

## Morphology and histochemistry of glandular trichomes of *Cordia verbenacea* DC. (Boraginaceae) leaves

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**ABSTRACT** – (Morphology and histochemistry of glandular trichomes of *Cordia verbenacea* DC. (Boraginaceae) leaves). The objective of this work was to study, using light and fluorescence microscopy and scanning electron microscopy, the morphology and secretory products of glandular trichomes of *Cordia verbenacea* DC. (Boraginaceae), known as ‘baleeira’, a species used in folk medicine as anti-inflammatory, analgesic, anti-ulcerogenic and healing agent. Two classes of glandular trichomes were recognized, globular and reniform. A morphological study of the secretory head and the characterization of the secretory product are also presented. Secretory products of globular trichomes consisted of essential oils, whereas reniform trichomes consisted basically of phenolic compounds such as flavonoids. No pre-established regions for releasing secretory products were found.

Key words - ‘baleeira’, essential oil, phenolic compounds, secretory product

**RESUMO** – (Morfologia e histoquímica de tricomas glandulares de folhas de *Cordia verbenacea* DC. (Boraginaceae)). O objetivo deste trabalho foi estudar, usando microscopia de luz e de fluorescência e microscopia eletrônica de varredura, a morfologia e o material secretado de tricomas glandulares de *Cordia verbenacea* DC. (Boraginaceae), conhecida como erva-baleeira, uma espécie utilizada na medicina tradicional como anti-inflamatório, analgésico, anti-úlceras e cicatrizante. Duas classes de tricomas glandulares foram reconhecidas, globular e reniforme. O estudo morfológico da cabeça secretora e a caracterização do material secretado também são apresentados. A secreção dos tricomas globulares caracteriza-se como óleo essencial, enquanto que nos reniformes consiste basicamente de compostos fenólicos, como flavonóides. Nenhuma região pré-estabelecida para a liberação da secreção foi encontrada.

Palavras-chave - compostos fenólicos, erva-baleeira, óleo essencial, secreção

### Introduction

Plants used worldwide in traditional medicine have been studied for its biological activities and secondary metabolites used as active principles for drugs or as chemical models for the synthesis and semi-synthesis of drugs (Balandrin *et al.* 1985, Svoboda *et al.* 1998/1999).

*Cordia verbenacea* DC. (Boraginaceae), known as ‘baleeira’, is a shrubby species native to sandbank environments along the Brazilian coast. Its aromatic leaves are used in folk medicine as anti-inflammatory, analgesic, anti-ulcerogenic and healing agent, in the form of teas or infusions for internal or topical use (Lorenzi & Matos 2002, Souza *et al.* 2004). Several studies have provided foundation for the popular use of this species, by confirming its antimicrobial (Carvalho Jr. *et al.* 2004, Hernandez *et al.* 2007), anti-inflammatory (Basile *et al.* 1989, Sertié *et al.* 1988, 2005, Ticli *et al.* 2005, Passos *et al.* 2007) and light analgesic (Sertié *et al.* 2005) activities. Phytochemical studies of *C. verbenacea* aerial parts

identified the presence of monoterpenes, sesquiterpenes (Carvalho Jr. *et al.* 2004), triterpenes (Velde *et al.* 1982) and flavones (Velde *et al.* 1982, Lins *et al.* 1990). Fresh leaves of *C. verbenacea* produce 0.23% (v/w) of essential oil, having  $\alpha$ -pinene as main constituent (29.69%), trans-caryophyllene (25.27%), alloaromadendrene (9.99%) and alpha-humulene (4.64%) (Carvalho Jr. *et al.* 2004).

Studies have demonstrated that the plant secretory products are generally mixed, located in only one secretory structure or compartmentalized in different secretory structures, in vegetative or reproductive organs (Fahn 1979, Ascensão & Pais 1987, Fahn & Shimony 1996, Serrato-Valenti *et al.* 1997, Ascensão *et al.* 1999, Bisio *et al.* 1999, Corsi & Bottega 1999, Monteiro *et al.* 2001, Machado *et al.* 2006, Combrinck *et al.* 2007). In the family Boraginaceae, the glandular trichomes present in the leaves have different morphological patterns and are the typical secretory structures of the group (Solleder 1908, Metcalfe & Chalk 1957).

The use of *C. verbenacea* in traditional medicine, the phytochemical studies and the proven pharmacological activities show the necessity of better knowledge of the secretory structures related to the production of secondary metabolites in this species. The objective of the present

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work was to characterize morphologically and histochemically the glandular trichomes, using techniques of light, fluorescence and scanning electron microscopy, seeking to establish relationships between trichome morphology and secretory product composition.

### Material and methods

**Plant material** – The work was carried out at the Plant Anatomy Laboratory, Department of Plant Biology (DBV), and at the Center for Microscopy and Microanalysis (NMM), both located in the Federal University of Viçosa (UFV), Viçosa-MG. Plant material of *Cordia verbenacea* DC. (Boraginaceae) was collected from a sandbank environment, at the Castelhanos beach, municipal district of Anchieta-ES, for vegetative propagation by stem cuttings. A voucher specimen was deposited at the VIC Herbarium of the Federal University of Viçosa, under number 26.789. The propagated plants were grown under field conditions at the Botanical Garden of the UFV (20°45' S, 42°55' W, 690 m a.s.l.) and fresh leaves were collected for the morphological and histochemical analyses.

