MICROMORPHOLOGY AND HISTOCHEMISTRY OF *LEONOTIS LEONURUS* L. TRICHOMES

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Abstract. *Leonotis leonurus* (Lamiaceae) (Wild dagga/Lion's ear) is an important medicinal plant known for its psychoactive and medicinal properties and used to treat several ailments such as fever, influenza, asthma, epilepsy, snakebite, tapeworm, and skin diseases. Various biological activities, including antifungal, antibacterial, antiprotozoal, anti-inflammatory effects, and modulation of immune cell functions, are exhibited by extracts and isolated compounds from *L. leonurus*. The purpose of this study was to examine the micromorphology, distribution, and chemical composition of the bioactive compounds in the trichomes of *L. leonurus*, using light and electron microscopy, along with histochemical analysis. Stereomicroscopy and scanning electron microscopy (SEM) revealed the presence of both glandular and non-glandular trichomes on the leaves and stems. The presence of peltate glandular trichomes, morphologically distinct glandular capitate trichome types (type I and II), and uniseriate, multicellular (two to five cells) pointed-shaped non-glandular trichomes were revealed using histochemistry and SEM. Trichomes on the stained leaves and stems revealed phenolics and alkaloids as the primary medicinal compounds. Secondary metabolites are essential for defense against herbivory, inhibiting and repelling microorganisms, and may also possess medicinal properties. To the best of our knowledge, this study offers updated insights into the key micromorphological characteristics of the secretory structures on the leaves and stems of *L. leonurus*, along with preliminary information on the composition of the secretions produced by these trichomes.
Keywords: *chemical compounds, microscopy, medicinal properties, secretory structures, plant anatomy*

Introduction

Leonotis leonurus (Lamiaceae) (Wild Dagga/Lion's ear) is a fast-growing shrub, which reaches up to 2-3 m tall and 1.5 m wide. This shrub features vertically branched stems, adorned with several bulbous inflorescences that bear orange, nectar-rich flowers. These flowers emerge from a spiny calyx that protects both the buds and, later, the seeds. Stems are distinctively square in cross-section, velvety, and woody at the base (Turner, 2001). This shrub is widespread throughout South Africa (KwaZulu-Natal, Limpopo, Mpumalanga, Eastern Cape and Western Cape) and grows amongst

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rocks and in grasslands (Turner, 2001; Nsuala et al., 2015). This species is also found in the tropical regions of America and was introduced to Europe as an ornamental (Ascensão et al., 1995). The genus *Leonotis*, often associated with Cannabis, is often believed to be narcotic as dried leaves are crushed and smoked or mixed with tobacco (Watt and Breyer-Brandwigk, 1962). *Leonotis leonurus* is famous for its psychoactive and medicinal properties (Hutchings et al., 1996). Africans (Zulus, Xhosas, and Hottentots) use the leaf extracts to treat various ailments such as fever, influenza, asthma, epilepsy, snakebite, tapeworm, and skin diseases (Ascensão et al., 1997; Scott et al., 2004). The aerial parts of *L. leonurus*, including leaves and flowers, can be administered in various forms, such as decoctions, infusions, or by smoking (Van Wyk et al., 1997). A range of biological activities, such as antibacterial, antifungal, and anti-inflammatory effects, have been shown to be demonstrated by extracts and isolated compounds from *L. leonurus* (Ojewole, 2005).

An important characteristic of the Lamiaceae family is the essential oil secreted by glandular trichomes (Fahn, 1988). Trichomes found in Lamiaceae are capitate glandular trichomes which contain one or two secretory cells, and peltate trichomes, which can have as many as eight cells. A subcuticular space, located outside the cell wall, accumulates the secretory substance found in glandular trichomes. Most of the essential oil is produced by peltate trichomes (Huang et al., 2008). Peltate, uniseriate glandular trichomes, and multicellular non-glandular trichomes were also found in *L. nepetifolia* (Giang et al., 2021). Several glandular trichomes (peltate and capitate) were found by Ascensão et al. (1995) on the vegetative and reproductive organs of *L. leonurus*. Phytochemical studies reported *L. leonurus* contained essential oils that were rich in terpene hydrocarbons (β-caryophyllene, α-pinene) (Pedro et al., 1991) labdane diterpenoids (Purushothaman and Vasanth, 1988), phenol, 2-methoxyl-4-(1-propenyl)-trans-isoeugenol (Tonisi et al., 2020). Trichomes may be uni- or multicellular structures with various morphologies, including spiral, straight, star-shaped, branched, or hooked and can be classified as either glandular or non-glandular (Werker, 2000; Li et al., 2023). A key role in sequestering and detoxifying metals is played by trichomes (Choi et al., 2001; Azmat et al., 2009; Li et al., 2023). Detoxification occurs when metal ions are inactivated by complexing with heavy-metal compounds such as phytochelatins and metallothioneins (Choi et al., 2001).

