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ORIGINAL PAPER

Vegetative anatomy and micromorphology of *Salvia divinorum* (Lamiaceae) from Mexico, combined with chromatographic analysis of salvinorin A

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Abstract *Salvia divinorum*—a species traditionally cultivated in Oaxaca, Mexico—possesses hallucinogenic properties. It is legally recognized as a controlled substance and prohibited in many countries. The proper identification of the plant, both in fresh and dried forms, is an important issue in crime-prevention campaigns. This paper provides a thorough anatomical description of leaves, petioles, and stems of *S. divinorum*. Detailed investigation of foliar trichomes was performed and illustrated. In addition, chromatographic analyses, including TLC and HPLC, were applied to fresh and dried plant material, together with the standard reference salvinorin A. A comprehensive identification method for *S. divinorum* based on a thorough anatomical examination is proposed, combined with chemical analysis for proper plant recognition.

Keywords *Salvia divinorum* · Anatomy · Microscopy · Chromatography · Salvinorin A

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Introduction

The use of *Salvia divinorum* Epling & Játiva as a popular hallucinogen has spread recently throughout the world, especially through e-commerce and among young people [1]. The plant was named *S. divinorum* after its use in divination by the Mazatec Indians. Other traditional uses for this species include the treatment of diarrhea, headache, and rheumatism [2]. *Salvia divinorum*—1 of about 1,000 species of *Salvia*—is a perennial herb belonging to the Lamiaceae family. It is traditionally cultivated for medicinal and magico-religious use by the Mazatec people of Oaxaca, Mexico.

The plant is found rarely growing outside of cultivation (never outside of the Mazatec region), but in most cases these appear to be feral, rather than truly wild. No definitively wild populations have been identified. The plant reaches over 1 m in height, has large green leaves, hollow square stems, and white corollas with purple calyces. In the Mazatec region, this sage is usually cultivated in forest ravines and other moist humid areas, usually between 750 and 1,500 m altitude. The plant was identified as a new species of *Salvia* by Carl Epling and Carlos D. Játiva at the University of California, Los Angeles, CA, in 1962 [2].

The principle substance responsible for the psychoactive effect of the plant is the neoclerodane diterpenoid salvinorin A. This compound has been recognized as an extremely potent and highly selective kappa-opioid receptor agonist [3]. Pharmacologically, salvinorin A is a unique compound, since it is the first naturally occurring, non-nitrogenous, opioid-receptor-subtype-selective agonist discovered.

In spite of the alarming rise in consumption, *S. divinorum* and salvinorin A are not currently controlled under the Controlled Substances Act of the United States, although, as of 1 September 2012, 33 states have enacted legislation

placing regulatory controls on *S. divinorum* and salvinorin A. Moreover, *S. divinorum* is currently a controlled substance in 24 other countries in Europe and Asia [4].

Due to the presence of an active marker, salvinorin A, *S. divinorum* can be identified easily by various analytical methods, including thin layer chromatography (TLC), gas chromatography/mass spectrometry (GC/MS) [5], high-performance liquid chromatography (HPLC) [6], ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) [7], and liquid chromatography/mass spectrometry (LC/MS) [8]; DNA fingerprinting has also been investigated [9]. However, the presence of salvinorin A alone is not entirely sufficient for species identification because other herbs and compounds are sometimes augmented with it. In this paper we identify anatomical and micromorphological diagnostic features of *S. divinorum* that can be useful for species determination.

A description of the morphology of *S. divinorum* and a brief account of the distribution of trichomes was published previously [10], but the micro-anatomical characterization of this plant has not been reported so far. The present study aimed to fill this gap. This paper provides detailed anatomical descriptions of leaf, petiole, and stem of *S. divinorum*. Detailed investigation of foliar trichomes was also performed and illustrated. In addition, chromatographic analyses, including TLC [10] and HPLC [11], were applied to fresh and dried plant material, together with standard reference for salvinorin A.

In this work we present a comprehensive method of identity confirmation for the *S. divinorum* species, based on anatomical description of the plant and chemical identification of the active substance.

Materials and methods

The plants used in this research as dry material were grown commercially in the Mazatec region of Mexico and shipped to the United States in the form of the dried leaves. Live plants (three samples) were purchased from Daniel Siebert (http://www.sagewisdom.org) and were grown indoors at the University of Mississippi. Both dry and live plants were authenticated botanically by Daniel Siebert. The voucher specimen (JKZ-SD-201203) is deposited in the Department of Pharmacognosy, School of Pharmacy at the University of Mississippi.

For light microscopy, the specimens were processed and paraffin blocks were prepared as per standard protocols [12]. Sections of 7–10 µm thick were made using a Leica RM3655 fully motorized rotary microtome (Leica Microsystem, Bannockburn, IL), stained with safranin and counterstained with Fast Green. These sections were examined and photomicrographs prepared using an Olympus BX41 light microscope with attached Olympus DP71 imaging system. In addition, hand-sections (stained with phloroglucinol reagent), surface preparations, isolated elements, and samples of powdered plant material were prepared for the purpose of studying the micromorphological and anatomical features. For scanning electron microscopy, leaf samples and hand-sections of petioles were fixed in 2 % glutaraldehyde solution [13] and dehydrated by passing through 25, 50, 75, 95, and 100 % acetone. The samples were then dried in a critical point dryer (Denton Vacuum, Moorestown, NJ) using liquid CO_2 as a transitional fluid. The dried samples were mounted on aluminum stubs using carbon tapes (Electron Microscopy Sciences, Hatfield, PA) and coated with gold using a Hummer 6.2 Sputter Coater (Anatech USA, Union City, CA) supplied with argon gas. The coated samples were then observed and photographed using JSM-5600 SEM (JEOL, Tokyo, Japan).

For chromatographic analyses, acetonitrile extracts were prepared. Whole fresh leaves as well as shredded dried leaves were extracted with acetonitrile for 30 min (10 ml solvent per 2 g dried material, and 100 ml solvent for 2 g fresh leaves, concentrated later to 1 ml volume).