**Light microscopy (LM)** – Fresh leaf blades at different developmental stages were sectioned transversally in a table-microtome (LPC Rolemberg & Bherring). The following histochemical reactions were carried out: Sudan red B (Brundrett *et al.* 1991) and black B (Lison 1960) for lipids, Nile blue sulphate (Cain 1947) for acid and neutral lipids, Nadi reagent (David & Carde 1964) for essential oils and oleoresins, osmium tetroxide (OsO<sub>4</sub>) (Ganter & Jollés 1969) for unsaturated lipids, periodic acid/Schiff (PAS) reagent (Feder & O'Brien 1968) for polysaccharides with vicinal glycol groups, Ruthenium red (Johansen 1940) for pectins, Alcian blue (Pearse 1985) for acid mucopolysaccharides, mercuric bromophenol blue (Mazia *et al.* 1953) and xylydine Ponceau (Vidal 1977) for proteins, ferric trichloride (Johansen 1940) and potassium dichromate (Gabe 1968) for phenolic compounds, hydrochloric vanillin (Mace & Howell 1974) for tannins, Wagner and Dittmar reagent (Furr & Mahlberg 1981) for alkaloids, anthimonium trichloride (Mace *et al.* 1974) for terpenes containing steroids, sulphuric acid (Geissmen & Griffin 1971) and Abraham reaction (Caniato *et al.* 1989) for sesquiterpenic lactones, 2-4-dinitrophenylhydrazine (Ganter & Jollés 1969) for terpenoids. Standard control procedures were carried out simultaneously. The observations were made in light microscope (Olympus AX-70). For the fluorescence microscopy investigation, fresh material was treated with: aluminium trichloride, magnesium acetate and lead neutral acetate (Charrière-Ladreix 1976) for flavonoids, and neutral red (Kirk 1970) for lipids. A epifluorescence microscope (Olympus BX60) equipped with a UV filter (WU: 330-385 nm), a dichroic mirror (400 nm) and a barrier filter (420 nm) were used. The autofluorescence investigation was carried out using the same instrument.

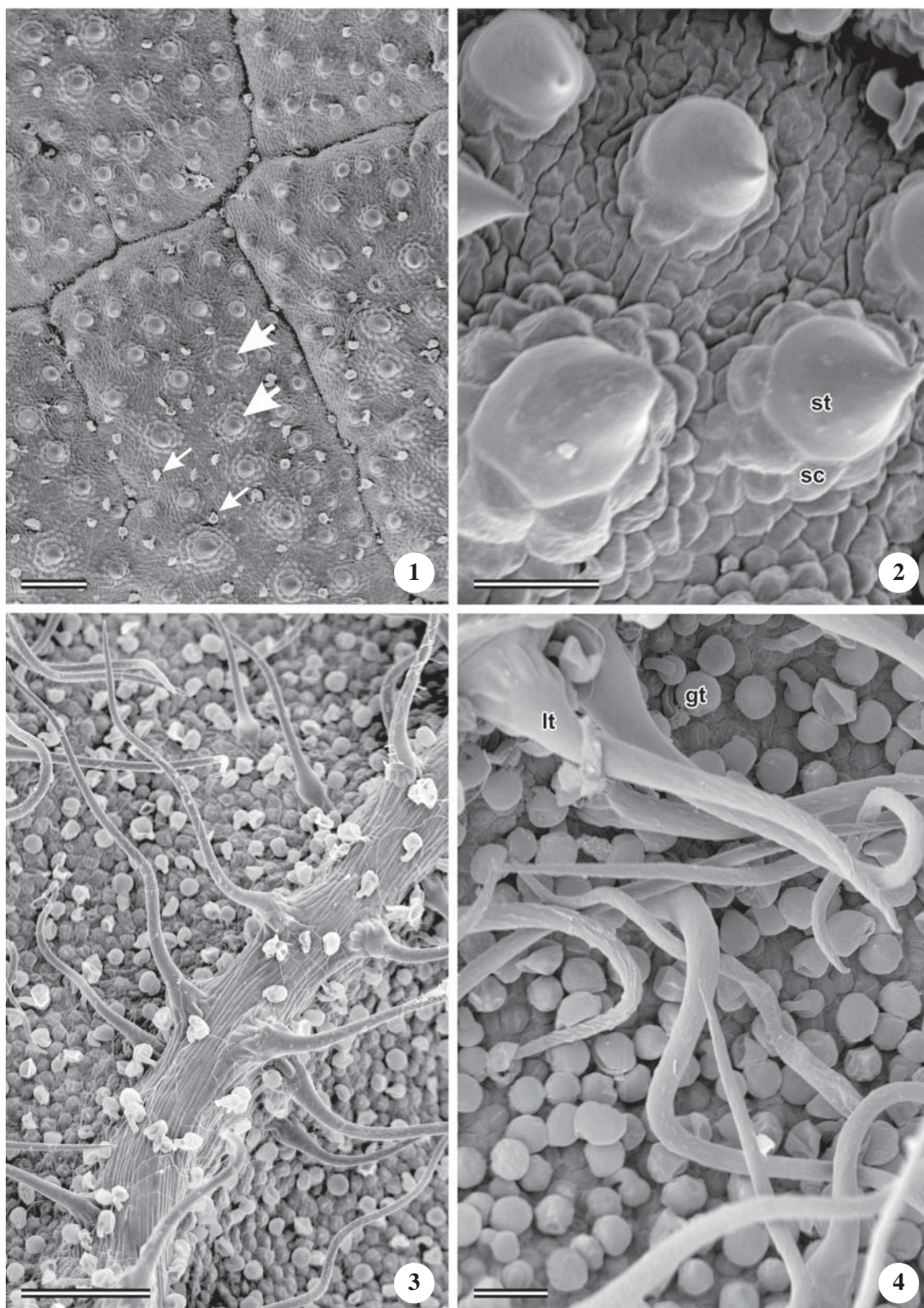
Schematic drawings and head measurements of glandular trichomes were obtained from transversal sections of fresh, young and mature leaves, under light microscope coupled with a camera lucida. Data from 100 measurements per trichome were statistically analyzed.