The micromorphology of the secretory structures, which are involved in synthesizing and secreting phytochemicals, has been seldom studied in this species. The distribution, micromorphology, and composition of chemicals located in the trichomes on the leaves and stems of *L. leonurus* are what this study seeks to investigate. Furthermore, previous studies (Ascensão et al., 1995, 1997) conducted on *L. leonurus* secretory structures were from 1995-1998 (26 years ago) in Portugal. This study further aims to provide updated information on any micromorphological differences that may have happened due to genetic changes, climate change, or the different geographic regions. The preliminary research conducted in this study will add valuable information to the South African medicinal plant database.

Materials and methods

Plant materials

Plant material was gathered from the University of KwaZulu-Natal, Durban, South Africa. The plant material was taxonomically identified, and a voucher sample was stored

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in the BEWS Herbarium at the University of KwaZulu-Natal, Pietermaritzburg Campus (Accession no. UDW23004). Different developmental stages of the leaves and stems were analyzed, including emergent, young, and mature stages. The stages of leaf developmental were based on characteristics such as color, texture and length. Based on the length of the leaves, material was categorized as emergent (6–8 mm), young (15–20 mm), and mature (40–45 mm) based on their length. Approximately three *L. leonurus* plants were sampled for the analysis, with leaves and stems collected in triplicate.

Stereomicroscopy

A stereomicroscope, Nikon AZ100 (Nikon Corporation, Yokohama, Japan) fitted with a Fiber Illuminator, was used to examine fresh plant material (leaves and stems). The specimens were photographed and analyzed using NIS-Elements Software (version D 3.00). Images were captured of the fresh leaves (both adaxial and abaxial surfaces) at different developmental stages.

Scanning electron microscopy (SEM)

Leaf sections (approximately 5 mm²) at three developmental stages, along with stems, were subjected to chemical fixation using standard protocols (Gangaram et al., 2020). Using a Quorum K850 Critical Point Dryer (Quorum Technologies Ltd., Laughton, East Sussex, UK), the samples were critical point-dried after dehydration. Using a Quorum 150 RES sputter coater (Quorum Technologies Ltd.), sections were placed onto stubs (aluminum) using adhesive double-sided carbon tape and then sputter-coated with gold. Sections were examined with the LEO 1450 SEM, and imaged with the SmartSEM imaging software (Zeiss, Jena, Germany).

Morphological analysis of trichomes

Using the ImageJ software program (Schindelin et al., 2012), selected SEM images of trichomes were analyzed. The diameter of each type of trichome head was measured.

Leaf sections (approximately 2 mm²) at each developmental stage was removed and fixed chemically and dehydrated using standard protocols (Gangaram et al., 2020). Transferred to propylene oxide for 15 min, the dehydrated samples were infiltrated with increasing concentrations of Spurr's low-viscosity epoxy resin in propylene oxide (25%, 50%, 75%, and 100%). Embedded in a mixture of equal parts Spurr's resin and acetone for 4 h, the samples were then placed in 100% resin at 70°C for 24 h (Spurr, 1969). Transferred to silicon molds after embedding, the samples were polymerized at 70°C for 8 h. Glass knives were prepared using standard protocols (Gangaram et al., 2020) and used to section the polymerized resin blocks. A Reichert Jung ultramicrotome (Leica, Wetzlar, Germany) was used to obtain ultrathin leaf and stem sections. Stained with 1% Toluidine Blue, the sections were mounted on microscopy slides and viewed using a Nikon Eclipse 80i light microscope (Nikon Corporation) (Protocol adapted from the Microscopy and Microanalysis Unit).