TLC was performed using Whatman® silica gel plates (catalog no. 4410222). The chromatogram was visualized by spraying the plates with anisaldehyde reagent and heating at 100 °C. Salvinorin A becomes visible as a violet spot on the chromatogram. The obtained chromatogram was photographed with a Panasonic Lumix DMC–LX3 camera and the color contrast and saturation were increased using Adobe Photoshop CS 5.1.

HPLC was performed using a Waters Delta Prep 4000 HPLC system (Milford, MA) with UV–VIS detector (Waters 2487) and Empower computer software. The HPLC column was Luna C18 column 250 × 4,6 mm i.d.; 5 µl particle diameter (Phenomenex, Torrance, CA). The chromatographic studies were carried out in the presence of a standard solution of salvinorin A (1 mg/ml), which was previously isolated following a known procedure [14]. The structure of the isolated salvinorin A was confirmed by spectroscopic methods (NMR).

Results

Anatomy of the plant

The leaf

The leaf is dorsiventral in transverse section (Fig. 1a). The upper epidermis consists of one-layer of squarish or irregularly rectangular cells, which are polygonal in

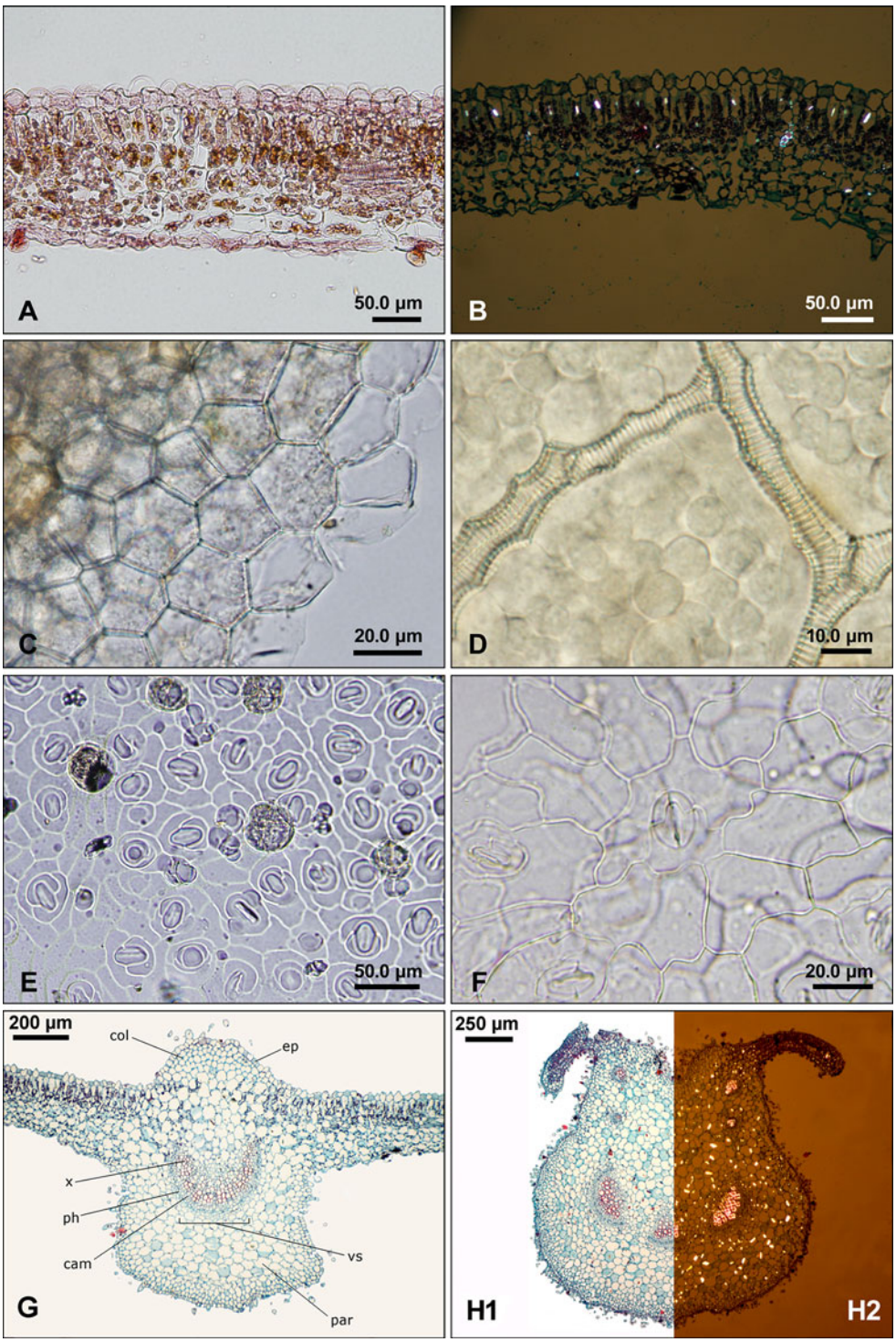


Fig. 1 *Salvia divinorum*: anatomy of leaf and petiole. **a**, **c**–**h1** light micrographs; **b**, **h2** polarized light; **g**, **h1**, **h2** safranin + fast green). **a**, **b** Portion of lamina transverse section (TS) showing palisade with calcium oxalate crystals. **c** Surface view of upper epidermis showing polygonal cells. **d** Veinlets showing constricted vessels. **e** Lower epidermis (impression made with clear nail-polish) showing stomata and glandular trichomes. **f** Diacytic stomata. **g** TS of leaf through midrib: *ep* epidermis, *col* collenchyma, *par* parenchyma, *vs* vascular bundles, *ph* phloem, *x* xylem, *cam* cambium. **h1**, **h2** Petiole TS showing raphide crystals of calcium oxalate (illuminated white)

surface view (Fig. 1c), measuring 10–30 µm high and 15–25 µm long, with papillate outer walls and straight anticlinal walls. The cuticle is thin and straight; stomata are absent. Numerous capitate glandular trichomes and short conical non-glandular trichomes are present on the adaxial surface.