**Scanning electron microscopy (SEM)** – Portions of leaves at different developmental stages were fixed in FAA in ethanol 50% for 48 hours, stored in 70% ethanol (Johansen 1940). After dehydration in a graded ethanol series, the material was critical-point dried with CO<sub>2</sub>, sputter-coated with a thin layer of gold (20 nm) and, finally, examined in a scanning electron microscopy (LEO 1430 VP) at an acceleration voltage of 10.6 kV.

### Results

**Trichome morphology and distribution** – Non-glandular and glandular trichomes were present on both leaf surfaces (figures 1-4). Non-glandular trichomes were conical, unicellular, with thick and warty wall and calcified apical portion. Surrounding this trichome base were special subsidiary cells protruding above the level of the other epidermic cells (figures 2, 8). Two types of non-glandular trichomes were found: 1) short, with the development of a body similar to a bulbous cystolith in the basal portion (not shown), on the leaf adaxial surface (figures 1, 2); 2) long, with inconspicuous cystolith bodies, leaning over the epidermis, mainly on the major veins of the leaf abaxial surface (figures 3, 4).

Glandular trichomes (figure 5) consisted of an epidermic basal cell, a uniseriate stalk and a unicellular secretory head. The stalk can be short, formed by a single cell, or long with up to ten cells; and the upper cell showed a special type of differentiation, as a neck cell. The form and type of insertion of the secretory head allowed the distinction of two classes of glandular trichomes: 1) globular type (figures 5E-F, 6, 9), with spherical secretory head, attached to the stalk by the usual way, having  $33.68 \pm 3.64 \mu\text{m}$  mean diameter, regardless of the stalk cell number, and short neck cell; 2) reniform type (figures 5A-D, 7, 8), with elipsoidal head attached to the stalk by one end, measuring  $35.79 \pm 5.02 \times 24.15 \pm 3.0 \mu\text{m}$  when the stalk was short (just one cell), and  $50.72 \pm 5.66 \mu\text{m} \times 30.48 \pm 3.45 \mu\text{m}$  when the stalk was long (2-10 cells); neck cell long and curved. Globular and reniform glandular trichomes occurred side by side on both leaf surfaces, being much more abundant on the abaxial surface. The short globular and reniform trichomes were more abundant and located in intervenal regions, whereas the long ones were scarcer and located along the veins, mainly on the abaxial surface. Globular and reniform trichomes with broken cuticle in the equatorial



Figures 1-4. Scanning electron micrographs of *Cordia verbenacea* leaves. 1-2. Adaxial surface showing short non-glandular trichomes (large arrows) and scattered glandular trichomes (narrow arrows). Note the subsidiary cells bearing the hair and protruding above the level of the other epidermal cells. 3-4. Abaxial surface, showing long non-glandular trichomes restricted to the veins and a large amount of glandular trichomes. (gt = glandular trichome; lt = long non-glandular trichome; sc = subsidiary cell; st = short non-glandular trichome). Bar = 200  $\mu$ m (1, 3); 50  $\mu$ m (2, 4).