Histochemistry

Fresh leaves and stems at all stages of development were hand-sectioned to a thickness of 90–150 µm for histochemistry. The sections of interest were stained with various reagents to identify and localize key chemical compounds. Stained cross-

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sections were examined and imaged using a Nikon Eclipse 80i compound light microscope. Phenolic compounds were observed using Ferric trichloride (McCracken and Johansen, 1940; Demarco, 2017), while alkaloids were identified with Wagner's and Dittmar's staining reagents (Furr and Mahlberg, 1981). Lignin, cutin, and suberin were stained with safranin, polyphenols were visualized using toluidine blue, and lipids were detected with Sudan Black B (Demarco, 2017). Mucilage and pectin were stained with Ruthenium red (Demarco, 2017).

Fluorescence microscopy

Sections of material (80–100 µm) were examined using a Nikon DS-Fi1 compound microscope (Nikon, Japan). To assess cell viability, Acridine orange was used to stain the sections (Demarco, 2017).

Results and discussion

Leaf and stem micromorphology

The emergent leaf surfaces of *L. leonurus* were characterized by a thick covering of both non-glandular and glandular trichomes (*Fig. 1A, B*). The distribution and density of these trichomes varied across both the leaves and stems. Emergent leaves are noted for being nutrient-dense, therefore requiring enhanced protection, potentially provided by the trichomes. These leaves are particularly vulnerable to attacks by insects and pathogens (Chaurasiya et al., 2007; Gangaram et al., 2020), and a higher density of trichomes may play a role in influencing secondary metabolite production (Agrawal et al., 2009). Furthermore, a high density of trichomes may reduce the absorbance of light (including UV), creating a denser boundary layer (Ehleringer and Björkman, 1978). This, in turn, reduces leaf overheating and photochemical injury, allowing for increased carbon accumulation at lower transpiration rates, which improves water-use efficiency (beneficial in hot and arid environments) (Ripley et al., 1999; Bickford, 2016). Non-glandular trichomes (uniseriate, multicellular, and pointed in shape, consisting of two to five cells) were densely concentrated along the midrib and veins of the emergent leaves (on both the adaxial and abaxial surfaces) and on the abaxial surface of the young leaves (*Fig. 1A, B, D*). The density of both glandular and non-glandular trichomes were observed to decrease as the leaves developed (*Fig. 1A-F*). Research describes the expansion of leaf cells during plant development, resulting in the number of trichomes deteriorating with growth (Werker et al., 1993). Furthermore, leaf trichome density, described as the number of trichomes per unit of leaf area, influences plant performance in both wild and crop ecosystems. (Gupt et al., 2021; Vinod et al., 2023). There is a lack of research on the leaf and stem micromorphology in species within the genus *Leonotis*, which provides an opportunity for further studies.

Secretory structures observed using SEM

Trichome density decreased as leaf development progressed (*Fig. 2A-D*). Two types of capitate trichomes, grouped as I and II, were identified. These trichomes, present at all stages of leaf and stem development, differed in size and shape. Trichomes were most densely distributed along the midvein at every stage of leaf development, while being sparse along the leaf margins. The non-glandular trichomes, due to their greater length and proximity to the glandular trichomes, likely offers physical protection.

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Ascensão et al. (1995) and Ascensão and Pais (1998) reported the presence of both capitate and peltate trichomes on the leaf adaxial and abaxial surfaces, with the highest concentration occurring in the interveinal areas. Type I capitate trichomes were characterized by a secretory head consisting of four cells (25.48 ± 3.44 µm) and a short stalk, which is sometimes not visible, and are embedded within the epidermal layer (*Fig. 3A, B*). Type II capitate trichome comprises a 4-celled secretory head (36.38 ± 1.54 µm), a long stalk, with a smooth surface and cuticle striations at the base (*Fig. 3C*). The production of different chemical compounds that perform specific functional roles may be associated with the varied morphological characteristics of certain capitate trichomes (Werker et al., 1985). Peltate trichomes consist of a short stalk cell (unseen), cutinized lateral walls, and a broad secretory head with eight cells (47.43 ± 1.85 µm) (*Fig. 3D*). Stomata were observed on both the leaf and stem surfaces (*Fig. 3D*). The closely packed non-glandular trichomes are assumed to provide protection to the stomata from excessive heat during dry and hot seasons. Ascensão et al. (1995) found both types of capitate trichomes and peltate trichomes on the leaves and flowers of *L. leonurus*. The morphology, distribution, and occurrence of glandular and non-glandular trichomes in the lamiaceae family can be used as descriptive characters at sub familiar level (Ascensão et al., 1995). Updated research on the secretory structures of the genus *Leonotis* and the species *L. leonurus* is lacking.