The mesophyll The palisade is one-, two-, rarely three-layered. Cells of the upper layer are columnar, 20–40 µm high and 12–18 µm in diameter. The second and third layer, if present, possess shorter, oblong cells measuring 33–41 µm in height and 16–18 µm in diameter. The palisade is interrupted at the midrib and lateral veins (Fig. 1g).

Spongy tissue is made up of two to three layers of loosely arranged, polygonal parenchyma cells measuring 15–30 μm high and 10–40 μm in diameter, with wide air spaces ending in stomata. Palisade and spongy parenchyma cells contain acicular crystals of calcium oxalate measuring 5–20 μm long. The palisade ratio is 2.0 : **2.22** : 2.5.

The lower epidermal cells are smaller, tabular, with curved anticlinal walls, measuring 10–20 μm high, 30–45 μm long, and 25–35 μm wide in surface view. Stomata are raised above the surface and form an arch over the stomatal chamber. Numerous peltate glandular trichomes, capitate glandular trichomes, and various types of non-glandular trichomes are present, especially along the midrib and lateral veins. Stomata are diacytic, frequent, measuring 14 μm long and 21 μm wide (Fig. 1e, f). The stomatal index is 20.0 : **21.6** : 24.5.

Midrib The upper epidermis consists of polygonal, tabular cells followed by a patch of collenchyma tissue of four to five cells high and six to eight cells wide; the stele is 'U' shaped, consisting of radially arranged collateral vascular bundles, with xylem pointed towards the adaxial surface and phloem as an arch towards the abaxial surface, enclosed by several layers of polygonal parenchyma cells. The cambium is made up of two to three layers of short, tangentially elongated cells, separating xylem and phloem. Medullary rays are uni- or bi-seriate, made up of polygonal parenchymatous cells. Xylem vessels show spiral thickening. Most of the parenchymatous cells of the ground tissue contain calcium oxalate crystals, including raphides, prisms of different shapes, microsphenoidal crystals, and minute druses (Fig. 1g). The vein-islet index is 16.5.

The petiole

The epidermis is formed of a single layer of isodiametric, polygonal cells with straight anticlinal walls (as seen in surface view), measuring 7–17 μm in diameter. The epidermal cells are covered with a thin cuticle. Stomata are absent. Few layers of lacunar collenchyma are located under the upper epidermis. The vascular tissue is composed of three main collateral vascular bundles and four small ones above the main strands and near the adaxial surface of the leaf. Cambium is distinct and formed of three to four layers of tangentially elongated cells in the main bundles and two to three layers of tangentially elongated thin-walled cells in the secondary bundles. No sclerenchyma was found in the petiole. Trichomes similar to those found on the midrib were observed also on the epidermis of petiole. Several types of calcium oxalate crystals were observed in the parenchymotous ground tissue of the petiole. The crystals include single columnar, microsphenoidal, needle-like, prisms, and bounded forms of raphides (Fig. 1h1, h2).

The stem

In transverse section, the stem is four-angled, square or rectangular in outline (Fig. 2a, b). It measures 5–10 mm

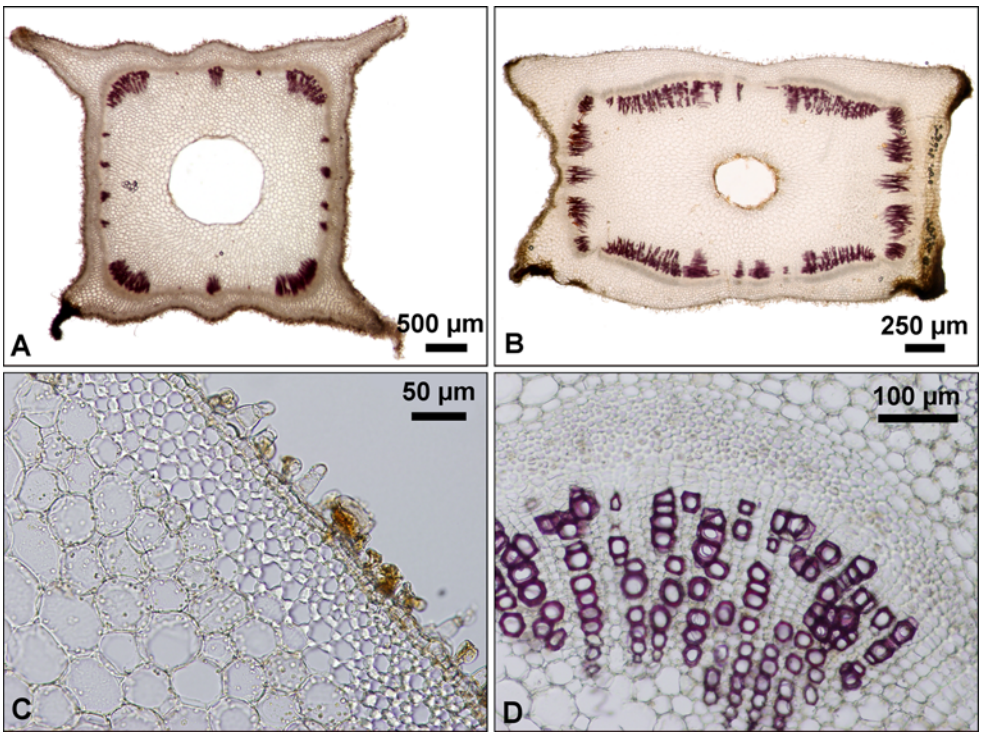


Fig. 2 *S. divinorum* anatomy of stem (hand sections). **a** TS of stem at internode shows square shape with the angles extend into short wings, and a large cavity in the middle of the pith. **b** Node TS shows rectangular shape of stem. **c** Portion of stem TS showing epidermis with trichomes, lacunar collenchyma, and a portion of cortex. **d** Stem vascular bundle

across, with a central cavity measuring up to 6 mm wide. The angles extend into short wings.

The epidermis consists of a single layer of squarish or tabular cells measuring 7–13 μm long, 8–16 μm high, covered on the outer wall with a thin cuticle and numerous non-glandular and glandular trichomes. In surface view, the epidermal cells are polygonal, with straight or slightly curved anticlinal walls, measuring 7–28 × 10–27 μm. Collenchyma occurs underneath the epidermis as a continuous ring, except at the wings in older stems, or as isolated patches alternating with chlorenchyma in younger stems. Collenchyma occupies four to five layers, and is thickened prominently at the intercellular spaces (Fig. 2c).