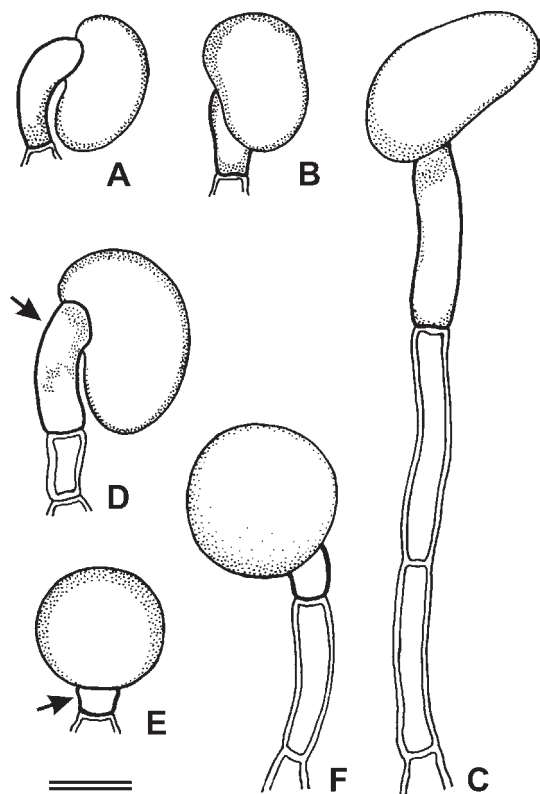


Figure 5. Schematic drawings of different types of glandular trichomes of *Cordia verbenacea* leaves. A-D. Reniform trichomes. E-F. Globular trichomes. The number of stalk cells varies in both globular and reniform trichomes, but the upper stalk cell is always distinct, like a neck-cell (arrows). Bar = 25 µm.

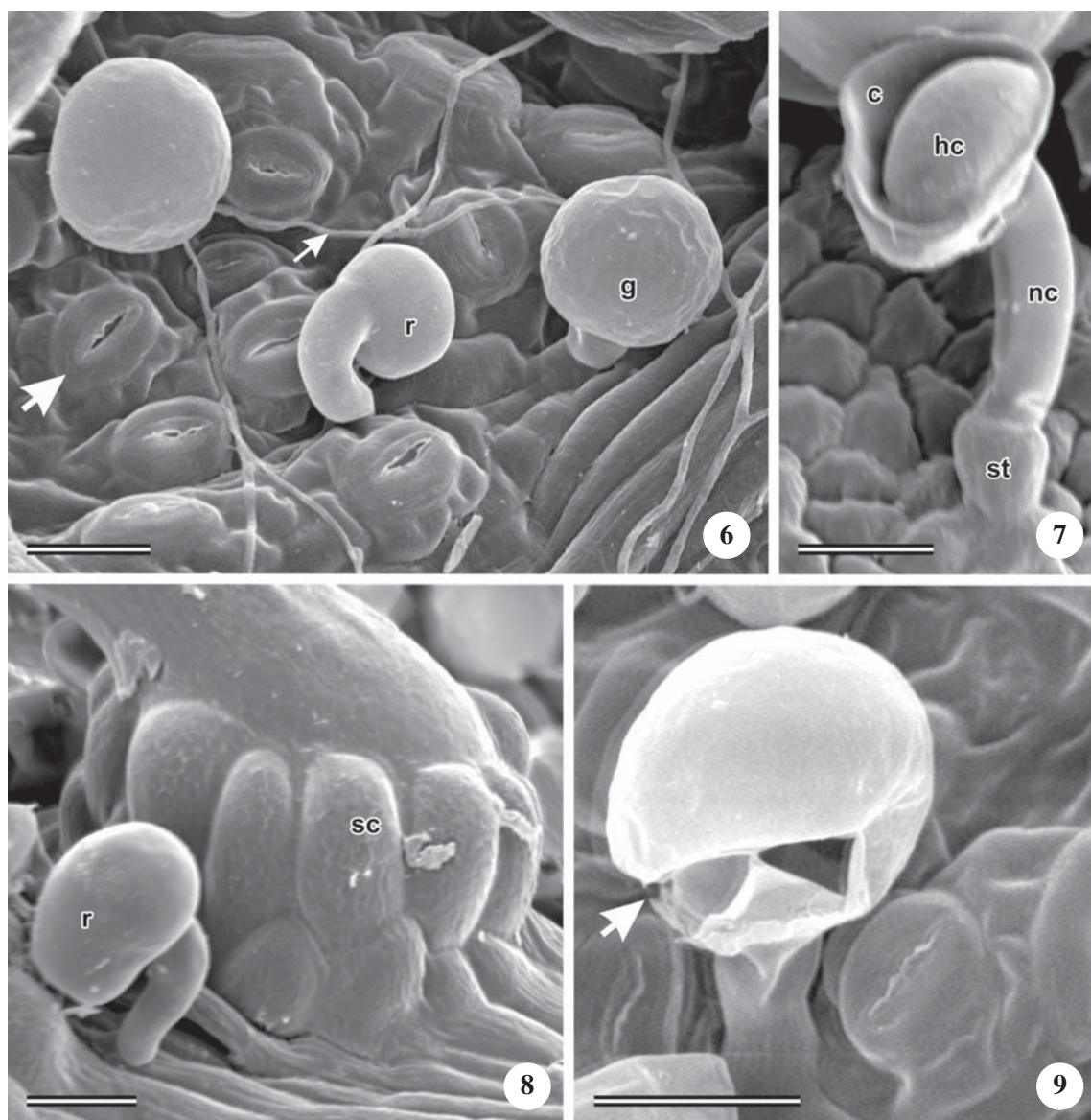
region of the secretory head cell were found (figures 7, 9), but neither pores nor pre-established regions for secretion release were observed. Globular and reniform trichomes were already present and fully differentiated at early stages of leaf development. On expanded mature leaves the glandular trichomes were still active and occurred in a sparse distribution.

Histochemistry of glandular trichomes – Table 1 shows the results of histochemical tests for globular and reniform trichomes.

