vibration (Beattie *et al.* 2004). The bands at 1282 and 1312 cm^{-1} are consistent with the in-phase CH_2 twisting vibration (Beattie *et al.* 2004). Lower intensity bands in the 1080–1135 cm^{-1} range (Fig. 9) may indicate in-phase aliphatic C–C stretches all-trans (Potcoava *et al.* 2021). The C-C stretching mode due to terminal C-C(=O), which occurred in the 800–900 cm^{-1} range, was thought to be uncoupled from the C-C stretching modes of the hydrocarbon chain skeleton, which occurred in the 1050–1150 cm^{-1} range. Bands at 907, 930 and 979 cm^{-1} (Fig. 9) were due to an out-of-plane =C-H bending vibration (Miranda *et al.* 2014). The band at about 850 cm^{-1} might indicate a mixture of stretches and rocks at the acyl and methyl terminals or pectin (Oakes *et al.* 2002). The Raman bands up to 800 cm^{-1} probably indicated C-C-C, C-C-O stretching and methyl rock vibration (Oakes *et al.* 2002; Miranda *et al.* 2014), such as the band on the cholesterol ring at 709 cm^{-1} (Potcoava *et al.* 2021).

The research conducted by Berenbaum & Zangeri (1986) on the diversity of the content of the fruit secretory structures was particularly interesting. They studied the localisation of furanocoumarins in ripe fruits of *Pastinaca sativa* and concluded that over 90% of its total content was present in the two inner vittae, while the four outer vittae on the noncommissural side consistently contained less than 10% of the total. The relative composition of furanocoumarins also differed between the vittae. In order to define the patterns of secondary metabolites in *L. aureus,* further and more detailed chemical characterisation is needed and recommended.

All phylogenetic studies agreed on the rather close relationship between species of *Malabaila, Leiotulus, Pastinaca* and *Trigonosciadium*, which have either been grouped into the *Pastinaca* Clade (Ajani *et al.* 2008; Logacheva *et al.* 2008) or the *Heracelum* Clade (Downie *et al.* 2010). Very interesting results were obtained in the study conducted by Menemen *et al.* (2016), in which the examined accessions of *Malabaila* (incl. *Leiotulus*)*, Pastinaca* and *Trigonosciadium* formed a well-supported clade (99%), within two groups provisionally recognised as *Pasinaca sativa* s.l. and *Malabaila* s.l. The species *Malabaila aurea* (= *Leiotulus aureus*) allied with *Tordylium elegans* and *T. maximum* near the base of all the phylogenetic trees. These *Tordylium* species shared similarities with *L. aureus* in terms of certain morphological features such as leaf and fruit shape, smooth mericarp margins, and the number of vittae (Al-Eisawi & Jury 1988) (Figs. 1, 6). A recent study on the chemical characterisation and biological propensities of the related species *Malabaila lasiocarpa* showed that different bark extracts demonstrated antioxidant potential, and inhibited enzyme activities (e.g. tyrosinase, an enzyme associated with hyperpigmentation, as well as α-amylase and α-glucosidase, enzymes associated with type 2 diabetes, Zengin *et al.* 2022). Current knowledge indicates that extracts from the aerial parts of *Leiotulus aureus* have antioxidative potential, and the essential oils of the aerial parts showed insecticidal activities and significant activity against *Staphylococcus aureus* and *Candida albicans* (e.g. Tzakou *et al.* 2008; Evergetis *et al.* 2013). Despite these features, there is no written evidence of the use of *L. aureus* in enthnobotany in the Dinaric region. According to Vučković *et al.* (2014), the most abundant volatile compounds in the methanol extracts of the roots, stems and leaves were apiole, myristicin and falcarinol, while the volatile constituents of the

fruits and flowers consisted mainly of octyl butyrate, octanol, and octyl hexanoate. The majority of Apiaceae contain apiole, which acts as an antioxidant, antifungal, anticancer, abortifacient, acaricidal, phytotoxic, antitumor, and antiproliferative agent (Tabassum 2021). Myristicin has been associated with anticarcinogenic, anti-inflammatory, antimicrobial, antioxidant, antidiabetic, analgesic, and hepatoprotective effects (Ramírez-Alarcón *et al.* 2023). Falcarinol exhibited biphasic effects on cell proliferation and cytotoxic and potential antitumor activity, which are concentration dependent (Hansen *et al.* 2003). Octyl butyrate expressed cytotoxic activity (Maggi *et al.* 2014), and octanol anticandidal activity (Işcan *et al.* 2004).

Considering the results of previous investigations on the biological properties of *L. aureus* and the suggested potential of the extracts (Tzakou *et al.* 2008; Vučković *et al.* 2014), future phytochemical investigations are recommended, particularly of the inhibition of enzyme activities.

CONCLUSIONS

Histo-anatomical and histo-chemical studies on *L. aureus* have confirmed the presence of specialised secretory structures related to the production of secondary metabolites. These structures were observed in all the plant parts, forming a network extending from the roots through the stem to the leaves, flowers, and fruits. The presence of phenolic compounds, flavonoids, fatty acids, and octyl-esters confirmed by histochemical staining, epifluorescence, and Raman spectroscopy, imply numerous potentials of their secretions. These include antioxidative, antifungal, anticancer, and phytotoxic effects, as well as insecticidal activities, thus highlighting the need for further investigations of secondary metabolite pathways.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Anatomske i histohemijske osobenosti endemične vrste *Leiotulus aureus* (Apiaceae)

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Leiotulus aureus je endemična štitonoša Balkanskog poluostrva i Turske. Pripada kompleksnom tribusu *Tordylieae*, čiji su rodovi karpološki detaljno okarakterisani, što nije i slučaj sa vegetativnim delovima biljke. Skorašnja hemijska istraživanja su ukazala na prisustvo sekretornih struktura koje su povezane sa stvaranjem sekundarnih metabolita, i njihova lokalizacija u biljci predstavlja fokus ovih istraživanja. Biljni materijal je sakupljen u Crnoj Gori. Anatomija biljnih organa je izučavana na parafinskim presecima, kao i na nativnim presecima bojenim standardnim reagensima koji se koriste u histohemijskim analizama, dok su sekreti u kanalićima plodova okarakterisani Ramanovom spektroskopijom. Sekretorne strukture su uočene u svim delovima ove monokarpne dvogodišnje ili kratkoviječe biljke, gdje formiraju svojevrsnu mrežu koja se širi kroz sve delove, počev od korena, kroz stablo, listove, cvetove i plodove. U korenu se nalaze isključivo u parenhimu primarne kore, a u nadzemnim vegetativnim delovima biljke u rebrima i to u liniji sa provodnim snopićima. Plodovi sadrže dobro razvijene dorzolateralne i komisuralne vite, i končaste i veoma teško uočljive središnje dorzalne vite i kanaliće uz provodne snopiće. Histohemijskim bojenjem, epifluorescencijom i Ramanovom spektroskopijom je potvrđeno prisustvo fitofarmaceutski važnih jedinjanja: fenolnih komponenti, flavonoida, masnih kiselina i oktil-estara, što predstavlja preporuku za dalja istraživanja sekundarnih metaboličkih puteva.

Ključne reči: *Malabaila aurea*, svetlosna mikroskopija, epifluorescencija, mikro-Raman, 532 nm, sekretorne strukture