The cortex is narrow, made up of polygonal parenchymatous cells, measuring 30–70 μm in diameter, containing chloroplasts. Most of the cells have bundles of acicular crystals of calcium oxalate measuring 20–29 μm. The stem is devoid of sclerenchymatous tissue. Endodermis is distinct and consists of one layer of thin-walled tubular cells measuring 14–37 μm long and 14–23 μm high, separating the cortex from the vascular tissue. The structure of the wings at the angles is composed of the extended cortical parenchymatous tissue.

Vascular tissue is represented by collateral bundles. In the internodes, these occur as four major groups at the angles and with a few small groups in-between the major strands, whereas, in the nodal region, the vascular tissue forms a more or less continuous ring. Phloem caps the xylem in internodes of young stems, forming a continuous ring in the nodes and mature stems. Phloem cells are small, more or less polygonal, or slightly tangentially elongated, with thickened walls. Xylem vessels are solitary or in groups of three to five or more and arranged in radial rows. The vessels are polygonal or rounded in sectional view, lignified and spirally or reticulately thickened with lumen 10–24 μm in diameter. Few of the metaxylem elements are filled with yellow contents. Medullary rays are one-, two-, three-, or several- celled wide with cells tangentially elongated, measuring 14–23 μm wide and 9–15 μm long, separating arches of xylem vessels (Fig. 2d).

The pith is wide, occupying a major part of the stem tissue, made up of thin-walled polygonal cells measuring 28–70 μm in diameter, abundant with starch grains. Most of the cells contain acicular crystals of calcium oxalate. Cells in the middle portion of the pith are degenerated and form a large cavity outlined by tangentially elongated epithelial cells filled with yellowish brown contents. The cavity is narrower, 190–275 μm in diameter in the nodal region, while it is 920–1,100 μm in diameter in the internodes of younger stems. The cavity is as wide as 3–6 mm in the older stems, making the hollow visible to the naked eye in a cut section.

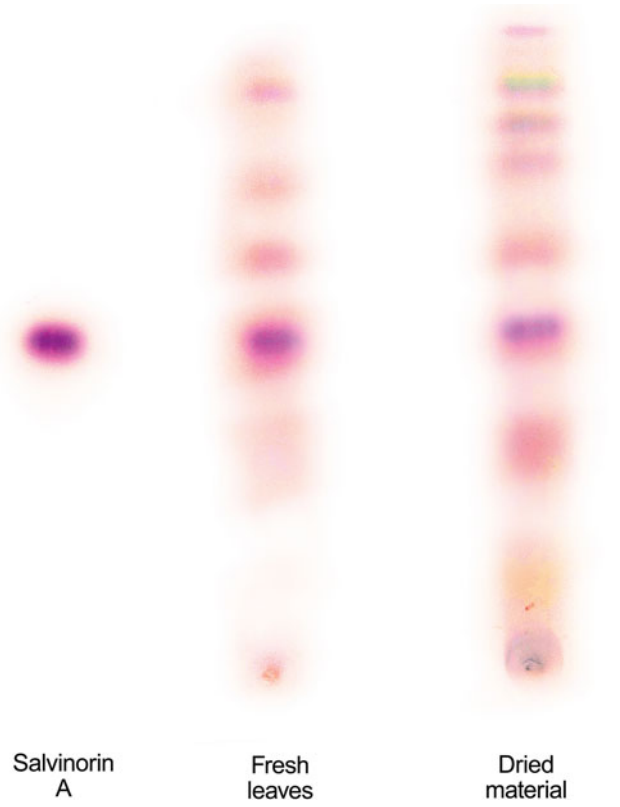


Fig. 3 TLC chromatogram for salvinorin A standard and extracts of fresh and dried leaves of *S. divinorum*

Chromatographic identification

TLC shows the salvinorin A spot in both extracts from fresh leaves and dried material (Fig. 3). R_f values for the standard solution equal 0.52, with values of 0.51 for the fresh leaves and 0.53 for the dried leaves extract.

HPLC chromatograms reveal the presence of salvinorin A in both solutions (Fig. 4). Retention times of the compound are 21.5 min for the extract of fresh leaves and 21.6 min for the extract from a dried material, in comparison with the standard solution, where the retention time of salvinorin A is 21.6 min.

Discussion

Salvia divinorum possesses hallucinogenic properties that have been attributed to salvinorin A—a neoclerodane diterpenoid. The plant is recognized legally as a controlled substance and prohibited in many countries. The proper identification of the plant, in both fresh and dried forms, is an important issue in a crime-prevention campaigns. The present paper provides a detailed anatomy of the vegetative parts of *S. divinorum* with investigation of the micromorphology of leaves. In addition, TLC and HPLC studies