Globular trichomes – The secretory product of globular trichomes was light yellow in color, abundant, and located in the subcuticular chamber (figure 10). Staining with Sudan red (figure 11) and Sudan black B (figure 12) showed positive lipid reaction in both the head cells and neck cell walls (figures 11, 12). The secretory product can be observed within the subcuticular chamber stained in red (figure 11) or dark blue (figure 12), characteristic of Sudan red B and Sudan black B respectively. The whole head of globular trichomes was intensely black stained by osmium tetroxide (figure 13), indicating the presence of unsaturated lipids in the whole secretory cell. Neutral lipids were identified by a pink color with Nile blue sulphate (figure 14). Essential oils were identified in the secretion composition by the violet-blue color with a Nadi reaction (figure 15), whereas oil-resins were absent. Polysaccharides, as pectin, only occurred within protoplasts of secretory cells, but the secretory product had no reaction for this group of compounds with

Table 1. Compounds identified by histochemical tests and/ or fluorescence microscopy in globular (G) and reniform (R) trichomes of *Cordia verbenacea* leaves.

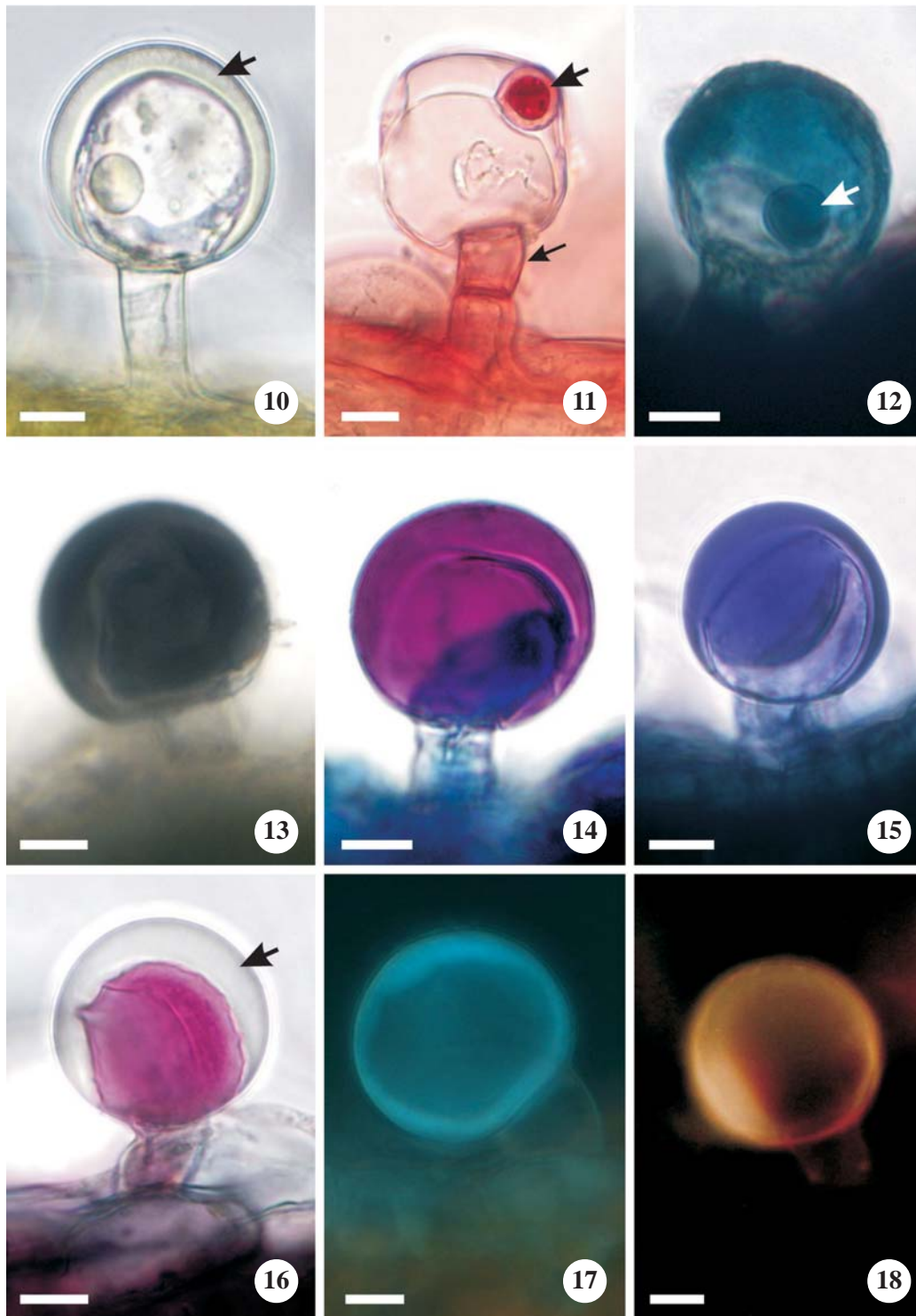
Test	Compound	Colour observed	Type of trichome	
			G	R
Sudan red B	lipids	red	*	
Sudan black B	lipids	dark blue	*	
Nile blue	acid lipids	blue		*
	neutral lipids	pink	*	
Nadi reagent	essential oils	violet-blue	*	
Osmium tetroxide	unsaturated lipids	black	*	*
Neutral red	lipids	yellow	*	
		blue		*
Ruthenium red	pectins	rose	*	*
Ferric trichloride	phenolic compounds	black		*
Potassium dicromate	phenolic compounds	brown		*
Aluminium trichloride	flavonoids	yellow-greenish		*
Magnesium acetate	flavonoids	yellow-greenish		*
Lead neutral acetate	flavonoids	yellow-greenish		*