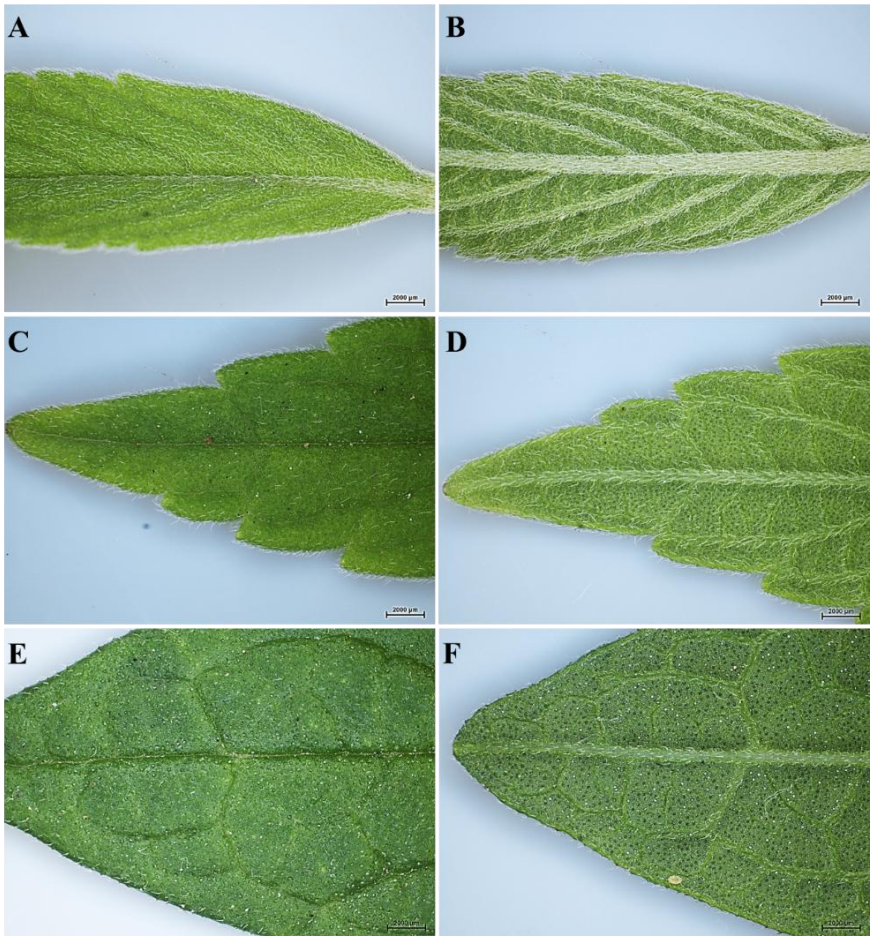

Figure 1. *Stereomicrographs showing trichome distribution on Leonotis leonurus. (A) Emergent leaf (Adaxial); (B) Emergent leaf (Abaxial); (C) Young leaf (Adaxial); (D) Young leaf (Abaxial); (E) Mature leaf (Adaxial); (F) Mature leaf (Abaxial)*

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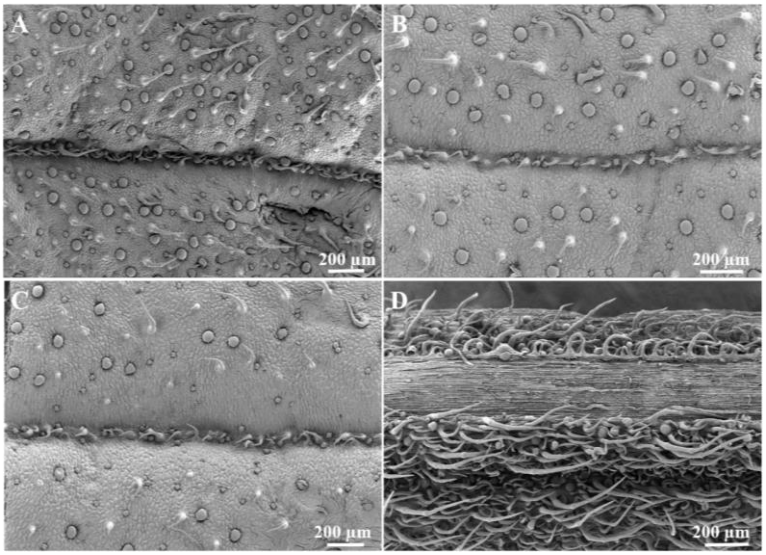