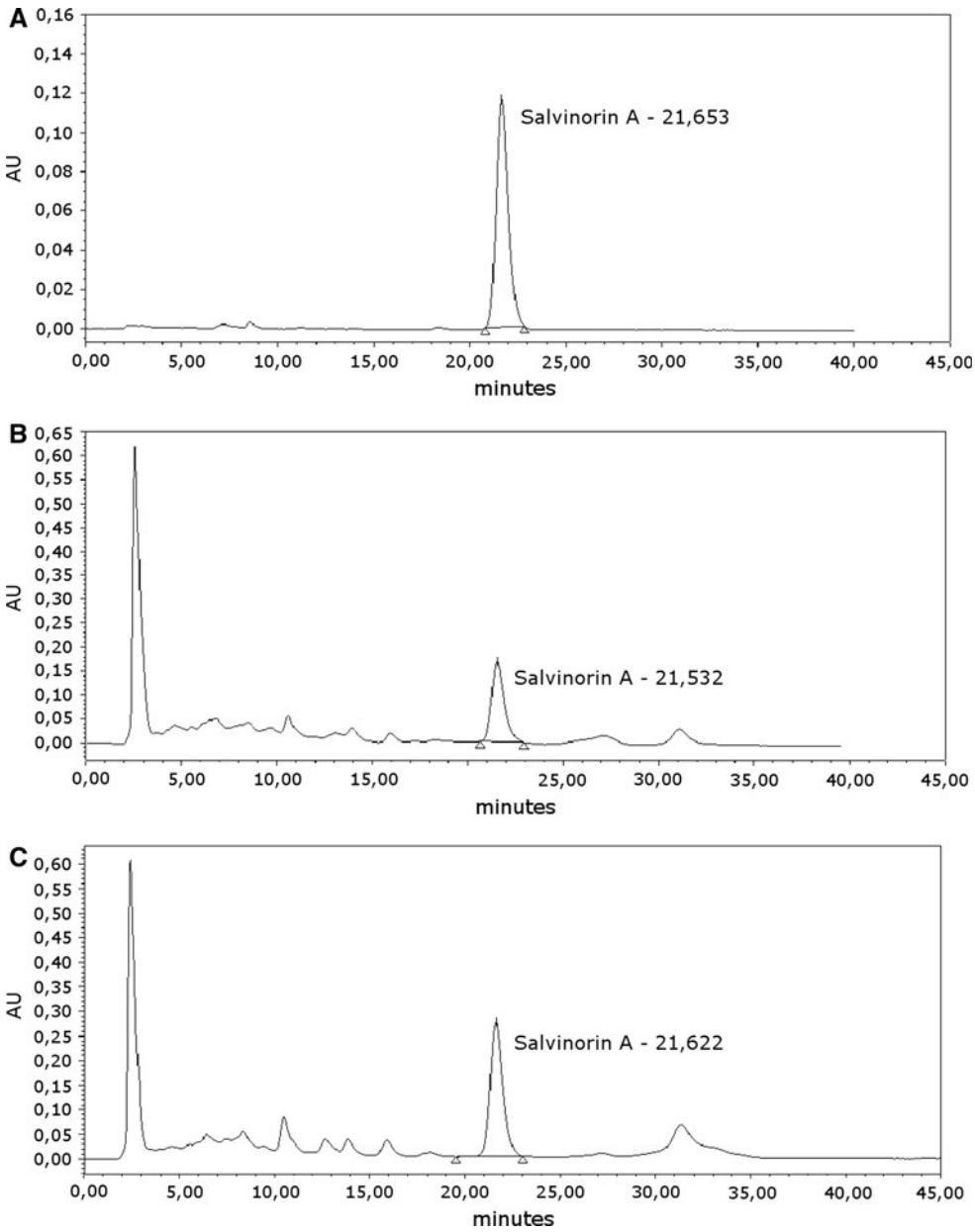


Fig. 4 HPLC chromatograms. **a** Salvinorin A standard. **b**, **c** Extract from fresh (**b**) and dried (**c**) leaves of *S. divinorum*

were carried out on fresh and dried leaf samples and compared with a standard reference for the detection of salvinorin A.

The presence of various types of trichomes, calcium oxalate crystals, and stomata are useful features for identification of *S. divinorum* leaves, in entire as well as powdered form. Equally important for identification and differentiation from other species is the arrangement of the different tissues of the leaf and petiole. Trichomes of the genus *Salvia* have been studied and classified previously by several researchers [15–21]. Singh et al. [22] reported 17 different kinds of trichomes from the floral appendages of 12 species of *Salvia*. Gupta and Bhambie [23] studied eight species of *Salvia* and recorded 11 types of non-glandular and five types of glandular trichomes. Siebert [10] recognized capitate, peltate and non-glandular types of trichomes in *S. divinorum*. However, during the present study, 33 subtypes of trichomes under five major types were observed on the leaves of *S. divinorum* and are reported here. The majority of these accumulate close to the midrib region. Capitate glandular trichomes occur on both adaxial and abaxial surface of the leaf, but peltate glandular trichomes, which contain the active principle, salvinorin A, are present only on the abaxial surface [10]. Non-glandular trichomes exist on both surfaces. Herein we report a few additional types of trichomes, such as branched non-glandular trichomes as well as various subtypes of capitate and peltate glandular trichomes, which have not been hitherto