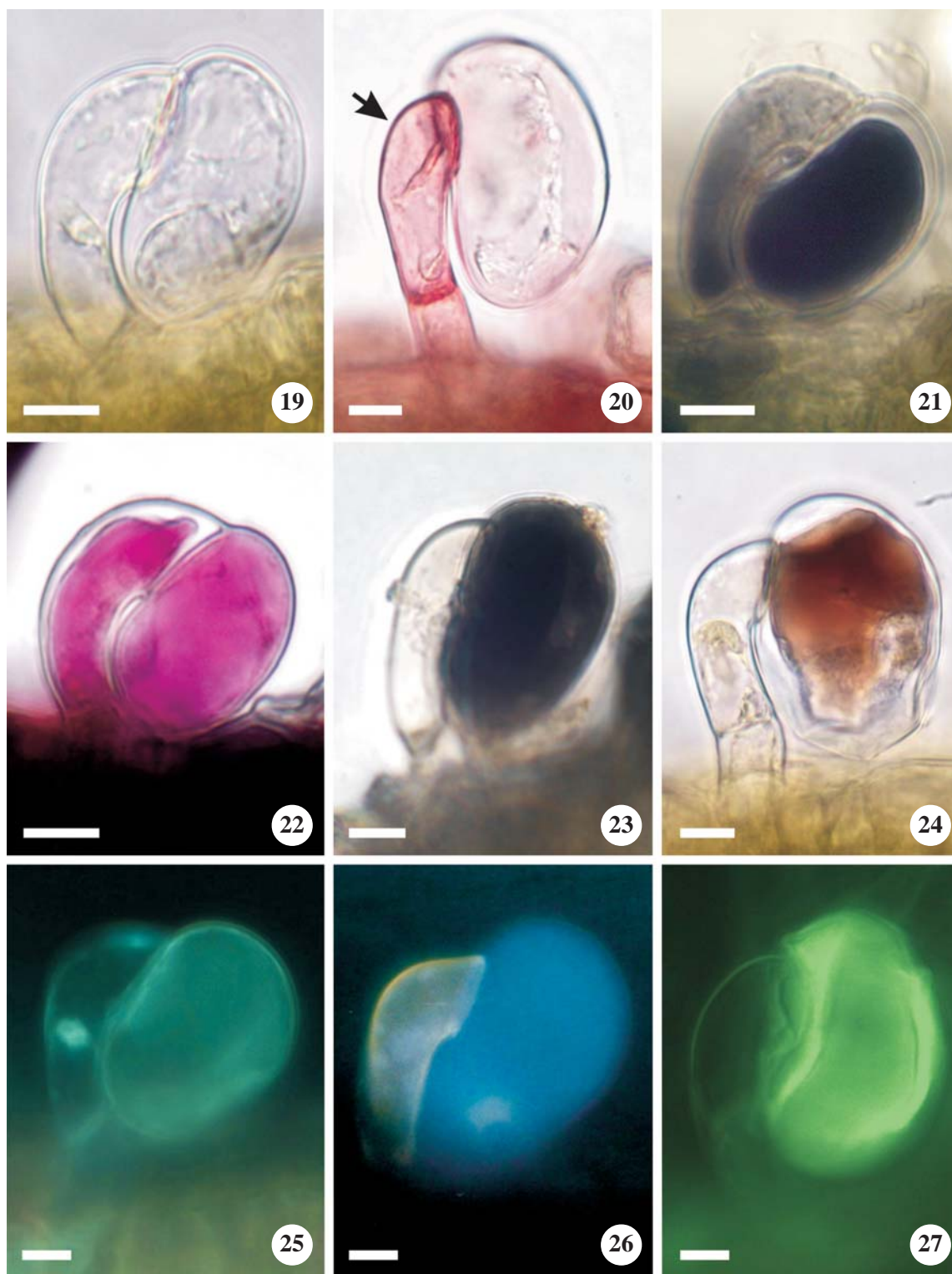
Figures 6-9. Scanning electron micrographs of *Cordia verbenacea* leaves. 6. Glandular trichomes on the abaxial surface, and stomata (large arrow) projected above the level of the other epidermal cells. Hyphae (narrow arrow) on epidermal surface. 7. Adaxial surface, reniform trichome showing broken cuticle and exposed secretory head cell; stalk consisting of two cells, where the apical cell differentiated into a long and curved neck cell. 8. Abaxial surface; note a reniform trichome and subsidiary cells surrounding the non-glandular trichome. 9. Globular trichome with broken cuticle (arrow) in the equatorial region of the head cell. (c = cuticle; g = globular trichome; hc = secretory head cell; nc = neck cell; r = reniform trichome; sc = subsidiary cell; st = stalk cell). Bar = 20  $\mu$ m.