Figure 2. *Scanning electron micrographs illustrating the distribution and density of glandular and non-glandular trichomes on the leaves and stems of Leonotis leonurus. (A) Adaxial surface of an emergent leaf; (B) Adaxial surface of a young leaf; (C) Adaxial surface of a mature leaf; (D) Abaxial surface of a mature leaf; (E) Stem surface*

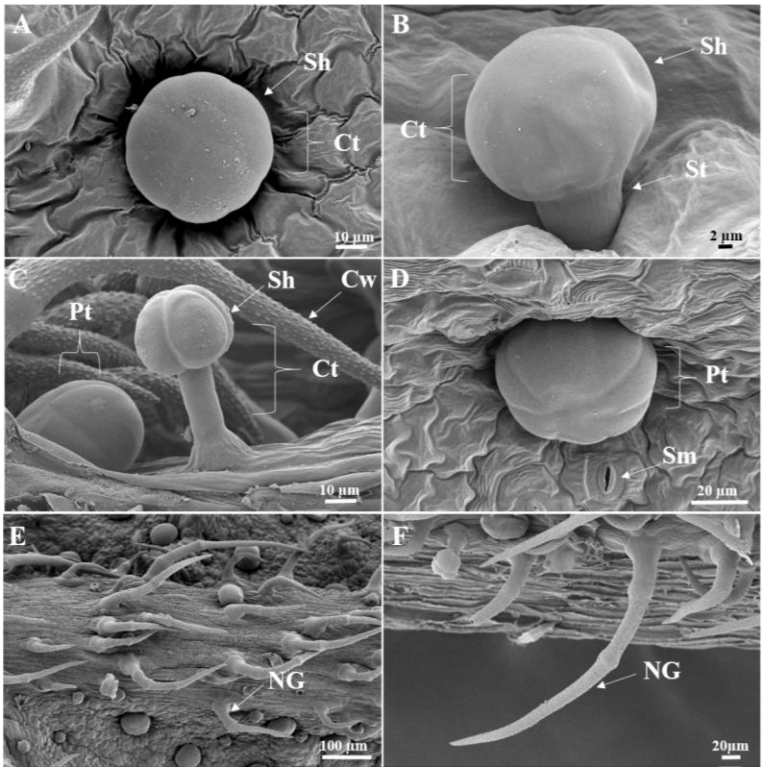

Figure 3. *Scanning electron micrographs of glandular capitate trichomes on the emergent, young, and mature leaves and stems of Leonotis leonurus. (A) Type I glandular capitate trichome comprising of a short stalk (St) and a broad secretory head (Sh); (B) Type I glandular capitate trichome with a short stalk (St); (C) Type II glandular capitate trichome with a secretory head (4-cells) and a peltate trichome (Pt); (D) Glandular peltate trichome with a seven-celled secretory head (Sh); (E-F) Uniseriate, multicellular (two to five cells), pointed-shaped non-glandular trichomes (NG)*

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Resin-embedded survey sections observed via light microscopy

The midrib's adaxial surface featured a short, wide protuberance, while the abaxial surface had a long, wide semicircular area (*Fig. 4A*). The leaf surface appeared dorsiventral, with the epidermal cells on the adaxial surface being wide and thick, while those on the abaxial surface were cylindrical and narrow. The cross-section of the leaf midrib reveals the palisade mesophyll cells, ground tissue, xylem, and phloem (*Fig. 4B-C*). Ground tissue consists of three types of tissue, collenchyma, sclerenchyma, and parenchyma cells (Crang et al., 2018). Palisade mesophyll cells were arranged in a single row on the adaxial surface, appearing long, thick, and wide. The vascular bundle appeared U-shaped and thick, composed of small layers of phloem elements and long, tightly packed rows of thick-walled xylem elements. Similar observations were noted in the leaves and stem survey sections of *L. nepetifolia* (Giang et al., 2021). Specialized, large epidermal cells known as lithocysts contained cystoliths (*Fig. 4A*, *C*). Lithocysts are enlarged epidermal cells comprising calcium carbonate in a cellulose matrix, with the calcium carbonate concretion known as a cystolith. Cystoliths vary in structure and are found in 620 angiosperm families (Crang et al., 2018). Uniseriate, multi-cellular pointed shaped non-glandular trichome was observed on the leaf and stem sections (*Fig. 4D*). Glandular peltate and capitate trichomes were observed on the survey sections. Capitate glandular trichomes were scattered only on the abaxial side of the leaves survey sections of *L. nepetifolia* (Giang et al., 2021).