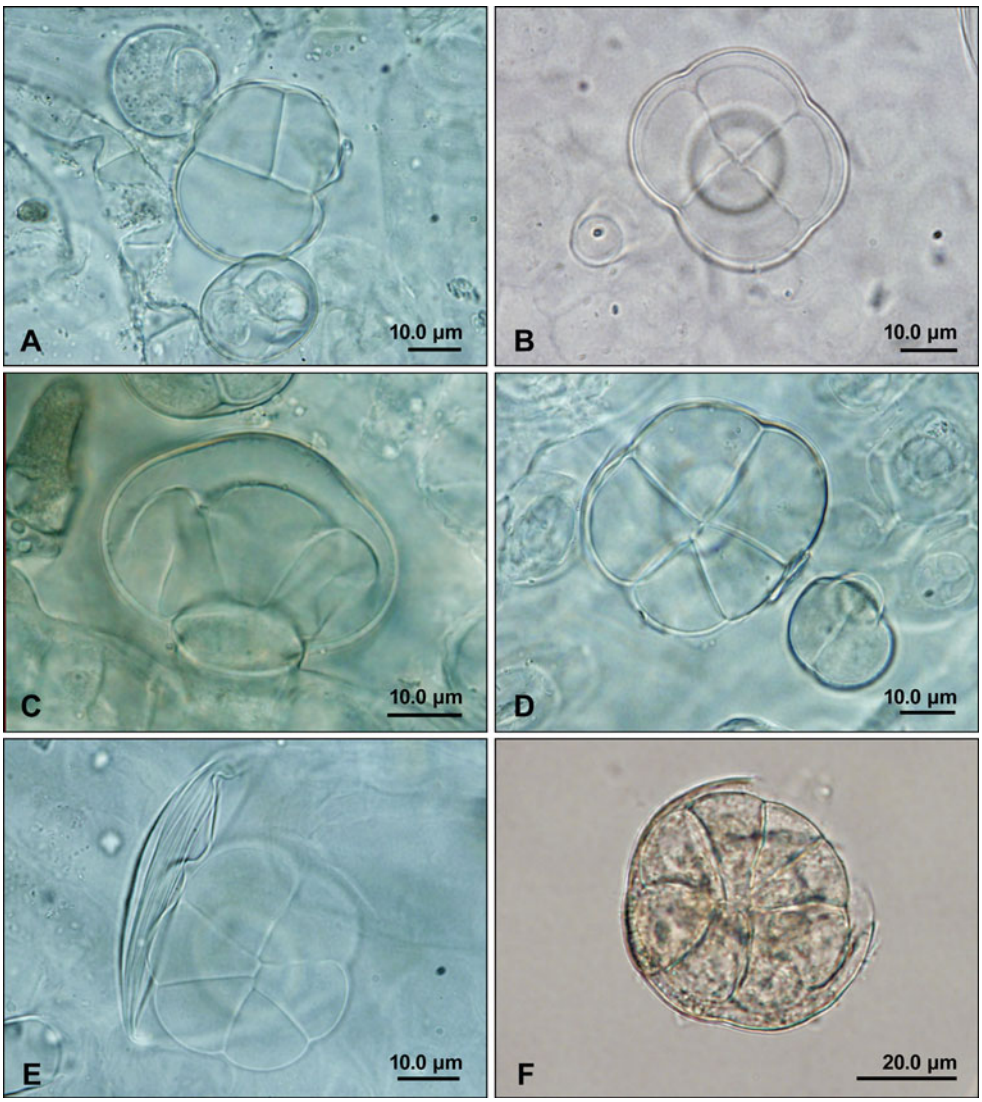


Fig. 5 *Salvia divinorum* peltate glandular trichomes (light microscopy). **a**–**e** Trichomes intact on a leaf clarified with chloral hydrate. **f** Detached trichome from powdered leaf. **c** Side view (all others aerial view). **a** Head three-celled. **b**, **c** Head four-celled. **d** Head five-celled. **e** Head six-celled. **f** Head eight-celled

reported in this species. Some trichome subtypes are not very common for the plant (i.e., peltate glandular trichomes with three, six, or eight head cells, branching non-glandular trichomes). The types and subtypes of trichomes observed from the leaves and stems of *S. divinorum* are listed below:

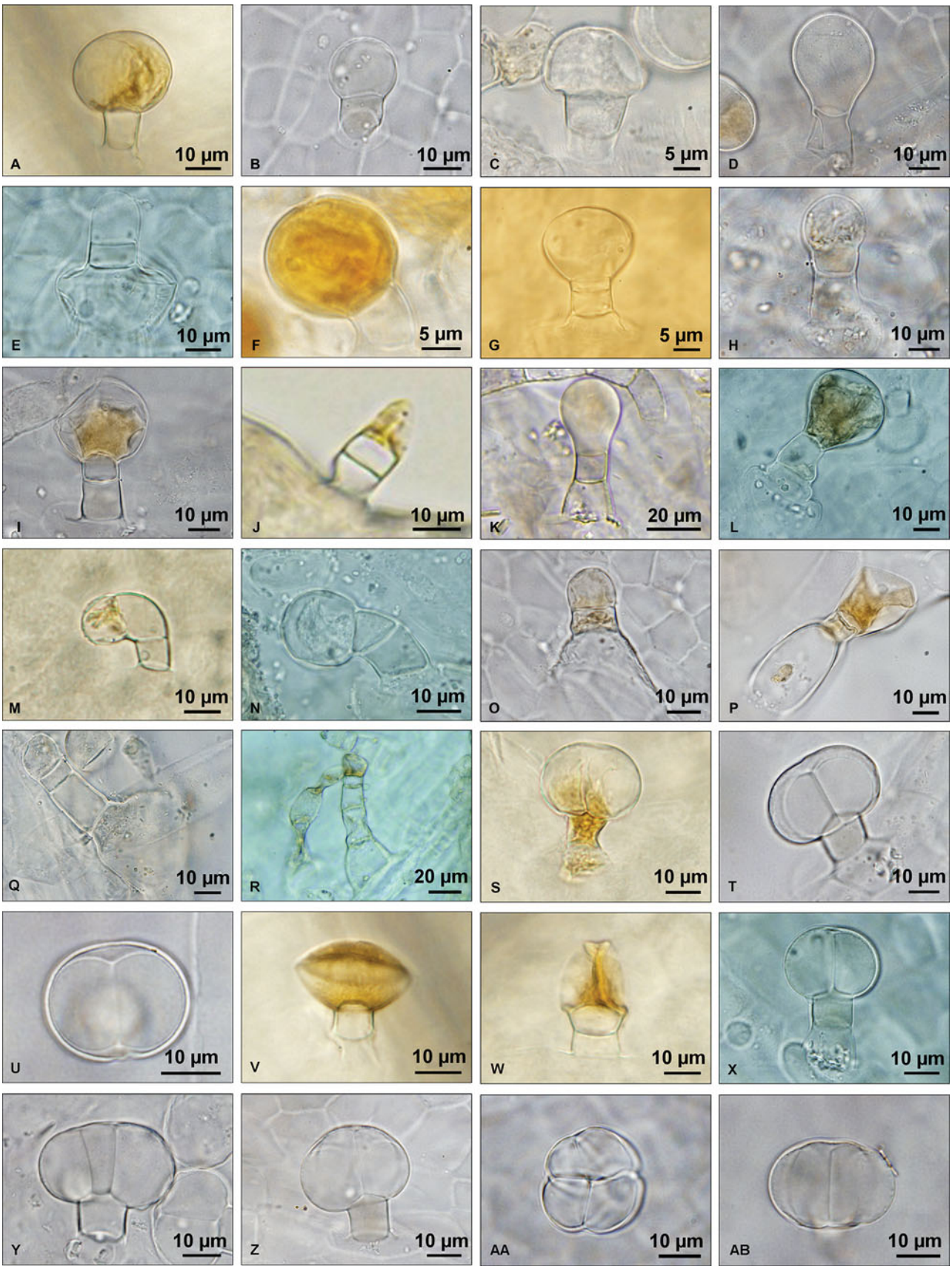
1. Glandular trichomes

   A. Peltate glandular trichomes

   - three-celled head, about 32 × 20 µm; one-celled stalk (Fig. 5a)
   - Four-celled head, about 35 µm across; one-celled stalk (Fig. 5b, c)
   - Five-celled head, about 39 µm across; one-celled stalk (Fig. 5d)
   - Six-celled head, about 39 µm across; stalk one-celled (Fig. 5e)
   - Eight-celled head, about 39 µm across; sessile (Fig 5f)