Ruthenium red, remaining clear (figure 16). Globular trichomes showed blue autofluorescence under ultraviolet light, mainly the secretory head (figure 17). The presence of lipids was confirmed by the intense yellow fluorescence with neutral red fluorochrome (figure 18). For the other histochemical tests, either under light or fluorescence microscopy, the reaction was negative or similar to the control.

Reniform trichomes – In these trichomes, the secretion was more scarce and lighter in color (figure 19), and subcuticular space formation was only observed through scanning electron microscopy, with broken cuticle (figure 7). The presence of structural lipids on neck cell walls was confirmed by the red staining with Sudan red (figure 20). Lipid classes were not found in the secretion of reniform trichomes, although the osmium tetroxide



Figures 10-18. Light (10-16) and fluorescence micrographs under UV light (17-18) showing the response of globular glandular trichomes of *Cordia verbenacea* leaves to histochemical tests. 10. *In vivo*. The secreted material appears to be oily; arrow shows the secreted material in a subcuticular space. 11. Characteristic red color of lipids stained with Sudan red B (large arrow). The neck-cell walls also stained red (narrow arrow). 12. Lipids stained dark blue with Sudan black B (arrow). 13.  $\text{OsO}_4$  test showing black staining of the unsaturated lipids. 14. Pink stained of neutral lipids with Nile blue test. 15. Essential oils stained violet-blue with Nadi reagent. 16. Pectins stained pink with Rutenium red at the secretory head cell. Note that the secretion in the subcuticular space (arrow) was not stained. 17. Blue autofluorescence of the head cell under UV light. 18. Intense yellow fluorescence with neutral red. Bar = 10  $\mu\text{m}$ .



Figures 19-27. Light (19-24) and fluorescence micrographs under UV light (25-27) showing the response of reniform glandular trichomes of *Cordia verbenacea* leaves to histochemical tests. 19. *In vivo*. 20. Sudan red B; positive reaction just in a neck-cell walls (arrow). 21.  $\text{OsO}_4$  test showing black staining in the secretory head cell. 22. Pectins stained pink with Ruthenium red in secretory head cell and neck cell. The neck cell walls have not reacted (arrow). 23. Phenolic compounds stained black with ferric trichloride test. 24. Brown color of phenolic compounds with potassium dichromate. 25. Weak green-blue autofluorescence under UV light. 26. Intense blue fluorescence with neutral red. 27. Intense green fluorescence of the head cell stained by aluminium trichloride. Bar = 10  $\mu\text{m}$ .

reaction stained the secretory head intensively black (figure 21). Ruthenium red showed pectin within the head and neck cells, which became rose (figure 22). Phenolic compounds were present in the secretion, confirmed by the black color when stained with ferric trichloride (figure 23) or by brown color with potassium dichromate (figure 24). Under ultraviolet light, the reniform trichomes showed weak green-blue autofluorescence (figure 25). With neutral red fluorochrome, these trichomes showed intense blue fluorescence (figure 26), indicating different chemical nature in comparison to globular trichomes, which fluoresce intense yellow under UV light. Flavonoids were identified with fluorochrome, fluorescing intense green under UV light, mainly when stained with aluminum chloride (figure 27). As for the other histochemical tests, either under light or fluorescence microscopy, the reaction was negative or similar to the control.

### Discussion

The non-glandular trichomes associated with cystolith bodies in the basal portion and with partially calcified walls, called cystolith-hairs, are widely distributed in Boraginaceae and responsible for the wrinkled leaf surface (Solereeder 1908, Metcalfe & Chalk 1957). In *C. verbenacea*, the development of cystolith bodies was more evident in short non-glandular trichomes with appear on the leaf adaxial surface.

The morphology and the distribution of glandular trichomes in *C. verbenacea* were similar to those described for other species of the genus (Solereeder 1908, Metcalfe & Chalk 1957), being always uniseriate, with unicellular secretory head and a typical shape. Despite of the variation in the number of stalk cells and in the size of the head cells, glandular trichomes could be classified as globular or reniform, according to the morphology of the head cell. Structural lipids, probably cutin or suberin, were present on neck cell walls, as shown by the positive reaction with Sudan red B and the negative reaction with Ruthenium red. This endodermal feature is common in oil-secreting trichomes, which prevent backward-flow of the secreted material (Fahn 1979, Werker 2000). Neck cells with cutinized lateral walls were also found in species of Scrophulariaceae (Sacchetti *et al.* 1999) and Lamiaceae (Bosabalidis & Tsekos 1982, Bisio *et al.* 1999), although in peppermint (Lamiaceae) they were not described as cutinized, but as suberized walls (Turner *et al.* 2000).