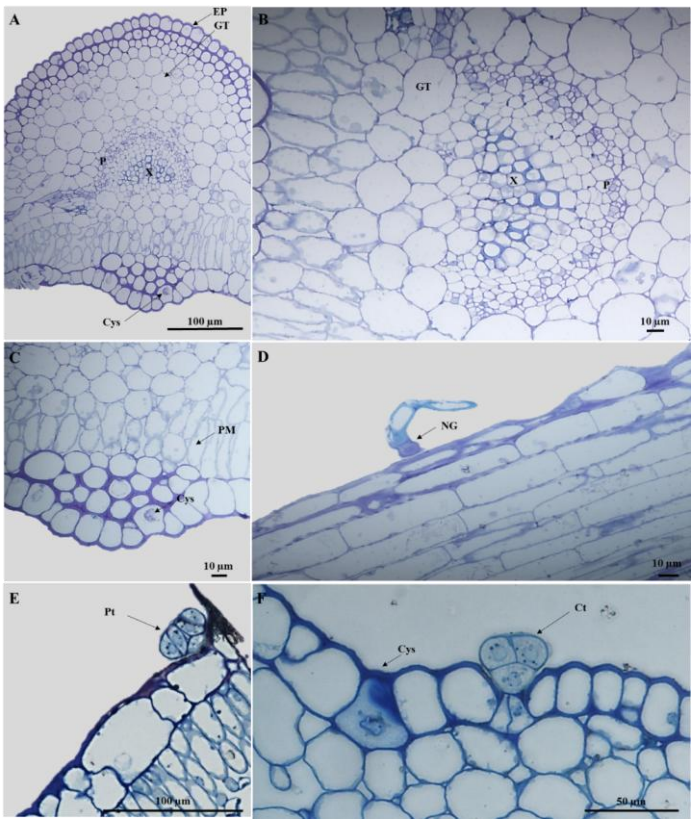

Figure 4. *Survey sections of resin-embedded plant material from Leonotis leonurus. (A) Midrib (emergent leaf); (B) Section of stem material; (C) Lower epidermal layer (young leaf); (D) Upper epidermal layer (mature leaf). Abbreviations: EP = Epidermis; GT = Ground tissue; PM = Palisade mesophyll; P = Phloem; X = Xylem; NG = Non-glandular trichome; Cys = Cystolith; Pt = Peltate trichome; Ct = Capitate trichome*

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Secretory structure's chemical composition

Histochemical tests are employed to identify and localize key chemical classes of metabolites in plant secretions (Ascensão et al.,1997). Glandular and non-glandular trichomes at all stages of leaf development were positively stained with Ferric trichloride (for phenolics), Wagner's and Dittmar's reagents (for alkaloids), Safranin (for lignin, cutin, and suberin) (*Fig. 5*), and Toluidine Blue (for carboxylated polysaccharides, phosphate groups, and polyphenols). Additionally, Sudan Black stained for lipids, cutin, and suberin, while Ruthenium Red was used to detect mucilage (*Fig. 6*). *Table 1* provides an overview of the reactions of different glandular trichome types to the various histochemical tests. No significant differences were observed in the results across trichome types. However, Ascensão et al. (1995) and Ascensão and Pais (1998) did not detect alkaloids, phenols, or tannins in the secretions of peltate trichomes. Phenols are crucial in defense and chemical protection and may be found in the cuticle, epidermis, and trichomes (Karabourniotis et al., 2020). Alkaloids observed in the trichome head cells may be related to the plant's chemical defense system, as these substances are poisonous to herbivores and insects (Wang et al., 2001; Nandi et al., 2022). Lignin provides structural support in plants and is located in the plant's cell walls of xylem vessels and sclerenchyma tissue (Boerjan et al., 2003; Swaminathan et al., 2022). The occurrence of cutin in the trichome head and stalk cells inhibits the backflow of exudates into the leaf (Fahn, 1988; Gangaram et al., 2020) and is assumed to contribute to structural support (Payne, 1978). Suberin found in trichomes cells has been associated with the controlled movement of metabolites (aqueous) to the apoplast. This movement enables the trichome stalk cells to control the directional flow of metabolites toward the head cells of the glandular trichomes (Werker, 2000; Lange and Turner, 2013). The development and secretion of mucilage noted in *L. leonurus* can provide further evidence of a protective function and may form a system that integrates the physical and chemical properties of the secretion with changes in water availability (Werker and Fahn, 1981; Ascensão et al., 1999; Waigwa et al., 2020). Trichomes (glandular and non-glandular) stained with acridine orange illustrated viability (*Fig. 7*). Acridine orange stains lysosomes and is used to determine cell viability (Pierzynska-Mach et al., 2014)