   B. Capitate glandular trichomes

   - one-celled head; one-celled stalk (Fig. 6a–c)
   - one-celled head, bulb like; one-celled stalk (Fig. 6d)
   - one-celled head, slightly swollen; one-celled stalk (Fig. 6e)
   - one-celled head; one-celled stalk, curved (Fig. 6f)
   - one-celled head; two-celled stalk (Fig. 6g)
   - one-celled head, slightly swollen; two-celled stalk (Fig. 6h)
   - one-celled head with the apical secretory cell is similar to the stalk cell, two-celled stalk (Fig. 6j)
   - one-celled head, bulb like; two-celled stalk (Fig. 6k, l)
   - one-celled head, slightly swollen; two-celled stalk curved (Fig. 6m)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◀ **Fig. 6** *Salvia divinorum* capitate glandular trichomes (CGT) (light microscopy). **a–f** Different forms of CGT having unicellular head and unicellular stalk. **g–p** Subtypes of CGT having unicellular head and bicellular stalk. **q** Unicellular head and three-celled stalk. **r** Unicellular head with four-celled stalk. **s–u** Head two-celled, common wall parallel to stalk, stalk unicellular (aerial view). **v** Head two-celled, common wall perpendicular to stalk, stalk unicellular. **w**, **x** Head bicellular, stalk bicellular. **y** Head three-celled, stalk one-celled. **z–ab** Head four-celled, stalk unicellular

- one-celled head; two-celled stalk, curved (Fig. 6n)
- one-celled head, slightly swollen; two-celled stalk with unequal cells (Fig. 6o)
- one-celled head, bulb like; two-celled stalk with unequal cells (Fig. 6p)
- one-celled head, slightly swollen; three-celled stalk with unequal cells (Fig. 6q)

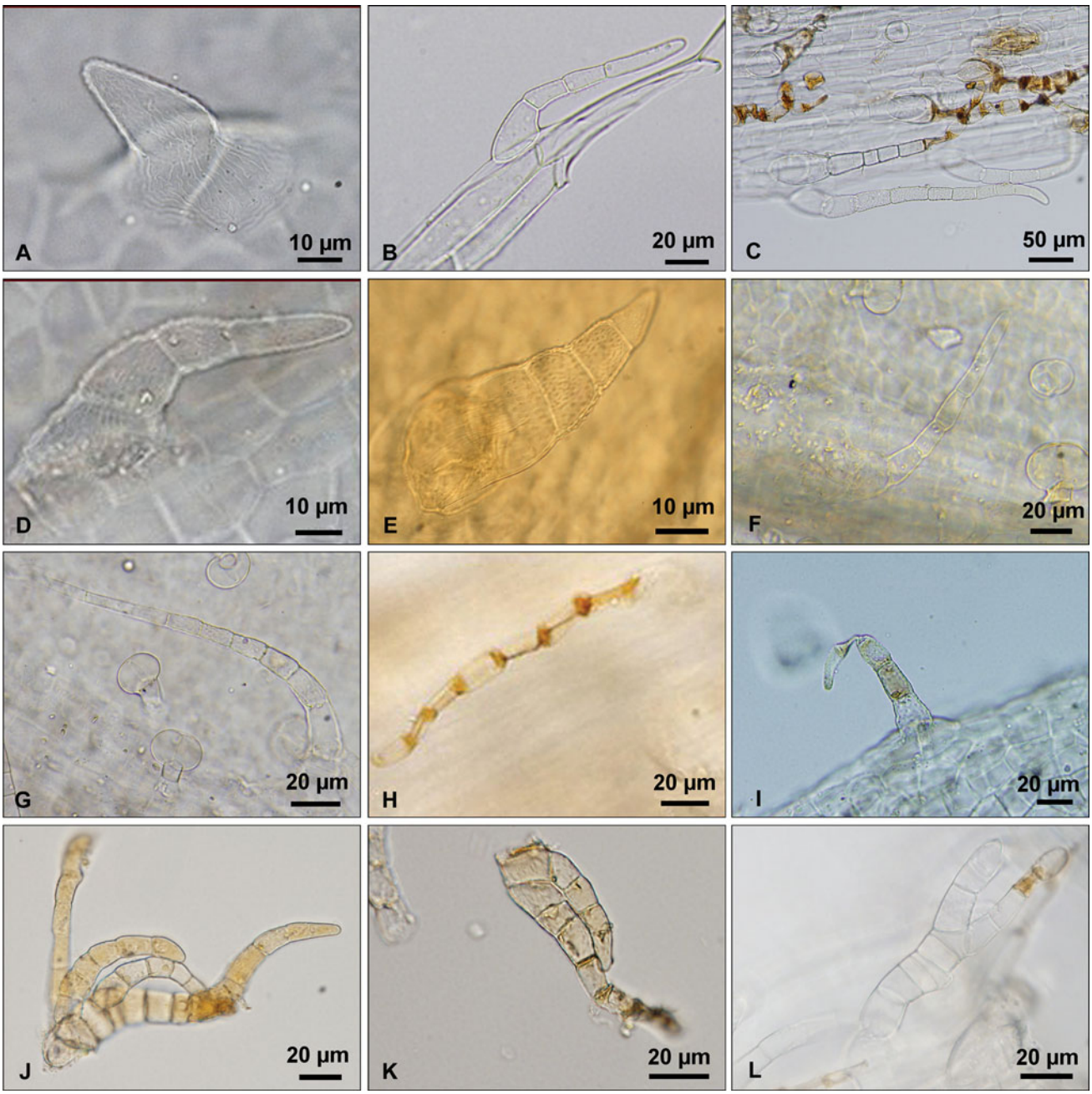

Fig. 7 *Salvia divinorum* non-glandular trichomes (light microscopy). **a** Unicellular conical. **b**, **c** Simple filiform. **d**, **f** Conical filiform. **g** Capitate filiform. **h** Collapsed filiform. **i** Collapsed conical. **j–l** Branched trichomes