Both glandular trichomes apparently accumulates their secretory product within the subcuticular space, as in glandular trichomes of Lamiaceae (Bosabalidis & Tsekos 1982, Werker *et al.* 1985, Ascensão *et al.* 1995,

1997, 1999, Bisio *et al.* 1999, Serrato-Valenti *et al.* 1997, Turner *et al.* 2000), Scrophulariaceae (Sacchetti *et al.* 1999), Asteraceae (Ascensão & Pais 1987, Figueiredo & Pais 1994) and Bignoniaceae (Machado *et al.* 2006). Secretion release in glandular trichomes can take place by the rupture of the cuticle in regions of greater fragility (Ascensão & Pais 1987, Figueiredo & Pais 1994, Ascensão *et al.* 1995, Serrato-Valenti *et al.* 1997, Bisio *et al.* 1999), through pores in the cuticle (Bisio *et al.* 1999, Ascensão *et al.* 1999) or even diffusion through the cuticle (Serrato-Valenti *et al.* 1997). Although trichomes with broken cuticle were found in *C. verbenacea*, no pores or regions indicating secretion release were observed, suggesting that the secretion is released to the environment through a rupture of the cuticle caused by mechanical damage.

The secretion of globular trichomes was oily and predominantly lipophilic, whereas reniform trichomes had hydrophilic secretion. The osmium tetroxide test indicated the presence of lipids in the composition of globular trichome secretion, with prevalence of unsaturated lipids, relatively large number of double bonds, and consequently low melting point, determining the fluidity of the secretion at room temperature. Although the osmium tetroxide test also indicated lipids in reniform trichome secretion, it should be considered that this is a broad-spectrum reagent, reacting not only with unsaturated lipids, but also with phenolic compounds, proteins and alkaloids (Jensen 1962, Machado *et al.* 2006), staining both secretion and protoplasts of secretory cells. Neutral lipids, such as essential oils, were restricted to the globular trichome secretion, and the positive reaction of reniform trichomes to osmium tetroxide can be attributed to phenolic compounds present in this class of trichomes. The difference in the autofluorescence pattern under light UV for the two types of trichomes also indicates differences in the secretory activity, with globular trichomes having blue autofluorescence and reniform trichomes showing weak autofluorescence in the head. When globular trichomes were stained with neutral red, they showed an intense yellow fluorescence, whereas reniform trichomes showed blue fluorescence, confirming the different chemical nature of their secretion. The blue fluorescence of reniform trichomes can be a consequence of this fluorochrome reaction with structural lipids, such as, plasma membrane phospholipids, but not with the secretion itself.

The occurrence of terpenes in the essential oil of globular trichomes was indicated by the positive reaction with Nadi reagent and confirmed by the characteristic scent of leaves and phytochemical studies that identified high proportions of mono- and sesquiterpenes, such as  $\alpha$ -pinene, trans-caryophyllene, alloaromadendrene and



$\alpha$ -humulene (Carvalho Jr. *et al.* 2004). Terpene-rich essential oils are commonly found in the secretion of glandular trichomes of different species (Werker *et al.* 1985, Ascensão & Pais 1987, Serrato-Valenti *et al.* 1997, Ascensão *et al.* 1999, Bisio *et al.* 1999, Sacchetti *et al.* 1999, Turner *et al.* 2000, Machado *et al.* 2006). Several biological activities are attributed to essential oils, which were recognized as phytoalexins, insect antifeedant, feromones, defensive agents, allelochemicals or signaling molecules (Harbone 1997). The *C. verbenacea* essential oil has antimicrobial activity (Carvalho Jr. *et al.* 2004), and two of its components,  $\alpha$ -humulene and trans-caryophyllene, were reported to have anti-inflammatory properties (Passos *et al.* 2007), which is one of the main pharmacological characteristics of the species (Sertié *et al.* 1988, Basile *et al.* 1989, Sertié *et al.* 2005).

The ferric trichloride test, which is based on the formation of intensively colored precipitate from the reaction of iron with ortho-dihydroxyphenols (Johansen 1940), and the potassium dichromate test, which is based on the formation of a product colored by the condensation of free phenolic hydroxyl groups with the chromium of the reagent (Gabe 1968), indicated that phenolic compounds were present only in reniform trichomes. The only class of phenolic compounds histochemically identified in these trichomes, by fluorochromes under UV light, was the flavonoids. Two flavones were already isolated and identified in *C. verbenacea*, 5,6'-dihydroxy-3,6,7,3',4'-pentamethoxyflavone and 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone, being the latter identical to artemetin that was initially isolated from *Artemisia* (Asteraceae) species (Velde *et al.* 1982, Lins *et al.* 1990). Approximately 8,000 phenolic compounds were already identified in plants, having biological role generally related to antifungal, antibacterial and antifeedant activities of some structures (Harbone 1997).

Although Ruthenium red indicated the presence of acid polysaccharides, as pectin (Johansen 1940), in both globular and reniform trichomes, this result must be analyzed with caution. First, because the positive reaction (rose color) did not extend to the secretion content of the subcuticular space of globular trichomes, and second because it was not specific to the head of reniform trichomes. Therefore, it may be concluded that this class of compound can be present in cell protoplasts as a precursor of the secretory product.

In resume, it can be concluded that the chemical composition of the secretion of glandular trichomes in *C. verbenacea* is related to the morphology of the secretory head, regardless of its size and the stalk cell number. The secretion of globular trichomes consists mainly of essential

oils, whereas the secretion of reniform trichomes consists basically of phenolic compounds, such as flavonoids.

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