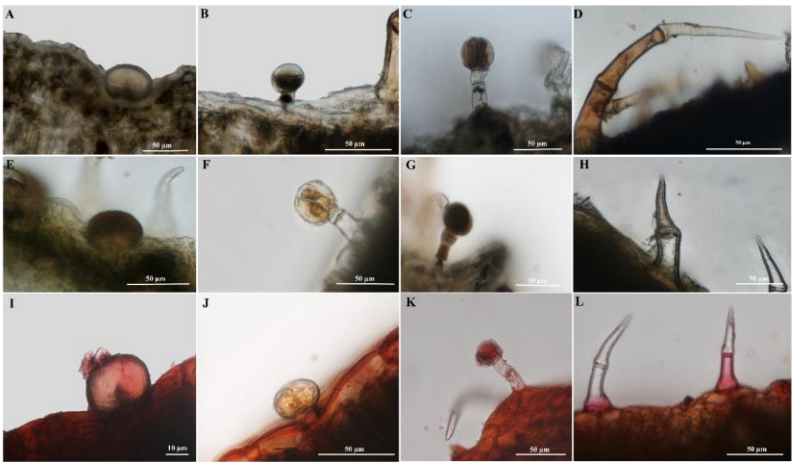

Figure 5. *Light micrographs of stained trichomes present on the leaves and stems of Leonotis leonurus. (A-D) Ferric trichloride staining black (phenolic deposits in the head cells); (E-H) Wagner's/Dittmar's reagent staining brown (alkaloids in the head and stalk cells); (I-L) Safranin staining red (cutin, lignin and suberin in the head and stalk cells)*

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Table 1. *Histochemical analysis of the glandular and non-glandular trichomes on the leaf and stem sections of L. leonurus*

Compound group	Stain/s	Trichomes				Reaction observed
		Peltate	Type I	Type II	Non-glandular	
Phenolic compounds	Ferric trichloride	+	+	+	+	Cells in the head and stalk stained dark black in the glandular trichomes, while the base and stalk of the non-glandular trichomes stained brown to black
Alkaloids	Wagner's and Dittmar	+	+	+	+	Cells in the head and stalk cells of the glandular trichomes stained orange, as well as the non-glandular trichomes, while the base stained brown
Lignin, cutin and suberin,	Safranin	+	+	+	+	Cells in the head and stalk stained pink-red in glandular and non-glandular trichomes (stalk and base cells)
Polysaccharides	Touldine blue	+	+	+	+	Cells in the head and stalk stained blue-purple in the glandular trichomes whereas the stalk and base cells of the non-glandular trichomes stained blue
Lipids, cutin and suberin	Sudan black B	+	+	+	+	Cells in the head and stalk stained dark black deposits in both glandular and non-glandular trichomes
Mucilage and polysaccharides	Ruthenium red	+	+	+	+	Cells in the head and stalk stained pink-red in both glandular and non-glandular trichomes