- one-celled small secretory head; four-celled stalk slightly collapsed (Fig. 6r)
- two-celled head; one-celled stalk (Fig. 6s–u)
- two-celled head, mushroom shape; one-celled stalk (Fig. 6v)
- two-celled head; two-celled stalk (Fig. 6w, x)
- three-celled head; one-celled stalk (Fig. 6y)
- four-celled head; one-celled stalk (Fig. 6z–ab)

2. Non-glandular trichomes

A. Unicellular conical trichome—the body cell is conical and papillate, the base of the hair has a radiating cuticule (Fig. 7a)

B. Multicellular, unbranched trichome

- Simple filiform-foot cell is slightly enlarged and curved, covered with the striated cuticule; body consists of 2 to 12 narrow cells (Fig. 7b, c)
- Conical filiform—consists of enlarged foot cell and from one to many elongated body cells (Fig. 7d–f)
- Capitate filiform—ten-celled filiform trichomes ending with the ovoid transparent head (Fig. 7g)
- Collapsed filiform—made up of two to many uniseriate collapsed cells containing yellow substance (Fig. 7h)
- Collapsed conical—basal cell is enlarged and conical, body of the hair consist of collapsed cells with yellow substance (Fig. 7i)
- Elongated-uniseriate, multicellular trichomes, longer than 2 mm, visible to naked eye. These hairs are irregularly curved and usually found on abaxial midrib and petiole

C. Multicellular, branched trichome

- Filiform- bi-, tri- or tertraforked (Fig. 7j, k)
- Capitate—made up of the filiform body with collapsed cell and ended with the ovoid head (Fig. 7l)

Many types of crystals, single crystals, columnar, prisms, needle-like or micro-sphenoidal, bundles of raphides, and small druses occur in leaves of *S. divinorum*. Crystals of calcium oxalate can be found also in trichomes. Such numerous and diverse occurrence of crystals provides important diagnostic features (Fig. 8a–c).

Trichomes and crystals of calcium oxalate are abundant also in the petiole. Collenchyma is located only under the upper epidermis of the petiole. A taxonomically significant feature in the *Lamiaceae* family is the arrangement of the vascular bundles in the petiole [24]. In *S. divinorum* the stele is composed of three large vascular bundles in the middle and two smaller ones near the wings, one on either side. Cambium is distinct in all vascular bundles, no sclerenchyma was seen.

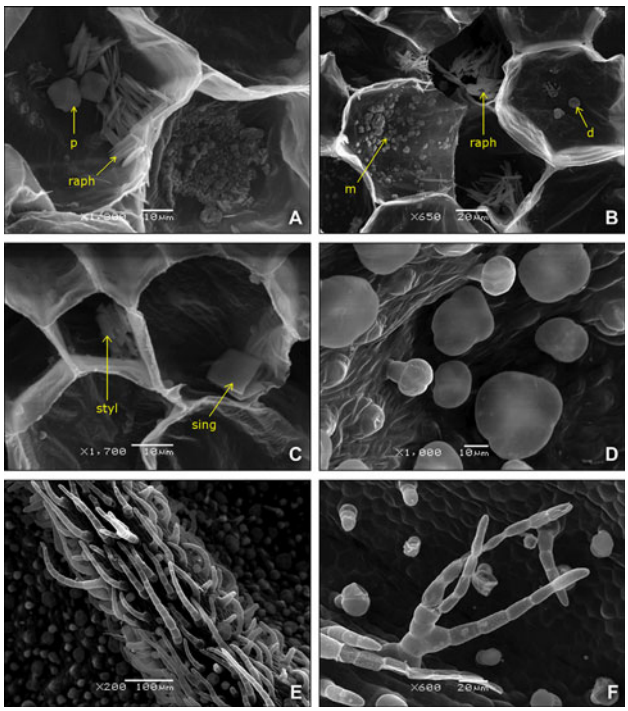

Fig. 8 Scanning electron microscopy of *Salvia divinorum*. **a–c** Stems TS showing different forms of calcium oxalate crystals. **a** Prisms (*p*), raphides in bundles (*raph*). **b** Microsphenoidal (*m*), raphides (*raph*), small druses (*d*). **c** Single crystals (*sing*), styloids (*styl*). **d** Glandular trichomes on the abaxial leaf surface. **e** Non-glandular trichomes on the midrib. **f** Branched trichome

In the stem, identifying the presence and type of calcium oxalate crystals plays a significant role as key elements. In addition, the outline of the stem sector, vascular bundle arrangement, presence of distinctive cambium, and distinguishable endodermis are considered as diagnostic characters. *S. divinorum* as a member of the Lamiaceae family possesses four-angled rectangular stems, but the angles extend into short wings with anatomy similar to leaf. Moreover, a cavity in the center of the pith occurs, determining a characteristic feature of the species. In older stems, the cavity is visible to the naked eye. In stem anatomy the pattern of arrangement of collenchyma can help distinguish *S. divinorum* from the other species. In contrast to other *Salvia* species, *S. divinorum* stem collenchyma does not exist in corners that are parenchymatous; however, it occurs underneath the epidermis in the remaining parts. The endodermis is distinct. Numerous cells of the cortex and pith contain crystals of calcium

oxalate; single and bounded, prisms, columnar, needle-like, or micro-sphenoidal. Several pith cells contain starch grains.

Chromatographic methods provide a fast detection of salvinorin A, a compound characteristic to only this plant. However, as mentioned before, proper plant identification cannot rely solely on physicochemical methods. Analytical techniques are especially useful when shredded or powdered material is being investigated. In our work, we found TLC and HPLC fast, simple and selective methods. The results obtained confirm the presence of an active marker—salvinorin A—in fresh and dried material.

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