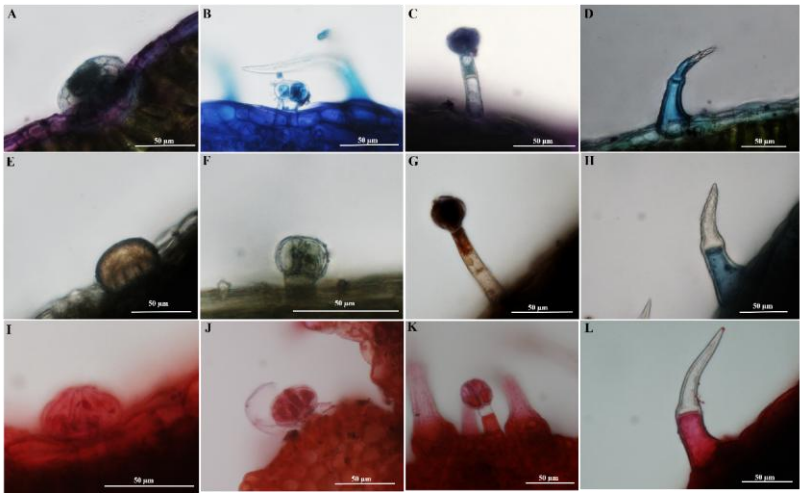

Figure 6. *Light micrographs of stained trichomes present on the leaves and stems of Leonotis leonurus. (A-D) Toluidine blue (polyphenols); (E-H) Sudan black (cutin, lipids and suberin); (I-L) Ruthenium red (mucilage)*

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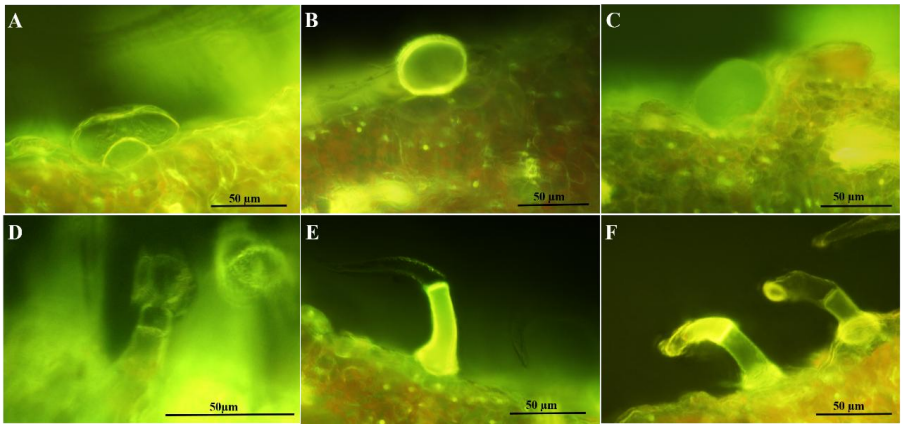

Figure 7. *Fluorescence micrographs of trichomes on Leonotis leonurus. (A-B) Peltate trichome; (C) Type II capitate trichome; (D) Type III capitate trichome; (E-F) Uniseriate, multicellular (two to five cells) pointed-shaped non-glandular trichomes*

Conclusions

Secretory structures identified in L. leonurus include glandular peltate and capitate trichomes, as well as non-glandular trichomes. Cystoliths were observed in resin-embedded sections of both leaf and stem, a distinctive feature of L. leonurus that is reported for the first time in this study. Histochemical staining of leaf and stem trichomes revealed the presence of phenolics and alkaloids, which are key medicinal compounds. The tests also highlighted various phytochemicals with medicinal properties that likely play a role in protecting the plant against pathogens and herbivores. Glandular trichomes are responsible for secreting bioactive compounds, which may contribute to the plant's healing properties. Classifying these medicinal compounds and understanding the mechanisms by which they are stored and secreted by the secretory structures is crucial. The results of this study enhance our understanding of the importance of these compounds in plant survival and defense. Although several studies on L. leonurus trichomes were published between 1995 and 1998, there remains a need for comprehensive, updated research and detailed visualization of these trichomes and their associated compounds using advanced microscopic techniques. There is a gap in updated research on the leaf and stem micromorphology of species within the Leonotis genus, presenting an opportunity for further investigation. This study, therefore, seeks to offer a deeper understanding and up-to-date information on the secretory structures of L. leonurus. The preliminary findings from this research will contribute to the South African medicinal plant database. Future research could explore the length of trichomes at various developmental stages, as this would provide valuable insights into trichome growth patterns. Further exploration of the micromorphology of the flowers and seedlings should be examined as these plant parts also contain trichomes.

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